Isoniazid-resistance conferring mutations in *Mycobacterium tuberculosis* KatG: Catalase, peroxidase, and INH-NADH adduct formation activities

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Received 27 August 2009; Revised 30 November 2009; Accepted 11 December 2009

DOI: 10.1002/pro.324

Published online 6 January 2010 proteinscience.org

Abstract: *Mycobacterium tuberculosis* catalase-peroxidase (KatG) is a bifunctional hemoprotein that has been shown to activate isoniazid (INH), a pro-drug that is integral to frontline antituberculosis treatments. The activated species, presumed to be an isonicotinoyl radical, couples to NAD⁺/NADH forming an isoniazid-NADH adduct that ultimately confers anti-tubercular activity. To better understand the mechanisms of isoniazid activation as well as the origins of KatG-derived INH-resistance, we have compared the catalytic properties (including the ability to form the INH-NADH adduct) of the wild-type enzyme to 23 KatG mutants which have been associated with isoniazid resistance in clinical *M. tuberculosis* isolates. Neither catalase nor peroxidase activities, the two inherent enzymatic functions of KatG, were found to correlate with isoniazid resistance. Furthermore, catalase function was lost in mutants which lacked the Met-Tyr-Trp crosslink, the biogenic cofactor in KatG which has been previously shown to be integral to this activity. The presence or absence of the crosslink itself, however, was also found to not correlate with INH resistance. The KatG resistance-conferring mutants were then assayed for their ability to generate the INH-NADH adduct in the presence of peroxide (*t*-BuOOH and H₂O₂), superoxide, and no exogenous oxidant (air-only background control). The results demonstrate that residue location plays a critical role in determining INH-resistance mechanisms associated with INH activation; however, different mutations at the same location can produce vastly different reactivities that are oxidant-specific. Furthermore, the data can be interpreted to suggest the presence of a second mechanism of INH-resistance that is not correlated with the formation of the INH-NADH adduct.

Keywords: catalase-peroxidase; KatG; isoniazid; INH; heme; crosslink

Introduction

Multi-drug resistant tuberculosis (MDR-TB), defined as strains which are resistant to more than one of the frontline antibiotics used in TB treatment, has been recorded at its highest levels ever, comprising

about 5% of the 9 million incident cases of TB in 2006.¹ MDR-TB is particularly difficult to treat, and has a high death rate of 50–80% within 4 months of diagnosis.¹ Extensively drug resistant (XDR) TB, which comprises about 7% of all MDR cases, is virtually untreatable.^{1,2} Thus, as the prevalence and severity of drug-resistant tuberculosis (TB) are on the rise, new efforts to understand the fundamental molecular basis of drug resistance are needed.

It is now well-established that the catalase-peroxidase (KatG) enzyme of *Mycobacterium tuberculosis* (*Mtb*) is responsible for activating the pro-drug

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: NIH/NIAID; Grant number: N01 AI-75320.

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Figure 1. Schematic representation of INH-NADH adduct formation as catalyzed by KatG via a putative isonicotinoyl radical.

isoniazid (INH). Although the details of this chemical transformation are still the subject of ongoing investigations, it is hypothesized that the activation of INH leads to an isonicotinoyl acyl radical (Fig. 1) that then combines with NAD^+/NADH to form what has been termed the isoniazid-NADH adduct (INH-NADH).^{3–5} This species was found to be a potent inhibitor of InhA, an enoyl acyl-carrier protein reductase involved in the production of mycolic acids, which are the key structural components of the mycobacterial cell wall.^{6–9} Although the phenomenological observation is that mutations in KatG can give rise to INH-resistance due to their inability to activate the INH pro-drug,^{10–13} an increased understanding of the interplay between an INH-conferring mutation and its consequences on the mechanism of isoniazid activation is necessary so that novel drug therapies can be developed to target these drug-resistant TB strains.

Several mechanisms have been proposed for INH activation.^{3,14,15} As KatG belongs to the Class I family of peroxidases,¹⁶ the enzyme is thus capable of utilizing hydrogen peroxide (or alkyl hydroperoxides) to catalyze the oxidation of various substrates via upwards of two consecutive one-electron oxidation steps, and it has been proposed that KatG oxidizes isoniazid in a similar manner (Fig. 2).^{15,17} Both, a two-electron oxidized KatG intermediate termed Compound I (ferryl porphyrin π -cation radical) and a one-electron oxidized form called Compound II (evidence suggests a ferric heme coupled with a protein radical in KatG rather than the traditional ferryl intermediate found in the monofunctional peroxidases, although this is the subject of current debate), have been observed in wild-type (WT) KatG,^{18,19} whereas an iron(IV)-oxo Compound II intermediate has been identified in KatGs con-

taining active site mutations.^{19–22} Both, Compound I and the ferryl Compound II, species are intermediates in the traditional peroxidase cycle, whereas Compound I is the key intermediate in the traditional catalase cycle, which is the other major enzymatic activity of KatG. Additional catalytic activities for KatG, such as NADH-oxidase,²³ peroxynitritase,²⁴ and Mn^{2+} -dependent peroxidase,²⁵ have also been reported.

The heme species, Compound III, has also been proposed as a critical intermediate in KatG linked to INH activation and drug susceptibility.^{14,26–28} Such an oxyferrous intermediate may be formed *in vivo* upon the binding of superoxide to the active site heme-iron of resting (ferric) KatG, by the addition of dioxygen to the ferrous form of the enzyme, or by the addition of a large excess of hydrogen peroxide to the ferric heme center (Fig. 2). Previous *in vitro* studies correlated the attenuation of INH-NADH adduct formation when catalyzed by KatG in the presence of superoxide to several mutants that were known to give rise to drug-resistance *in vivo*.¹⁴ Furthermore, it was observed in earlier pulse radiolysis studies that oxyferrous WT KatG underwent reaction with isoniazid, whereas the oxyferrous form of the resistance mutant KatG(S315T) was unable to do so.²⁸ Additional evidence in support of an oxyferrous mechanism for INH activation came from superoxide consumption studies which showed that superoxide was consumed by WT KatG in the presence of INH and NADH, but not by those KatG active site mutants that were linked to drug-resistance in clinical *Mtb* isolates.¹⁴

To date, no systematic study exists in which KatG mutations that confer isoniazid-resistance are correlated to three variables: enzymatic activity, strain fitness, and TB transmission. As the first step towards such a larger and more comprehensive informatics-styled interdisciplinary study in which isoniazid-resistance is fully correlated to those three factors, the principal goal of this current investigation is to generate a more extensive library of mutants that have been shown clinically to be related to INH resistance (Table I and Fig. 3), and identify

Figure 2. Proposed reactions and putative intermediates of KatG involved in the oxidation of isoniazid.

Figure 3. Active site of *Mtb* KatG showing the heme prosthetic group, as well as the Met-Tyr-Trp crosslink. Coordinates (1SJ2) were obtained from the Protein Data Bank. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

potential trends between mutant residue location, enzymatic activity, INH-NADH adduct formation as a function of heme intermediate (oxidant), and INH resistance. To probe the role of key heme intermediates in INH oxidation, the ability to form the INH-NADH adduct was assayed in the presence of peroxide (*t*-BuOOH and the H₂O₂-generating system glucose/glucose oxidase) and superoxide (xanthine/xanthine oxidase), and compared to air-only background controls

Table I. *KatG* Mutations Investigated in this Study

Location	Mutation
INH-resistance conferring mutations	
N-terminal domain	D63E
Active site	R104L, W107R, H108E, H108Q, N138D, N138S, Y229F, W300G
Proximal side	T262R, T275P, W328G, Y337C, A350S
Substrate access channel	S315G, S315I, S315N, S315R, S315T
C-terminal domain	R463L, L587M, G629S, D735N
Lab mutations	W107F, M255C, M255I, M255Y, T275V, W321F, W328F, R418L

Figure 4. Crystal structure of the *Mtb* KatG dimer. The heme prosthetic group (red) and mutations examined in this study (Table I) are highlighted. Coordinates (PDB ID: 1SJ2) were obtained from the Protein Data Bank.

(no exogenously added oxidant). In addition, peroxidase and catalase activities of each mutant were also assayed and correlated with the above INH-NADH adduct formation studies. Finally, we also investigated whether the presence of the structural feature unique to KatG, namely the Met-Tyr-Trp crosslink in which three non-sequential residues M255, Y229, and W107 are covalently linked together through their side chains (Fig. 4), can be correlated with INH-resistance. As will be demonstrated, the results of this study strongly suggest multiple mutation-specific pathways that, either separately or in combination, can affect INH-NADH adduct formation. Interestingly, several resistance-conferring INH mutations were identified which do not alter INH-NADH adduct formation, possibly suggesting a yet-to-be identified mechanism for INH-resistance in TB.

Results

Site-directed mutagenesis and overexpression of *KatG* mutants

The plasmid encoding wild-type KatG with an N-terminal poly-His tag (pMRLB11) was obtained from Colorado State University under the TB Research Materials and Vaccine Testing Contract (NIH, NIAID NO1 AI-75320). PCR amplification of pMRLB11 using mutagenic primers yielded the mutant plasmid(s) as confirmed by DNA sequencing. Hemin (30 mg L⁻¹, dissolved in 10 mL 0.1M NaOH) was added to the culture medium before autoclaving. In this fashion, no insoluble hemin was observed. The addition of hemin (or, alternatively, aminolevulinic acid, ALA^{18,29}) assures stoichiometric incorporation of the heme cofactor during overexpression in *Escherichia coli* for maximal holoenzyme isolation.³⁰ Purification using immobilized metal affinity chromatography yielded KatG with an acceptable optical purity ratio (Reinheitzahl or *R_z*, defined as *A_{Soret}*/

Table II. Kinetic Parameters for the Catalase Activity of WT KatG and Mutants

Mutant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} \text{s}^{-1}$)	Crosslink	Ref.
WT	6000 ± 70	2.5 ± 0.2	$(2.4 \pm 0.028) \times 10^6$	Yes	14
D63E	2260 ± 40	1.8 ± 0.3	$(1.26 \pm 0.21) \times 10^6$	Yes	^a
R104L	3.6 ± 0.2	10.5 ± 2.1	346 ± 22	No	14
W107R	n/d	n/d	n/d	No	^a
H108E	n/d	n/d	n/d	No	^a
H108Q	1.5 ± 0.1	26.5 ± 3.5	58 ± 4	Tyr-Trp	14
N138D	827.8 ± 29.8	3.5 ± 0.5	$(2.37 \pm 0.34) \times 10^5$	Yes	^a
N138S	127.3 ± 3.1	0.6 ± 0.1	$(2.12 \pm 0.36) \times 10^5$	Yes	^a
Y229F	0.1 ± 0.05	39.8 ± 6.4	2.5 ± 1.3	No	14
T262R	111.7 ± 2.6	4.1 ± 0.4	$(2.73 \pm 0.27) \times 10^4$	n/d	^a
T275P	n/d	n/d	n/d	Yes	^a
W300G	n/d	n/d	n/d	Yes	^a
S315G	711 ± 22	1.7 ± 0.3	$(4.18 \pm 0.76) \times 10^5$	Yes	^a
S315I	739.3 ± 4.9	2.2 ± 0.06	$(3.36 \pm 0.10) \times 10^5$	Yes	^a
S315N	326.5 ± 4.0	2.1 ± 0.2	$(1.55 \pm 0.15) \times 10^5$	Yes	^a
S315R	727 ± 77	5.7 ± 2.1	$(1.28 \pm 0.49) \times 10^5$	Yes	^a
S315T	2106 ± 47	3.9 ± 0.3	$(5.40 \pm 0.43) \times 10^5$	Yes	^a
W328G	468 ± 11	4.8 ± 0.4	$(9.75 \pm 0.85) \times 10^4$	Yes	^a
Y337C	1236 ± 13	1.7 ± 0.09	$(7.27 \pm 0.40) \times 10^5$	Yes	^a
A350S	1397 ± 28	2.3 ± 0.3	$(6.07 \pm 0.80) \times 10^5$	Yes	^a
R463L	1996 ± 43	2.2 ± 0.2	$(9.07 \pm 0.85) \times 10^5$	Yes	^a
L587M	3180 ± 100	2.6 ± 0.4	$(1.22 \pm 0.19) \times 10^6$	Yes	^a
G629S	3070 ± 510	9.2 ± 4.6	$(3.33 \pm 1.76) \times 10^5$	Yes	^a
D735N	1600 ± 220	33.6 ± 8.7	$(4.76 \pm 1.38) \times 10^4$	Yes	^a
W107F	n/d	n/d	n/d	No	^a
M255C	20.6 ± 1.0	20.1 ± 2.4	1026 ± 49	Cys-Tyr-Trp	^a
M255I	1.1 ± 0.1	40.2 ± 6.1	27.0 ± 4.1	Tyr-Trp	22
M255Y	1.9 ± 0.4	13.1 ± 7.4	144 ± 86	Tyr-Tyr-Trp	^a
T275V	36.1 ± 0.84	6.3 ± 0.4	5730 ± 397	No	^a
W321F	1490 ± 130	4.4 ± 1.4	$(3.38 \pm 1.11) \times 10^5$	Yes	^a
W328F	940 ± 30	1.7 ± 0.6	$(5.54 \pm 1.96) \times 10^5$	Yes	^a
R418L	33 ± 4	16.7 ± 4.4	1988 ± 210	Yes	14

^a This work.

n/d, not determined.

A_{280}) of greater than 0.5. Table SIII provides the relevant UV-visible spectral features for each mutant reported herein. Typical yields of isolated, purified KatG ranged from 20 to 80 mg L^{-1} .

Crosslink identification

Tryptic digestion studies were performed as previously described.^{19,22} For each of the KatG mutants, at least 40+ peptide fragments were well resolved, representing the majority of the 72 theoretical fragments calculated for KatG proteolytic cleavage by trypsin, and an even higher percentage of the 54 fragments of size $n > 4$ (n = number of amino acid residues). In the region (r.t. ~ 180 min), which we have previously^{19,22} assigned as containing the Met-Tyr-Trp or Tyr-Trp CLPFs (crosslinked peptide fragment), the majority of the KatG mutants also exhibited a similar pattern of peaks in a tight cluster which were absent from the KatG(Y229F) (no CLPF present¹⁹) digest (data not shown). The similarity with WT KatG and difference with KatG(Y229F) suggested that these peaks represented either Met-Tyr-Trp or Tyr-Trp

crosslinked peptide fragments, predicted from trypsin cleavage sites to be comprised of ¹⁰⁵MAWHAAG-TYR¹¹⁴ [105–114], ²¹⁵DLENPLAAVQMGLIYVN-PEGPNGNPDPMMAAVIDR²⁴⁹ [215–249], and ²⁵⁵MAMNDVETAALIVGGHTFGK²⁷⁴ [255–274]. As the HPLC chromatograms were monitored at $\lambda = 220$ (peptide backbone), 280 (tyrosine/tryptophan), and 330 nm (crosslinked peptides), Met-Tyr-Trp [$\lambda_{\text{max}} = 252$ (sh), 296 nm] or Tyr-Trp [$\lambda_{\text{max}} = 252$ (sh), 296 nm] CLPF assignments were made using the characteristic spectral features and retention time for each (Table II). As we have discussed in detail for the Met-Tyr-Trp and Tyr-Trp crosslinks,¹⁹ the presence of these large bathochromic shifts in the UV-visible spectra of the CLPFs is suggestive of the presence of peptide crosslinks, which were previously confirmed by mass spectrometry for both the Met-Tyr-Trp ([105–114][215–249][255–274]) and Tyr-Trp ([105–114][215–249]) CLPFs.

The *Mtb* KatG mutants investigated in this study that did not exhibit the previously observed pattern of CLPFs included W107R/F, M255C, M255Y, T262R, and T275V. Regarding the

KatG(W107R/F) mutants, it has been shown that the mutation of the tryptophan analog in *Synechocystis* PCC 6803 (Trp122) blocks Met-Tyr-Trp crosslink formation.³¹ The first step in the autocatalytic formation of the Met-Tyr-Trp crosslink has been proposed to be the formation of the Tyr-Trp bond which occurs upon the simultaneous one electron oxidation of both the indole and phenol rings of Trp¹⁰⁷ and Tyr²²⁹, respectively, by KatG Compound I.^{19,22} We thus posit that the absence of a redox active side-chain in position 107 is likely the reason as to why the KatG(W107R/F) mutants did not exhibit a CLPF here. KatG(M255C) forms a Cys-Tyr-Trp crosslink and will be the subject of a future report.³²

The remaining KatG mutants (M255Y, T262R, and T275V) contain mutations within the peptides that comprise the CLPF, which may lead to either (i) an altered retention on our HPLC column used in detecting these fragments due to a change in peptide sequence that comprises the CLPF structure or (ii) a change in the location of the protease cleavage site, making detection by our previously established HPLC method (via retention) tentative. We thus further investigated the possible formation of cross-linked peptide fragments in these mutants using LC-MS of the tryptic digests. We were able to rule out the presence of either Met-Tyr-Trp or Tyr-Trp CLPFs in KatG(T275V). In KatG(T262R), we were unable to conclusively rule out or identify the presence of a Met-Tyr-Trp crosslink containing ²⁵⁵MAMNDVER²⁶² (in lieu of 255–274 due to the incorporation of a new protease cleavage site upon mutation of the threonine residue at position 262) due to difficulty with the sample.

However, KatG(M255Y) was found to form both the Tyr-Trp CLPF as well as a Tyr-Tyr-Trp crosslinking containing structure. Crosslinked peptide [105–114]–[215–249] was detected at *m/z* 1206.3373(4+), 965.2747(5+), and 804.5616(6+), corresponding to a neutral monoisotopic mass of 4821.299 that is in very good agreement (within 2 ppm) with the calculated value: 4821.309 Da. This molecule was also detected singly and doubly oxidized, that is, containing methionine sulfoxide(s). The Tyr-Tyr-Trp crosslink was detected between peptides [105–114], [214–249], and [255–274] at *m/z* 1179.0804(6+), 1010.7838(7+), and 884.5629(8+) corresponding to neutral monoisotopic mass of 7068.436 that is in good agreement (within 3 ppm) with the calculated value of 7068.419 Da. CID analysis of *m/z* 1179(6+) confirmed the identity of this molecule (Fig. S1).

As only five of the isoniazid-resistance conferring mutants have been found to conclusively lack a Met-Tyr-Trp crosslink, the results are in agreement with our previous¹⁴ (but less expansive) study which noted no correlation between drug susceptibility/resistance and the presence/absence of the crosslink.

Catalase activity

Kinetic parameters (k_{cat} , K_m , and catalytic efficiency, k_{cat}/K_m) for the catalase activity of the KatG mutants are presented in Table II. All KatG mutants exhibited saturable catalase activity under the conditions used for this kinetic study. However, the kinetic constants reported here for catalase activity are “apparent” values as conventional catalases do not follow typical Michaelis-Menten kinetics.^{33,34} None of the mutant KatGs exhibited a catalytic efficiency for catalase activity greater than that of WT KatG [$k_{\text{cat}}/K_m = (2.4 \pm 0.028) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$], primarily due to the fact that k_{cat} was lower for all mutants when compared to WT KatG, more than offsetting any improvements in K_m . As the catalase activity of KatG has been linked to the presence of the Met-Tyr-Trp covalent crosslink,^{20,35–37} these activity results are best considered in conjunction with the above crosslink-identification studies. The results presented in Table II clearly indicate that for each mutant that exhibited a catalytic competency of $\sim k_{\text{cat}}/K_m > 10^3 \text{ M}^{-1} \text{ s}^{-1}$, an intact Met-Tyr-Trp crosslink was required, consistent with literature observations.^{20,36,37} However, not all mutants with a fully formed Met-Tyr-Trp crosslink were catalase active, suggesting that while the crosslink is needed for activity, it is not the only requirement. This observation was most apparent in KatG(T275P) and KatG(W300G), both of which lacked any detectable catalase activity under the conditions used in this study. Thr²⁷⁵ is found within the large loop 2 (LL2), is within Van der Waals contact of heme propionate D, follows K²⁷⁴, which is involved in the active site H-bonding network, and is adjacent to His²⁷⁶ which forms an H-bond with the propionate C group of the heme cofactor.³⁵ Given the conformational rigidity of proline, we surmise that the Thr→Pro mutation at position 275 significantly perturbs the H-bonding network necessary for catalase activity, either through altering the propionate arm directly (itself bonded to water and Arg¹⁰⁴), or by altering the entire conformation of the LL2 loop itself. We rule out constriction or blockage of the active site substrate access channel as to why the catalase activity is lost in the KatG(T275P) mutation as this mutant possesses significant peroxidase activity ($0.23 \pm 0.01 \text{ s}^{-1}$) and a K_m ($18.4 \pm 3.6 \text{ mM}$) only 2-fold greater than that determined for the WT enzyme. By contrast, mutation at position 275 to valine lowers ($5730 \pm 397 \text{ M}^{-1} \text{ s}^{-1}$), but does not completely abolish, catalase activity. Valine differs from threonine by replacement of the hydroxyl substituent with a methyl group, a less drastic mutation when compared to that found in KatG(T275P), and is a plausible explanation as to why the T275V mutant retains some measureable activity and the T275P mutant does not. The significant loss of catalase activity in KatG(T275V) is likely due to the lack of an intact

Met-Tyr-Trp crosslink (*vide infra*). Similarly, we surmise that the Trp→Gly mutation in W300G also disrupts the hydrogen bonding network obligatory for catalase activity. This is a plausible hypothesis given that the added conformational flexibility of this achiral mutation coupled with the large structural difference between tryptophan and glycine likely alter the LL2 region significantly, as this mutation is found within the interhelical insertion between the F and G loops which is not present in monofunctional peroxidases.³⁸

KatG(R418L) ($1988 \pm 210 M^{-1} s^{-1}$) was found to also possess the Met-Tyr-Trp crosslink, yet exhibits greatly attenuated catalase activity. Arg⁴¹⁸ forms two hydrogen bonds with the Met-Tyr-Trp crosslink, specifically between δ -NH₁ and the phenolic oxygen of Tyr²²⁹, as well as ω -NH₂ and the backbone carbonyl oxygen of Met²⁵⁵.³⁵ Several studies have commented on the role of Arg⁴¹⁸ as a pH sensitive molecular switch that moves between two conformations, the so-called Y conformation (H-bond/ionic association between Arg⁴¹⁸ and Tyr²²⁹ present) that activates heme for oxidation via H₂O₂, and the R conformation (H-bond/ionic association between Arg⁴¹⁸ and Tyr²²⁹ absent), which appears to be involved in ferryl heme reduction by H₂O₂.^{39–41} These studies, however, may need to be re-interpreted in light of the novel mechanism proposed by Magliozzo and coworkers which involves a non-traditional (for catalase activity) oxyferrous heme/protein-based radical intermediate as a catalytically competent species in KatG.^{42,43} One putative role of Arg⁴¹⁸ may be to aid in the formation (or stabilization) of this crosslink-centered radical species for subsequent turnover in the proposed catalase mechanism,⁴² and the use of this mutation may facilitate the trapping of key intermediates for further study.

Although most KatG active site mutations do negatively affect catalase activity, comparison of the catalytic efficiencies (k_{cat}/K_m) for catalase activity to MICs for INH (Table SII) do not demonstrate any apparent correlation between catalase activity and isoniazid resistance.

Peroxidase activity

All KatG mutants exhibited saturable peroxidase activity for the one-electron oxidation of ABTS to the corresponding radical cation, ABTS^{•+}, in the presence of *tert*-butylhydroperoxide, and the corresponding kinetic parameters (k_{cat} , K_m , and catalytic efficiency, k_{cat}/K_m) are reported in Table III. With the exception of the previously studied mutants H108Q ($0.029 \pm 0.003 s^{-1}$) and S315T ($0.051 \pm 0.003 s^{-1}$),¹⁴ the k_{cat} values for peroxidase activity for the new mutants reported herein all increased by 2-fold to 35-fold over that of WT KatG ($0.062 \pm 0.001 s^{-1}$). The R104L, W107F, H108E/Q, Y229F, M255C/I/Y, and S315T mutants all exhibited lower K_m values

Table III. Kinetic Parameters for the Peroxidase Activity of WT KatG and Mutants

Mutant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} s^{-1}$)	Ref.
WT	0.062 ± 0.001	8.44 ± 0.45	7.3 ± 0.4	14
D63E	1.41 ± 0.07	62.6 ± 8.2	22.5 ± 3.2	a
R104L	0.124 ± 0.002	4.48 ± 0.21	28 ± 2	14
W107R	1.49 ± 0.04	18.4 ± 1.8	80.9 ± 8.2	a
H108E	0.53 ± 0.01	7.3 ± 0.5	72.8 ± 5.1	a
H108Q	0.029 ± 0.003	2.06 ± 0.64	14 ± 3	14
N138D	0.62 ± 0.03	50.3 ± 8.2	12.4 ± 2.1	a
N138S	0.25 ± 0.01	35.4 ± 3.4	7.0 ± 0.7	a
Y229F	0.843 ± 0.056	2.66 ± 0.66	316 ± 39	14
T262R	0.25 ± 0.01	12.8 ± 1.9	19.3 ± 3.0	a
T275P	0.23 ± 0.01	18.4 ± 3.6	12.7 ± 2.5	a
W300G	0.45 ± 0.03	18.0 ± 5.0	25.0 ± 7.1	a
S315G	0.81 ± 0.02	26.5 ± 2.8	30.5 ± 3.3	a
S315I	0.61 ± 0.03	12.6 ± 2.6	48.1 ± 10.2	a
S315N	0.41 ± 0.02	20.7 ± 2.9	20.0 ± 2.9	a
S315R	0.64 ± 0.02	12.4 ± 2.4	51.9 ± 10.2	a
S315T	0.051 ± 0.003	2.06 ± 0.64	5.7 ± 1	a
W328G	2.15 ± 0.02	49.3 ± 2.0	43.5 ± 1.8	a
Y337C	1.18 ± 0.05	61.8 ± 6.6	19.0 ± 2.2	a
A350S	1.28 ± 0.09	51.8 ± 10.2	24.7 ± 5.1	a
R463L	1.04 ± 0.05	85.7 ± 10.8	12.2 ± 1.7	a
L587M	1.88 ± 0.09	39.8 ± 5.9	47.2 ± 7.3	a
G629S	0.67 ± 0.03	25.6 ± 4.3	26.3 ± 4.6	a
D735N	0.28 ± 0.01	36.1 ± 5.1	7.7 ± 1.1	a
W107F	0.47 ± 0.04	2.9 ± 1.2	163 ± 69	a
M255C	0.170 ± 0.004	2.8 ± 0.2	60.7 ± 4.3	a
M255I	0.164 ± 0.002	3.40 ± 0.08	48.1 ± 1.2	22
M255Y	0.31 ± 0.02	4.7 ± 1.1	66.7 ± 16.2	a
T275V	0.64 ± 0.04	18.4 ± 4.4	35.0 ± 8.7	a
W321F	0.42 ± 0.02	21.9 ± 3.2	19.0 ± 3.0	a
W328F	0.68 ± 0.01	37.4 ± 0.9	18.1 ± 0.5	a
R418L	0.119 ± 0.002	11.05 ± 0.52	10.7 ± 0.5	14

^a This work.

than WT KatG. Generally speaking, the combined effects of an increase in k_{cat} and a decrease in K_m led to catalytic efficiencies 1-fold to 45-fold greater than that of WT KatG ($7.3 \pm 0.4 M^{-1} s^{-1}$) for all but the KatG(S315T) mutant ($5.7 \pm 1.0 M^{-1} s^{-1}$). Such an increase in peroxidase activity for *Mtb* KatG (Y229F) has been noted previously by Magliozzo and coworkers²⁰ who suggested that upon loss of the covalent adduct the increase in peroxidase activity (with concomitant loss of catalase function) is due to enhanced formation and/or increased stabilization of the iron(IV)-oxo containing Compound II intermediate, which plays a role in the peroxidase, but not catalase, cycle. The results here suggest an extension of that observation, with mutations generally affecting peroxidase activity regardless of the presence/absence of the Met-Tyr-Trp adduct. Loss of peroxidase function does not appear to correlate with INH-resistance, a similar finding to the one noted above for catalase activity. One possible interpretation of these results is that the KatG peroxidase activity measured using ABTS as the reducing substrate is not predictive of INH resistance. Instead, formation of the INH-NADH adduct in a direct

Table IV. *INH-NADH Adduct Formation as Catalyzed by WT KatG and Mutants in the Presence and Absence (Air-Only) of *t*-BuOOH^a*

Mutant	Air-only ^b	400 μ M <i>t</i> -BuOOH ^b
WT	4.64 $\pm$ 0.34 (100%)	26.78 $\pm$ 2.97 (100%)
D63E	4.42 $\pm$ 0.88 (95.2%)	27.03 $\pm$ 1.90 (100.9%)
R104L	0.36 $\pm$ 0.03 (7.7%)	3.17 $\pm$ 0.52 (11.9%)
W107R	1.29 $\pm$ 0.09 (27.9%)	5.19 $\pm$ 0.10 (19.4%)
H108E	0.19 $\pm$ 0.08 (4.1%)	4.51 $\pm$ 0.08 (16.8%)
H108Q	1.41 $\pm$ 0.14 (30.4%)	6.47 $\pm$ 0.21 (24.2%)
N138D	1.68 $\pm$ 0.09 (36.3%)	6.24 $\pm$ 1.68 (23.3%)
N138S	0.00 $\pm$ 0.00 (0.0%)	4.55 $\pm$ 0.69 (17.0%)
Y229F	0.60 $\pm$ 0.13 (12.9%)	4.42 $\pm$ 1.24 (16.5%)
T262R	1.20 $\pm$ 0.09 (25.8%)	1.51 $\pm$ 0.39 (5.6%)
T275P	0.54 $\pm$ 0.12 (11.6%)	4.23 $\pm$ 0.39 (15.8%)
W300G	0.00 $\pm$ 0.00 (0.0%)	2.29 $\pm$ 0.78 (8.6%)
S315G	1.10 $\pm$ 0.36 (23.7%)	11.21 $\pm$ 1.12 (41.8%)
S315I	1.72 $\pm$ 0.05 (37.1%)	17.38 $\pm$ 0.34 (64.9%)
S315N	0.00 $\pm$ 0.00 (0.0%)	8.09 $\pm$ 0.30 (30.2%)
S315R	0.09 $\pm$ 0.05 (2.0%)	6.82 $\pm$ 0.52 (25.5%)
S315T	0.50 $\pm$ 0.07 (10.7%)	6.92 $\pm$ 0.31 (25.9%)
W328G	0.43 $\pm$ 0.02 (9.2%)	11.20 $\pm$ 0.34 (41.8%)
Y337C	3.06 $\pm$ 0.43 (65.9%)	14.51 $\pm$ 5.09 (54.2%)
A350S	3.61 $\pm$ 0.17 (77.8%)	24.54 $\pm$ 0.24 (91.7%)
R463L	12.93 $\pm$ 0.99 (278.9%)	51.88 $\pm$ 2.68 (193.7%)
L587M	5.97 $\pm$ 0.37 (128.7%)	0.44 $\pm$ 0.09 (1.7%)
G629S	4.16 $\pm$ 0.19 (89.7%)	7.58 $\pm$ 2.88 (28.3%)
D735N	17.87 $\pm$ 2.33 (385.4%)	8.65 $\pm$ 0.86 (32.3%)
W107F	1.70 $\pm$ 0.10 (36.6%)	5.94 $\pm$ 0.38 (22.2%)
M255C	6.20 $\pm$ 0.11 (133.7%)	1.40 $\pm$ 0.17 (5.2%)
M255I	3.80 $\pm$ 0.27 (82.0%)	4.01 $\pm$ 0.75 (15.0%)
M255Y	3.13 $\pm$ 0.08 (67.6%)	2.49 $\pm$ 0.78 (9.3%)
T275V	0.57 $\pm$ 0.10 (12.2%)	2.13 $\pm$ 0.17 (8.0%)
W321F	8.10 $\pm$ 0.25 (174.7%)	4.63 $\pm$ 1.14 (17.3%)
W328F	0.80 $\pm$ 0.19 (17.2%)	5.01 $\pm$ 0.59 (18.7%)
R418L	20.42 $\pm$ 0.69 (440.3%)	11.04 $\pm$ 2.50 (41.2%)

^a Concentration of adduct formed in μ M.

^b (%) = Normalized to the value obtained for WT KatG as 100%.

comparison of wild-type KatG to a resistance-conferring mutant in an oxidation state(s) (e.g., Compounds I, II, or III) competent for the oxidation of the drug may be (vide infra).

INH-NADH adduct formation studies

The data on the formation of the INH-NADH adduct (InhA-inhibitor) as catalyzed by WT KatG, isoniazid-resistance conferring mutants, and lab mutants are reported for the following specific oxidant conditions: background control [no exogenously added oxidant; Table IV, Figs. 5 and S2(A)], *t*-BuOOH [Table IV, Figs. 5, 6(B), and S2(B)], the H₂O₂-generating system glucose/glucose oxidase [Table V, Figs. 5, 6(C), and S2(C–E)], and the superoxide-generating system xanthine/xanthine oxidase [Table VI, Figs. 5, 6(D), and S2(F)]. Identification and quantification of the INH-NADH adduct was performed by HPLC using published protocols^{14,44,45} with only slight modifications. The INH-NADH adduct exhibited a characteristic^{44,46} UV-visible absorption spectrum, with absorption maxima at 260 and 330 nm, and an A_{330/260} ratio of 3.2. The integration of the area under INH-NADH peaks was assigned as 100% for WT KatG for each set of conditions, and the yields reported for the KatG mutants were normalized to this value. The results for each oxidant are divided into three categories: Category I: mutants that generate near wild-type amounts of the adduct; Category II: mutants whose adduct formation is approximately more than two-fold attenuated; and Category III: mutants whose adduct formation is approximately greater than 2-fold enhanced. Refer to Table SIV for a summary of mutations listed by Category and as a function of reaction conditions used.

As an air-only background control, the amount of INH-NADH adduct formed as catalyzed by KatG in the absence of an exogenously added oxidant (i.e., air-only) was determined [Table IV, Figs. 5 and S2(A)]. Compared with WT KatG (4.64 $\pm$ 0.34 μ M), five resistance mutations (D63E, 4.42 $\pm$ 0.34 μ M; Y337C, 3.06 $\pm$ 0.43 μ M; A350S, 3.61 $\pm$ 0.17 μ M; L587M, 5.97 $\pm$ 0.37 μ M; G629S, 4.16 $\pm$ 0.19 μ M) and three lab mutations (M255C, 6.20 $\pm$ 0.11 μ M; M255I,

Figure 5. Formation of the INH-NADH adduct as catalyzed by WT KatG and resistance mutations in the presence of air-only (orange), 400 μ M *t*-BuOOH (blue), 225 μ M superoxide (red), 400 μ M H₂O₂ administered over 1 h (black), 400 μ M H₂O₂ administered over 3.3 h (gray), and 120 μ M H₂O₂ administered over 1 h (cyan). The resistance mutations are color coded by their putative roles in KatG (refer to Fig. 3 and Table I). See “Experimental” section for reaction conditions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6. INH-resistance mutations, which give rise to significantly lower reactivity compared to WT KatG. A: Catalase activity attenuated $\geq 1000\times$ (red). INH-NADH adduct formation attenuated $\geq 10\times$ using: (B) $400\ \mu\text{M}$ *t*-BuOOH (green), (C) $400\ \mu\text{M}$ H_2O_2 over 1 h (G/GO_x, blue), (D) $225\ \mu\text{M}$ O_2^- (X/XO, pink). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

$3.80 \pm 0.27\ \mu\text{M}$; M255Y, $3.13 \pm 0.08\ \mu\text{M}$) fall under Category I, the majority (19 out of 31) of the resistance and lab mutations belong to Category II, and two resistance mutations (R463L, $12.93 \pm 0.99\ \mu\text{M}$; D735N, $17.87 \pm 2.33\ \mu\text{M}$) and two lab mutations (W321F, $8.10 \pm 0.25\ \mu\text{M}$; R418L, $20.42 \pm 0.69\ \mu\text{M}$) fall under Category III. Closer examination of the Category II mutants reveals 10 isoniazid resistance mutants which are ~ 10 -fold attenuated (R104L, H108E, N138S, Y229F, T275P, W300G, S315N, S315R, S315T, and W328G), yet lack a correlation with loss of either catalase or peroxidase activities (*vide supra*). Activation of INH and formation of the INH-NADH adduct by KatG in the presence of air may be mediated through either the classical peroxidase intermediate Compound I, or via oxyferrous KatG (*vide infra*). Formation of Compound I may occur upon reaction with hydrogen peroxide, itself

generated by either the spontaneous disproportionation of superoxide in solution,⁴⁷ or by the NADH-oxidase activity of KatG.²³ Pathways for the spontaneous formation of oxyferrous KatG in solution include: (i) reduction of ferric (resting) KatG to the ferrous state by trace N_2H_4 present in solutions containing INH, followed by dioxygen binding^{26,27}; (ii) trace metal catalyzed oxidation of hydrazines in the presence of dioxygen; (iii) trace metal catalyzed autooxidation of INH at alkaline pH^{48–50}; and/or (iv) the pH-dependent generation of superoxide or hydrogen peroxide via the NADH-oxidase activity of KatG (as observed for *Burkholderia pseudomallei*).²³ In the absence of KatG, the formation of the INH-NADH adduct is undetectable under these conditions, highlighting the necessity of the enzyme for this reaction.

Formation of the INH-NADH adduct as catalyzed by the heme intermediate Compound I (Fig. 2,

Table V. INH-NADH Adduct Formation as Catalyzed by WT KatG and Mutants in the Presence of Varying Fluxes of H₂O₂ Produced by the Enzymatic System Glucose/Glucose Oxidase^a

Mutant	400 μ M H ₂ O ₂ over 1 h	400 μ M H ₂ O ₂ over 3.3 h	120 μ M H ₂ O ₂ over 1 h
WT	58.07 $\pm$ 2.31 (100%)	20.01 $\pm$ 1.34 (100%)	18.61 $\pm$ 3.86 (100%)
D63E	14.45 $\pm$ 0.39 (24.9%)	7.22 $\pm$ 0.78 (36.1%)	15.55 $\pm$ 0.31 (83.6%)
R104L	4.77 $\pm$ 2.65 (8.2%)	3.28 $\pm$ 0.99 (16.4%)	4.37 $\pm$ 0.27 (23.5%)
W107R	6.31 $\pm$ 0.62 (10.9%)	6.15 $\pm$ 0.26 (30.7%)	2.22 $\pm$ 0.13 (11.9%)
H108E	2.25 $\pm$ 0.47 (3.9%)	1.12 $\pm$ 0.20 (5.6%)	0.96 $\pm$ 0.11 (5.2%)
H108Q	4.59 $\pm$ 0.27 (7.9%)	6.55 $\pm$ 0.53 (32.7%)	6.15 $\pm$ 0.95 (33.1%)
N138D	8.81 $\pm$ 1.02 (15.2%)	4.00 $\pm$ 0.32 (20.0%)	6.06 $\pm$ 1.44 (32.6%)
N138S	3.27 $\pm$ 0.24 (5.6%)	4.33 $\pm$ 0.20 (21.6%)	4.53 $\pm$ 0.13 (24.3%)
Y229F	10.22 $\pm$ 2.20 (17.6%)	6.97 $\pm$ 1.15 (34.8%)	8.55 $\pm$ 0.74 (45.9%)
T262R	5.89 $\pm$ 1.19 (10.2%)	3.26 $\pm$ 0.07 (16.3%)	4.91 $\pm$ 0.14 (26.4%)
T275P	7.45 $\pm$ 0.32 (12.8%)	6.44 $\pm$ 0.66 (32.2%)	3.92 $\pm$ 1.11 (21.1%)
W300G	3.25 $\pm$ 0.26 (5.6%)	2.51 $\pm$ 0.62 (12.6%)	2.00 $\pm$ 0.16 (10.8%)
S315G	20.57 $\pm$ 1.82 (35.4%)	18.26 $\pm$ 1.15 (91.3%)	19.13 $\pm$ 3.34 (102.8%)
S315I	25.97 $\pm$ 4.64 (44.7%)	21.63 $\pm$ 1.87 (108.1%)	19.86 $\pm$ 3.61 (106.7%)
S315N	3.13 $\pm$ 0.92 (5.4%)	2.62 $\pm$ 0.30 (13.1%)	2.70 $\pm$ 0.21 (14.5%)
S315R	1.76 $\pm$ 0.19 (3.0%)	1.79 $\pm$ 0.41 (9.0%)	1.21 $\pm$ 1.00 (6.5%)
S315T	4.29 $\pm$ 0.36 (7.4%)	6.78 $\pm$ 0.42 (33.9%)	4.42 $\pm$ 0.47 (23.8%)
W328G	14.62 $\pm$ 1.27 (25.2%)	14.25 $\pm$ 1.20 (71.2%)	12.88 $\pm$ 1.89 (69.2%)
Y337C	36.82 $\pm$ 3.60 (63.4%)	22.16 $\pm$ 0.99 (110.8%)	25.94 $\pm$ 2.83 (139.4%)
A350S	27.16 $\pm$ 1.09 (46.8%)	10.06 $\pm$ 2.30 (50.3%)	22.51 $\pm$ 2.09 (121.0%)
R463L	52.15 $\pm$ 3.71 (89.8%)	27.28 $\pm$ 1.95 (136.3%)	38.84 $\pm$ 0.90 (208.7%)
L587M	18.65 $\pm$ 2.81 (32.1%)	13.91 $\pm$ 1.29 (69.5%)	14.18 $\pm$ 2.93 (76.2%)
G629S	17.52 $\pm$ 0.60 (30.2%)	8.87 $\pm$ 0.23 (44.3%)	17.00 $\pm$ 0.92 (91.3%)
D735N	14.07 $\pm$ 4.08 (24.2%)	13.24 $\pm$ 1.73 (66.2%)	14.11 $\pm$ 1.01 (75.8%)
W107F	5.96 $\pm$ 1.00 (10.3%)	9.69 $\pm$ 0.37 (48.4%)	8.56 $\pm$ 0.66 (46.0%)
M255C	13.02 $\pm$ 0.82 (22.4%)	10.47 $\pm$ 0.58 (52.3%)	14.43 $\pm$ 0.89 (77.5%)
M255I	0.53 $\pm$ 0.04 (0.9%)	7.31 $\pm$ 0.27 (36.6%)	17.33 $\pm$ 2.06 (93.1%)
M255Y	0.85 $\pm$ 0.41 (1.5%)	1.83 $\pm$ 1.20 (9.2%)	6.79 $\pm$ 0.31 (36.5%)
T275V	4.65 $\pm$ 1.26 (8.0%)	4.20 $\pm$ 0.16 (21.0%)	4.66 $\pm$ 0.47 (25.0%)
W321F	32.57 $\pm$ 3.64 (56.1%)	26.50 $\pm$ 0.47 (132.4%)	23.78 $\pm$ 2.07 (127.8%)
W328F	9.21 $\pm$ 0.51 (15.9%)	5.10 $\pm$ 0.36 (25.5%)	9.01 $\pm$ 0.67 (48.4%)
R418L	0.30 $\pm$ 0.07 (0.5%)	0.21 $\pm$ 0.00 (1.1%)	0.34 $\pm$ 0.02 (1.8%)

^a Concentration of adduct formed in μ M.

^b (%) = Normalized to the value obtained for WT KatG as 100%.

step A) was performed using *tert*-butylhydroperoxide [Table IV, Figs. 5, 6(B), and S2(B)], the same reagent used in the peroxidase activity measurements. When compared to the air-only background control reactions, both WT KatG (26.78 $\pm$ 2.97 μ M) and all isoniazid-conferring resistance mutants exhibited an increase in the amount of the INH-NADH adduct produced by 1-fold to 100-fold in the presence of the *t*-BuOOH oxidant, except in the cases of KatG(L587M) (0.44 $\pm$ 0.09 μ M) and KatG(D735N) (8.65 $\pm$ 0.86 μ M) mutants, implying that Compound I may play a significant role in activating either INH or NADH for INH-NADH adduct formation. Specifically, Category I mutants include the four resistance mutants D63E (27.03 $\pm$ 1.90 μ M), S315I (17.38 $\pm$ 0.34 μ M), Y337C (14.51 $\pm$ 5.09 μ M), and A350S (24.54 $\pm$ 0.24 μ M). The bulk (26 out of 31) of the resistance and lab mutations belong to Category II, and only one resistance mutation (R463L, 51.88 $\pm$ 2.68 μ M) fell under Category III. Interestingly, only three Category II resistance mutants are $\sim$ 10-fold attenuated or more in their ability to generate the INH-NADH adduct under conditions favorable for Compound I, including T262R (1.51 $\pm$ 0.39 μ M),

W300G (2.29 $\pm$ 0.78 μ M), and L587M (0.44 $\pm$ 0.09 μ M), despite each possessing peroxidase activity that is significantly higher than WT KatG. Similarly, three Category II lab mutants are also $\sim$ 10-fold attenuated (M255C, 1.40 $\pm$ 0.17 μ M; M255Y, 2.49 $\pm$ 0.78 μ M; T275V, 2.13 $\pm$ 0.17 μ M), again with each exhibiting peroxidase activity that exceeds that of WT KatG.

As the *t*-BuOOH data above suggested a possible correlation of the Compound I activation pathway with INH-resistance, we used the hydrogen peroxide generating system glucose/glucose oxidase (G/GOx) in a similar procedure to that utilized by Magliozzo and coworkers^{51,52} to explore this chemistry in further detail. INH-NADH adduct formation (Table V) was examined under three hydrogen peroxide fluxes: 400 μ M over 1 h [Figs. 5 and S2(C)], 400 μ M over 3.3 h [Figs. 5 and S2(D)], and 120 μ M over 1 h [Figs. 5 and S2(E)]. For this latter condition of lowest total amount of hydrogen peroxide examined (120 μ M over 1 h; Fig. 2 step A, H₂O₂ "low-flux"), nine INH-resistance conferring mutants (D63E, S315G, S315I, W328G, Y337C, A350S, L587M, G629S, and D735N) and three lab mutants

Table VI. *INH-NADH Adduct Formation as Catalyzed by WT KatG and Mutants in the Presence of the Superoxide-Generating System Xanthine/Xanthine Oxidase^a*

Mutant	225 μM O_2^-	225 μM O_2^- (+ catalase)
WT	31.72 $\pm$ 2.57 (100%)	20.18 $\pm$ 1.82 (100%)
D63E	20.85 $\pm$ 0.97 (65.7%)	
R104L	2.63 $\pm$ 0.10 (8.3%)	0.40 $\pm$ 0.15 (2.0%)
W107R	2.10 $\pm$ 1.01 (6.6%)	0.57 $\pm$ 0.22 (2.8%)
H108E	2.09 $\pm$ 0.05 (6.6%)	0.78 $\pm$ 0.28 (3.9%)
H108Q	5.74 $\pm$ 0.10 (18.1%)	1.11 $\pm$ 0.23 (5.5%)
N138D	5.94 $\pm$ 0.99 (18.7%)	3.74 $\pm$ 0.63 (18.5%)
N138S	0.37 $\pm$ 0.02 (1.2%)	
Y229F	4.47 $\pm$ 0.70 (14.1%)	3.21 $\pm$ 0.25 (15.9%)
T262R	12.99 $\pm$ 0.43 (41.0%)	
T275P	4.33 $\pm$ 0.60 (13.6%)	1.20 $\pm$ 0.27 (5.9%)
W300G	2.35 $\pm$ 0.14 (7.4%)	2.37 $\pm$ 0.24 (11.8%)
S315G	0.00 $\pm$ 0.00 (0.0%)	
S315I	20.77 $\pm$ 0.76 (65.5%)	
S315N	1.62 $\pm$ 0.14 (5.1%)	
S315R	0.49 $\pm$ 0.30 (1.5%)	
S315T	0.67 $\pm$ 0.16 (2.1%)	
W328G	4.65 $\pm$ 0.17 (14.7%)	
Y337C	24.82 $\pm$ 1.66 (78.2%)	
A350S	21.13 $\pm$ 0.49 (66.6%)	
R463L	20.58 $\pm$ 1.22 (64.9%)	
L587M	16.01 $\pm$ 2.55 (50.5%)	
G629S	25.5 $\pm$ 0.38 (80.4%)	
D735N	24.57 $\pm$ 3.46 (77.4%)	
W107F	7.46 $\pm$ 0.67 (23.5%)	2.45 $\pm$ 0.19 (12.2%)
M255C	12.22 $\pm$ 0.66 (38.5%)	14.89 $\pm$ 0.54 (73.8%)
M255I	3.84 $\pm$ 0.18 (12.1%)	3.96 $\pm$ 0.05 (19.6%)
M255Y	14.47 $\pm$ 0.82 (45.6%)	6.08 $\pm$ 0.30 (30.1%)
T275V	2.27 $\pm$ 0.37 (7.2%)	
W321F	14.21 $\pm$ 1.04 (44.8%)	
W328F	7.82 $\pm$ 0.50 (24.7%)	
R418L	16.48 $\pm$ 1.02 (51.9%)	13.03 $\pm$ 0.56 (64.6%)

^a Concentration of adduct formed in μM .

^b (%) = Normalized to the value obtained for WT KatG as 100%.

(M255C, M255I, and W321F) were classified as Category I, a total of 13 INH-resistance mutants (R104L, W107R, H108E/Q, N138D/S, Y229F, T262R, T275P, W300G, and S315N/R/T) and five lab mutants (W107F, M255Y, T275V, W328F, and R418L) fell under Category II, while only one mutant, the INH-resistance phenotype of KatG(R463L) was classified as Category III. Only four Category II resistance mutants were $\sim$ 10-fold attenuated or more in their ability to generate the INH-NADH adduct under these G/GOx conditions, including W107R (2.22 $\pm$ 0.13 μM), H108E (0.96 $\pm$ 0.11 μM), W300G (2.00 $\pm$ 0.16 μM), and S315R (1.21 $\pm$ 1.00 μM) when compared to WT KatG (18.61 $\pm$ 3.86 μM).

Increasing the total amount of hydrogen peroxide to 400 μM administered over 3.3 h led to an increase in the attenuation of INH-NADH adduct formation [Figs. 5 and S2(D)] for the vast majority of the mutants, with seven INH-resistance conferring mutants (S315G, S315I, W328G, Y337C, R463L,

L587M, and D735N) and the one lab mutant W321F classified as Category I, a total of 14 INH-resistance mutants (D63E, R104L, W107R, H108E/Q, N138D/S, Y229F, T262R, T275P, W300G, S315N/R/T, and A350S) and seven lab mutants (W107F, M255C/I/Y, T275V, W328F, and R418L) falling under Category II, and no mutant classified as Category III. Importantly, administering this amount (400 μM ; Fig. 2, step F; H_2O_2 “high-flux”) of hydrogen peroxide over a shorter 1 h time period led to a significant increase in the Category II classifications [Figs. 5 and S2(C)]. Under these conditions, no Category III mutants were identified, only two mutants were classified as Category I (Y337C and R463L), and the rest (29 out of 31) fell under the Category II heading, 10 of which were $\sim$ 10-fold or more attenuated than WT KatG.

The above data unequivocally demonstrates a strong dependence between the amount of hydrogen peroxide administered (in terms of flux), and the formation of the INH-NADH adduct. This does not necessarily imply, however, that a Compound I pathway is invoked, particularly as high peroxide concentrations can lead to the formation of oxyferrous KatG in mutants that lack catalase activity.^{18,19} To investigate whether oxyferrous KatG is a competent oxidant for catalyzing the formation of the INH-NADH adduct, the assay conditions were altered by reacting the resting enzyme with superoxide (Fig. 2, step F; “ O_2^- ”) delivered by the enzymatic system xanthine/xanthine oxidase (X/XO).^{27,53} Under these conditions [Table VI, Figs. 5 and S2(F)], six Category I INH-resistance mutants (D63E, S315I, Y337C, A350S, R463L, G629S, and D735N) were classified, with the remaining 17 resistance mutations and all eight lab mutants (W107F, M255C/I/Y, T275V, W321F, W328F, and R418L) falling under the resistance-significant Category II (no Category III mutants identified). The interpretation of these results may be complicated by the fact that xanthine oxidase may be oxidizing NADH in the reaction mixture. However, we obtained similar results here for the X/XO system as were observed in the previous limited study that used SOTS-1 as the superoxide generating system.¹⁴ To preclude a minor amount of H_2O_2 produced by the X/XO enzymatic system as a contributing factor in the INH-NADH adduct formation studies, and to rule out the possibility that KatG mutants that exhibit attenuated levels of catalase activity could lead to the formation of intermediates different from those produced in the wild-type enzyme, the addition of exogenously added catalase to mutants which lacked native catalase activity was also performed (Table VI). However, no significant changes to the overall trends or classifications were observed. Furthermore, of the Category II INH-resistance conferring mutants, nine exhibited a 10-fold or greater attenuation of adduct formation when compared to WT KatG. Thus, superoxide was found to be a

competent oxidant for the formation of the INH-NADH adduct for WT KatG and other Category I mutants, but led to significantly attenuated rates of adduct formation for Category II mutants, yielding similar results to those observed for the high flux hydrogen peroxide studies.

Discussion

Mtb clinical isolates that exhibit isoniazid resistance have been associated with point mutations, deletions, insertions, or complete loss of the KatG gene.^{5,13,54} Our previous investigations^{14,28} have chosen to focus on the KatG mutations closest to the active site, as these would most likely relate to INH-activation processes governed by either the heme cofactor (i.e., oxidation by Compounds I, II, or III), or by the Met-Tyr-Trp crosslink. However, to further understand the origins of INH-resistance, we have expanded our studies described herein to include a total of 23 point mutations in the *katG* gene spanning the N-terminal domain, the active site (both proximal and distal sides of the heme pocket), the putative substrate access channel, and the C-terminal domain (see Table I). D63E, R104L, H108Q, H108E, N138S, T262R, T275P, W300G, S315T, S315G, S315N, S315I, S315R, W328G, A350S, L587M, and G629S have been classified as single mutations within *Mtb* which give rise to isoniazid resistance.^{13,14,54–59} W107R, N138D, Y229F, Y337C, and D735N are listed as isoniazid-resistance conferring mutations⁶⁰; however, the presence of other mutations within *Mtb* is not known for these KatG mutants. R463L has been listed both as a single site mutation and as a polymorphism in the *katG* gene of strains in certain populations that contain other mutations responsible for INH resistance.^{61–64} Although the isoniazid-resistance conferring mutants were selected to provide for a direct causality between mutation and resistance-associated phenotype, it is important to note that the occurrence of some of these point mutations in KatG may be associated with other biological traits that may influence interpretation of the results presented herein.

Using a combination of biochemical assays and enzymology, we have examined the catalase and peroxidase activities of each mutant, identified whether the Met-Tyr-Trp crosslink is present in each case, and quantified their ability to catalyze the formation of the putative mycobactericidal INH-NADH adduct in the presence of oxidants such as superoxide, organic hydroperoxides (*t*-BuOOH), and varying fluxes of hydrogen peroxide. Regarding the latter INH-NADH adduct formation reactions, several assumptions based on previous findings have been made to allow for a simplified analysis of the data. Foremost, Magliozzo and coworkers^{18,65} have shown that the reduction of Compound I is accelerated several fold in the presence of isoniazid, with formation of the

ferric state occurring in the absence of an identifiable Compound II intermediate. We, therefore, assume that our Compound I reaction pathway follows a similar trajectory in which isoniazid accelerates the return of the enzyme to a ferric state, at which point the catalytic cycle is free to re-commence. Second, our pulse radiolysis study²⁸ suggested that formation of a catalytically competent Compound III intermediate is rate-limiting when compared to its subsequent reaction with isoniazid. Finally, we assume that the Compound III intermediate is unable to further react with H₂O₂, leading to a catalytically inactive state in the case of resistance-conferring mutants.¹⁴ This latter assumption is necessary given that hydrogen peroxide forms as a result of the spontaneous disproportionation of superoxide produced by slow-fluxes of xanthine/xanthine oxidase.

In general, the more comprehensive study here of resistance-conferring mutations supports our previous conclusion that there is not a correlation between catalase or peroxidase activities and isoniazid resistance in KatG.^{14,28} Interestingly, virulence of drug-resistant TB strains was thought to be related to the catalase activity of KatG as this function is to protect the mycobacterium from oxidizing species, notably hydrogen peroxide, produced by neutrophils of the infected host.⁶⁶ A bacterium that possesses a relatively high catalase activity is, therefore, believed to be more virulent because it is able to overcome the host's defense mechanism. It was postulated that since the KatG(S315T) mutant possesses a high level of catalase activity, strains containing it were able to grow in a host more rapidly than strains containing other mutants, and at levels near that of WT KatG.⁶⁶ This is supported by the fact that, except for the possible polymorphism KatG(R463L), KatG(S315T) is the most commonly occurring resistance mutation, being found in ~40% of INH-resistant clinical isolates.³ The findings of this work showed at least four other resistance mutations (D63E, Y337C, A350S, and R463L) that possess higher catalase activity than S315T. This suggests that while the catalase activity of KatG may play a role in virulence, it is likely not the only factor related to it. The ability of some KatG mutants to consume superoxide¹⁴ (as an oxidant) to a greater extent than others would also provide added protection to those strains from the harmful oxidizing environment produced by neutrophils.

We also explored the correlation between the presence of the Met-Tyr-Trp crosslink and catalase activity. Indeed, in all mutants examined which possess catalase activity approaching that of the wild-type enzyme, the presence of the crosslink was observed. However, not all crosslink containing mutants exhibited catalase activity, consistent with previous observations that an intact Met-Tyr-Trp

crosslink is required, but not solely responsible, for catalase activity.^{19,22} It has been suggested that the elaborate hydrogen-bonding network within the KatG active site, involving the crosslink and supported by the LL1 loop as well as helices E and F, is also a necessity for catalase activity given the inherent need for precise (de)protonation events involved in the disproportionation of two molecules of hydrogen peroxide into water and molecular oxygen.^{40,41,67,68} We have hypothesized that the crosslink confers catalase activity via a “ferry-avoidance” mechanism which circumvents the catalase-inactivating Compound II (iron-oxo) intermediate that may arise during peroxidase activity, which is the other inherent function of KatG.^{19,22} More recently, however, Magliozzo and coworkers have posited an elegant mechanism for catalase activity in KatG.^{42,43} In this novel catalase reaction pathway, formation of a radical on the Met-Tyr-Trp crosslink (specifically Y229) is then followed by the transfer of an electron from oxyferrous KatG, leading to the release of dioxygen and a return to the resting form of the enzyme. Although not specifically intended to address the mechanistic questions surrounding the origins of catalase activity in the bifunctional catalase-peroxidases, our results here provide further support for the necessity of both the Met-Tyr-Trp crosslink and the extended H-bonding network for maintaining catalase activity in KatG.

As neither the catalase nor peroxidase activities directly correlated with INH resistance in the mutations examined within this study, the ability of each mutant to form the putative mycobactericidal INH-NADH adduct was investigated as a function of oxidant. The results presented herein suggest that while residue location may play a significant role in conferring isoniazid resistance via attenuated adduct formation, the underlying resistance mechanisms for each mutation that govern INH activation are likely different. This is most easily seen by grouping the mutations into different categories based on location. The mutations at residues 104, 107, 108, and 138 can be classified as active site mutations given their proximity to the heme cofactor, and interestingly all of these active site mutations exhibited attenuated INH-NADH adduct formation compared to WT KatG regardless of the oxidant employed. These mutations may affect either activation of the heme group by hydrogen peroxide, binding of superoxide, or reduction of the heme intermediates responsible for INH activation. As was previously observed for KatG(R104L) and KatG(H108Q), these mutations may also lead to rapid heme bleaching in the absence of a reducing substrate.¹⁴

As one well-studied example of INH-resistance in KatG, mutations of the native serine residue at position 315 may cause conformational changes in the substrate access channel leading to the inability of the enzyme to bind INH under physiologically rel-

evant conditions. Specifically, the serine-to-threonine mutation of KatG(S315T) has been shown to constrict the substrate access channel from 6 Å in the WT enzyme to 4.7 Å in the mutant.¹⁷ The added steric hindrance of the additional methyl group in threonine results in a substantial increase in K_d (INH) of 400 μ M for the mutant, compared to 2.5 μ M for WT KatG.⁶⁹ Remarkably, however, despite possessing an even more sterically-constraining Ser→Ile mutation, the mutant KatG(S315I) does not significantly attenuate (<2-fold) INH-NADH adduct formation under any conditions studied. Although these results suggest that amino acid size may not necessarily dictate substrate channel accessibility, they could also be interpreted to suggest that adduct formation may not be the only factor to consider when examining the underlying mechanisms of INH-resistance in KatG. In addition, this mutant possesses significant catalase and peroxidase activities, and as such, loss of enzyme function cannot be correlated with INH-resistance. Further studies of the KatG(S315I) mutant, including structural and spectroscopic investigations, will be necessary to determine the factors that contribute to isoniazid resistance in this mutation.

Magliozzo and coworkers have recently studied the mutant KatG(S315G).⁵² Although the Ser→Gly mutation was found not to disrupt INH binding ($K_d \sim 1 \mu$ M) nor alter catalase or peroxidase activities to a great extent, double-mixing stopped-flow experiments revealed that INH was a poor substrate for reducing the Compound I intermediate of KatG(S315G), and it was hypothesized that INH resistance in this mutant arose upon the shunting of catalytic intermediates normally responsible for INH activation toward other reaction pathways that did not lead to substrate oxidation. This bypass mechanism⁵² would allow for both catalase and peroxidase activities, but could be the underlying cause of reduced levels of INH-NADH adduct formation. Generally, our results here on KatG(S315G) with *t*-BuOOH and H₂O₂ (G/GOx), which show only slightly (2-fold or less) attenuated levels of INH-NADH adduct formation, are in agreement with those previously observed by Magliozzo and coworkers for this mutant,⁵² who also used the enzymatic H₂O₂-generating system. Of importance, however, is that when utilizing the superoxide-generating system xanthine/xanthine oxidase in this study, no detectable amount of the INH-NADH adduct was formed by KatG(S315G) (0 μ M), compared to 32 μ M for WT KatG. Thus, we hypothesize that a superoxide-dependent pathway for INH activation may be one possible explanation for the INH-resistance observed for *Mtb* isolates that harbor this mutation. We surmise that this mutation completely disrupts the ability of the enzyme to utilize superoxide as an oxidant involved in INH activation, although the molecular details of this disruption are unclear at this

time. One possibility is that oxyferrous KatG(S315G) is unable to oxidize INH, similar to our observations with KatG(S315T),^{14,28} for which we suggested that the underlying cause of the observed results may be mutation-induced (1) disruption of the active site hydrogen-bonding network, (2) differences in heme or side-chain redox potential, (3) increased stability of the oxyferrous species rendering it inactive, or (4) reduced affinity for INH, although this latter possibility is not supported by the INH-binding studies for KatG(S315G).⁵² It may also be that the bypass mechanism suggested by Magliozzo and coworkers⁵² for Compounds I/II circumvents oxyferrous KatG(S315G) reactivity, and further studies of the oxyferrous species in this mutant will be necessary to elucidate its lack of reactivity.

Resistance-conferring mutations further away from the heme active site also exhibited interesting reactivity patterns. Mutations at positions 262, 275, 328, 337, and 350 are located on the proximal side of the heme group, and may affect charge transfer pathways or lead to disruptions in loop regions that support vital hydrogen bonding networks, INH-binding, or govern protein–protein interactions. The status of KatG(R463L) as a resistance mutation is the subject of some debate as it is a commonly found mutation and has a relatively low minimum inhibitory concentration (MIC, Table SII).⁶² The high reactivity of this mutant towards isoniazid in the presence of all oxidants provides further evidence against the status of KatG(R463L) as a resistance mutation, and more likely as a polymorphism. However, this conclusion is predicated upon the assumption that resistance is correlated with attenuated formation of the INH-NADH adduct, which may not be the only possible resistance mechanism associated with KatG (see below). Mutations at positions 587, 629, and 735 all occur in the C terminal domain of KatG,⁷⁰ the origin of which has been ascribed to a gene duplication event.¹⁶ Interestingly, despite being considered an “inactive” domain due to its inability to bind a heme cofactor, the C-terminal domain has been shown to be necessary for catalase activity.⁷¹ These residues may play a role in governing KatG dimerization (KatG is a functional homodimer^{35,71,72}) or in protein stability.⁷³ Disruption of hydrogen-bonding networks or electron-transfer pathways may also occur as a result of these mutations, but specific structural studies addressing these issues are still lacking.

Our results for the mutations at positions 337, 350, 463, 587, 639, and 735 yield near wild-type levels (2-fold attenuated or less) of the INH-NADH adduct formed for the physiological oxidants superoxide and hydrogen peroxide, and are evidence that supports a possible second mechanism for INH-resistance that is not correlated with the formation of the adduct. Given that no physiological peroxidase substrate of KatG has been identified to-date, nor

whether there are any other protein or cofactor interactions known, it is possible that a resistance mechanism related to INH, but not the formation of the INH-NADH adduct itself, exists and is mutation-specific. For example, INH (or one of its oxidation products) could function as a competitive inhibitor of the naturally occurring substrate, INH activation could lead to another type of adduct being formed that is not NADH-based, or perhaps activated INH could lead to protein-INH adducts that interfere with downstream function. Although these conjectures are speculative, further investigations into these or other possibilities⁴ must be considered in light of the data presented herein. In addition, further elucidation of the mechanism of INH-NADH adduct formation in resistance mutation KatGs is also warranted. The key heme intermediate(s) involved appear to be oxyferrous KatG and Compound I, with the possibility of mutation-specific mechanisms involving superoxide and hydrogen peroxide that correlate, in part, to isoniazid resistance. Interestingly, we have identified a mutant, KatG(S315G), that exhibits a clear distinction between these two oxidants in its ability to generate the INH-NADH adduct, and provides direct evidence for a bifurcated pathway in these mutants in which isoniazid is able to reach the active site, but is unable to be oxidized by one or several key heme intermediates.

In conclusion, residue location plays a critical role in determining INH-resistance mechanisms associated with INH activation; however, different mutations at the same location can produce vastly different reactivities that are oxidant-specific. It is likely that a combination of several factors leads to isoniazid resistance, including oxidant type, isoniazid and/or NADH binding, protein/heme stability, and mutation induced alterations of the hydrogen bonding networks. The results of this study represent a first step towards a more comprehensive informatics-styled interdisciplinary study in which isoniazid-resistance is fully correlated to three variables: KatG activity, strain fitness, and TB transmissibility (virulence). Thus, by knowing any one variable, specific treatments may be tailored to the patient harboring a defined *Mtb* clinical isolate, with the ultimate goal of if an understanding of the individual causes of resistance in each mutant can be elucidated, novel drug therapies can be designed that are strain-specific to strengthen control of drug-resistant TB.

Materials and Methods

General

The QuikChange XL[®] site-directed mutagenesis kit and XL-10 Gold and BL-21(DE3)pLysS competent *E. coli* cells were purchased from Stratagene. Buffer salts and acetonitrile (HPLC grade) were purchased

from Fisher Scientific. All other reagents and biochemicals, unless otherwise specified, were purchased from commercial sources and were of reagent or molecular-biology grade.

Plasmid preparation, protein expression, and purification

Recombinant WT KatG, resistance mutants^{3,5,12,13,60} R104L, H108Q, Y229F, S315T, and lab mutants M255C, M255I, and R418L were overexpressed in *E. coli* as previously described.^{14,22,28} For the resistance mutants^{3,5,12,13,60} D63E, W107R, H108E, N138D, N138S, T262R, T275P, W300G, S315G, S315I, S315N, S315R, W328G, Y337C, A350S, R463L, G629S, and D735N, and lab mutants W107F, T275V, W321F, and W328F, mutagenesis was performed per manufacturer's protocols using the QuikChange XL site-directed mutagenesis kit from Stratagene using the primers listed in the Electronic Supporting Information (Table SI).

Parental DNA was digested with DPN1 (New England Biolabs) and the mutated plasmids were transformed into XL-10 Gold *E. coli* cells and grown on LB agar plates containing 100 mg L⁻¹ ampicillin. Cultures containing LB and 100 mg L⁻¹ ampicillin were inoculated with individual colonies, grown 16 h, and centrifuged. DNA was extracted and purified from the bacterial pellet using either the Promega Wizard Plus Minipreps DNA Purification system or the Qiagen QiaPrep Spin Miniprep kit. Sequencing of double-stranded plasmid DNA by the Sanger method was used to confirm the desired nucleotide substitution and the absence of secondary mutations (University of Michigan Sequencing Core Facility, Ann Arbor, MI). Following confirmation of the correct DNA sequence, the mutated KatG plasmid was transformed into BL-21(DE3)pLysS *E. coli* cells, and protein overexpression of each mutant was performed in a protocol identical to that previously used for WT KatG.^{14,22,28}

Enzyme assays

All measurements were performed with minor modification from published procedure.^{14,22,28} Specifically, measurements were performed in octiplet using a SpectraMax Plus384 UV-visible plate reader equipped with 96-well plates. Assays were carried out at 25°C in 100 mM NaP_i buffer (pH 7.5) containing 5 μM EDTA (200 μL total volume). Catalase activity was measured spectrophotometrically by following the decrease over 60 s (linear least-squares fittings) of the hydrogen peroxide concentration (1.5, 5, 10, 15, 30, and 50 mM) at 240 nm ($\epsilon_{240} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$)⁷⁴ with a fixed KatG concentration of 25 nM. Peroxidase activity was measured by following the increase (linear least-squares fittings) in absorb-

ance for 1.0 mM ABTS ($\epsilon_{405} = 36.8 \text{ mM}^{-1} \text{ cm}^{-1}$)⁷⁵ in the presence of *tert*-butyl hydroperoxide (1, 10, 25, 100, 200, and 500 mM) over 60 s at a fixed enzyme concentration of 1 μM. Kinetic parameters (K_m , V_{max}) were obtained from non-linear regression (least squares fitting) of Michaelis-Menton plots using the GraphPad Prism kinetics software package (<http://www.graphpad.com/>).

INH-NADH adduct formation

INH-NADH adduct formation assays were performed in triplicate at 37°C. The reaction sample (0.5 mL total volume) contained 100 mM NaP_i (pH 7.5), 100 μM EDTA, 6 μM WT or mutant KatG, 200 μM INH, 240 μM NADH, and the following oxidants: 400 μM *t*-BuOOH (1 h incubation), 400 μM H₂O₂ (delivered as either 16.7 mM glucose and 66.6 U mL⁻¹ glucose oxidase over 1 h, or 5 mM glucose and 20 U mL⁻¹ glucose oxidase over 3.3 h), 120 μM H₂O₂ (5 mM glucose and 20 U mL⁻¹ glucose oxidase over 1 h), or 225 μM O₂⁻ (500 μM xanthine and 0.012 U mL⁻¹ xanthine oxidase, 1 h incubation). After incubation, 10 μL of a 10 mg mL⁻¹ trypsin solution, corresponding to a final concentration of 0.84 μM, was added to halt all reactions.

HPLC analyses were performed using 350 μL injection volumes on a Waters 2796 High Pressure Liquid Chromatograph equipped with a Waters 2996 Photodiode Array detector using a reversed-phase C₁₈ column (Alltech, 10 μm particle size, 100 Å, 4.6 mm i.d. × 250 mm, Nucleosil). A linear gradient was applied from 2.5 to 15% acetonitrile in 70 mM ammonium acetate solution (pH 4.5) over 15 min (flow-rate: 1 mL min⁻¹; detection: 210–400 nm). INH-NADH adducts were identified by their characteristic UV-visible absorption spectrum and characteristic A_{260}/A_{326} ratio as described in the literature. Under these conditions, three peaks were identified with retention times between 10 and 15 min, which correspond to the INH-NADH adduct. In a previous study that used a different elution method,¹⁴ four peaks were seen which corresponded to the four possible isomers of the adduct.^{44,76,77} In the method described here, two of the isomers co-elute together, accounting for the difference in observable peaks. The total integration under all three peaks was calculated in units of μV*s, and converted to INH-NADH adduct concentration using a previously known calibration standard.¹⁴

Tryptic digests and HPLC/MS analysis

Typically, a 50 μM solution (400 μL total) of KatG in 100 mM NaP_i (pH 7.5) was incubated with 8 μg (200:1 protein/protease ratio) of sequencing grade modified trypsin (Promega) for 3 h at 37°C. Following proteolytic digestion, the peptide fragments were separated by HPLC as previously described,^{19,22} and

the presence of the Met-Tyr-Trp crosslinked peptide fragment (CLPF) was confirmed by both its retention time and its characteristic UV-visible features.

The following mutations are found within or immediately adjacent to the CLPF, rendering the above HPLC method inaccurate for identifying its presence by altering either the trypsin digestion sites or retention time: KatG(M255Y), KatG(H108E), KatG(T262R), and KatG(T275V). For KatG(M255Y), fractions (0.5 mL) were collected and concentrated (SpeedVac), and mass spectrometry analyses of these fractions were performed by nanoLC/MS/MS on a linear ion trap Orbitrap hybrid tandem mass spectrometer (LTQ Orbitrap, Thermo) in a data dependent fashion, precursor ions were measured in the Orbitrap, multiply charged precursor ions were selected for CID experiments, peptide fragments were also measured in the Orbitrap. For KatG(H108E), KatG(T262R), and KatG(T275V), the tryptic digests were subjected to LC/MS/MS analysis on a QoaTOF mass spectrometer (QSTAR Pulsar, MDS Sciex) similarly in a data dependent fashion, 1 s mass surveys were followed by 3 s CID experiments on computer-selected multiply charged ions. Collision energy was adjusted to the charge and m/z of the precursor ion selected. For the LC/MS experiments, samples were injected onto reversed phase nanoHPLC columns, the flowrate was ~ 300 nL min⁻¹, and a linear gradient was developed from 5% B to 60% B over 35 min, with solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile.

Electronic supplementary material

Mutagenic primers used in the site-directed mutagenesis of WT KatG (Table SI), minimal inhibitory concentration (MIC) data for TB strains harboring WT and selected KatG mutations that confer INH resistance (Table SII), mass spectrometric characterization of the Tyr-Tyr-Trp crosslink in KatG(M255Y) (Fig. S1), and the comparison between WT KatG and mutants for the formation of the INH-NADH adduct using various oxidants including air-only (background control), 400 μ M *t*-BuOOH, 400 μ M H₂O₂ delivered over 1 and 3.3 h (via glucose/glucose oxidase), 120 μ M H₂O₂ delivered over 1 h, and 225 μ M O₂⁻ (xanthine/xanthine oxidase) (Fig. S2).

Acknowledgments

The authors acknowledge the Colorado State University for supplying the KatG-encoding plasmid pMRLB11. Support for this research was provided by the North Carolina State University (R.A.G.) and the Department of Education Graduate Assistance in Areas of National Need (GAANN) Molecular Biotechnology Fellowship (C.E.C.). KFM and SPSC were supported by NIH grants NCRR P41RR001614 and RR012961 to the Bio-Organic Biomedical Mass Spec-

trometry Resource at UCSF (Director A. L. Burlingame).

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