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Superoxide Reactivity of KatG: Insights into Isoniazid Resistance Pathways in TB

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Tuberculosis (TB) is one of the leading causes of death due to a single disease, claiming 2−3 million lives annually.[1] Efforts to control TB infection have been hampered by the rise of multiple-drug-resistant strains, which include resistance to isoniazid (INH), the most widely prescribed antibiotic used to treat TB. While it is now generally accepted that INH is a pro-drug that is activated by the catalase peroxidase KatG, the specifics of this activation process have to date proven to be elusive, and further study is necessary for rational drug design of antibiotics to combat TB.

To gain insight into the mechanism of INH activation by KatG and to subsequently understand resistance pathways derived from single point mutations in KatG, we have employed pulse radiolysis as the means to generate oxyferrous KatG and study its reactivity with INH, as roles for both oxyferrous KatG and superoxide in INH activation have been previously suggested.[2−5]

Pulse radiolysis (PR) experiments were performed as previously described.[6] Upon radiolysis of a solution containing only WT KatG,[7] a new species is formed [$k_1 = (4.47 \pm 0.91) \times 10^5$ M^{-1} s^{-1}], whose absorption spectrum (415 (Soret), 545, 580 nm) is consistent with compound III "oxyferrous" KatG.[8,9] However, in the presence of a saturating amount of INH (200 μM),[10] a different intermediate is observed whose UV−visible spectral features match those previously seen for compound II (410 (Soret), 540, 575 nm).[9] Formation of the compound II intermediate occurs with a rate [$k_1 = (4.43 \pm 0.69) \times 10^5$ M^{-1} s^{-1}] comparable to that seen for compound III, suggesting that compound III forms initially, but is rate-limiting when compared to the subsequent (and presumably faster) reaction with INH yielding compound II.

Radiolysis of a solution sample of the mutant KatG(S315T) (see Supporting Information), whose Ser → Thr mutation leads to INH resistance in *Mycobacterium tuberculosis* (*Mtb*) clinical isolates,[11] in the absence of isoniazid also led to the formation [$k_1 = (4.51 \pm 1.38) \times 10^5$ M^{-1} s^{-1}] of compound III, with an equivalent rate to that found for WT KatG, suggesting that the S315T mutation does not significantly alter the binding of small molecule diatomic substrates such as superoxide. In contrast to the results observed for WT enzyme, KatG(S315T) in the presence of isoniazid (2.25 mM) still forms [$k_1 = (4.72 \pm 0.99) \times 10^5$ M^{-1} s^{-1}] compound III and not compound II, indicating that INH is not capable of donating an electron to "oxyferrous" KatG(S315T), thereby leaving the O−O bond intact.

On the basis of the results obtained from these PR experiments and through modification of previously established mechanisms for the general function of catalase peroxidases,[12] we propose the following mechanism for the in vitro superoxide-dependent oxidation of isoniazid by *Mtb* KatG (Scheme 1). First, superoxide binds to the resting ferric KatG (step A), forming an oxyferrous compound III intermediate (in vivo it is also conceivable that dioxygen binds to ferrous KatG). Oxidation of isoniazid by compound III, and subsequent O−O bond cleavage and protonation of the leaving oxo moiety, yields compound II (step B), which ultimately returns to the resting state upon further reaction with isoniazid (step C). For KatG(S315T), however, the oxidation of INH does not occur by compound III, thereby resulting in an intermediate incapable of activating isoniazid.

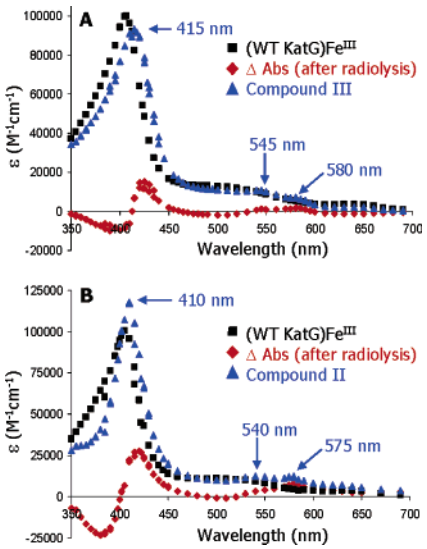

Figure 1. UV−visible spectra of the intermediates obtained after pulse radiolysis of WT KatG in the (A) absence and (B) presence of isoniazid.

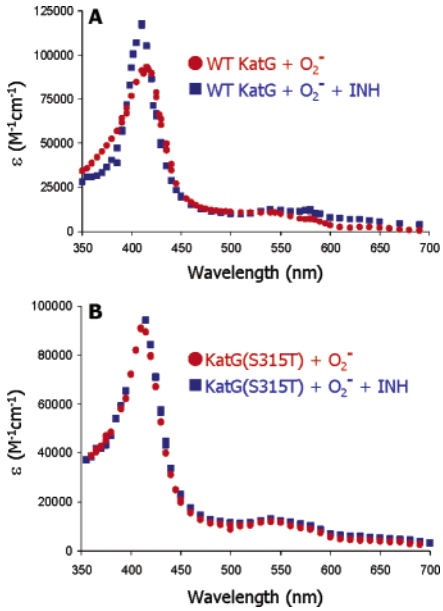

Figure 2. Overlaid UV−visible spectra of the intermediates observed in the radiolysis reaction of (A) WT KatG and (B) KatG(S315T) in the (■) presence and (●) absence of isoniazid (INH).

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Scheme 1. Proposed Mechanism for INH Activation by KatG.

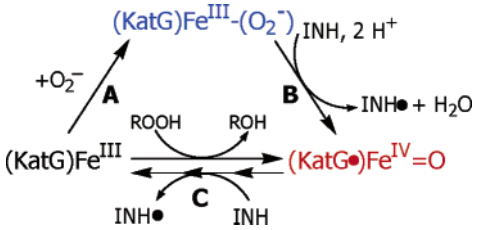

Table 1. Rates of Formation of the InhA-Inhibitor (INH−NADH Adduct) by WT KatG and KatG(S315T) Using Various Oxidants[a]

	WT KatG ($v \pm$ SD) $\times 10^4$		KatG(S315T) ($v \pm$ SD) $\times 10^4$	
[INH]	200 μM	2.25 mM	200 μM	2.25 mM
air	7.18 ± 1.25	7.41 ± 0.98	0.74 ± 0.39	0.82 ± 0.28
superoxide[b]	9.22 ± 1.10	9.17 ± 1.07	n.d.[c]	0.13 ± 0.11
t-BuOOH	20.5 ± 1.13	20.14 ± 1.34	10.15 ± 0.19	10.42 ± 0.22

[a] Velocities (three measurements ± SD) reported are in mol INH−NADH formed·mol enzyme^{-1}·s^{-1}. Reaction conditions: 6 μM KatG, INH, 240 μM NADH, 50 mM sodium−phosphate (pH 7.5), 400 μM *tert*-butylhydroperoxide (*t*-BuOOH) or 1 mM SOTS-1 (equivalent to 267 μM O_2^-), 1 h incubation at 37 °C. [b] Generated as SOTS-1. [c] n.d. = none detected (minimally detectable rate estimated to be ~0.1 mol INH−NADH formed·mol enzyme^{-1}·s^{-1}). No INH−NADH adduct formation was detected for WT or KatG(S315T) (2.25 mM INH) when H_2O_2 (400 μM) was employed, presumably due to $k_{catalase}$ (ref 10) $\gg k_{INH-NADH}$ (~10^7 faster).

The results of the PR study suggest that the origin of isoniazid resistance in KatG(S315T) is the inability of the oxyferrous intermediate to accept an electron from INH, thereby preventing isoniazid oxidation ("activation"), presumably to the isonicotinyl acyl radical. It is this radical which is believed to couple to NADH, forming a covalent adduct which has been found to be a potent inhibitor of InhA, an enoyl reductase essential for mycolic acid biosynthesis.[13,14] To test this hypothesis that KatG(S315T) will be unable to catalyze the formation of the INH−NADH adduct utilizing superoxide as an oxidant, we have undertaken kinetic studies aimed at following the formation of the INH−NADH adduct (InhA−inhibitor complex).

The INH−NADH adducts were identified by HPLC as described elsewhere,[15] with only slight modification.[16] In the absence of an exogenously added oxidant,[17] the rate of INH−NADH formation as catalyzed by WT KatG was found to be ~10-fold higher than that for KatG(S315T) (Table 1), representing the first observed correlation between suppressed INH−NADH adduct formation and INH resistance by a mutant KatG. This finding supports the theory[13,14] that InhA inhibition is the primary means by which KatG-activated INH confers antitubercular activity. Even more disparate rates of adduct formation between WT KatG and KatG(S315T) are observed when superoxide (as SOTS-1)[18] is employed as an oxidant: WT KatG-catalyzed INH−NADH adduct formation is slightly accelerated (over air), whereas that for KatG(S315T) is completely abolished (WT/S315T ≈ 90-fold). This is noteworthy, given that the MIC for KatG(S315T) is approximately 20−200-fold higher than that for WT KatG,[11] with both results (oxidation by air and exogenous superoxide) being consistent with our proposed mechanism (Scheme 1). By circumventing the oxyferrous pathway through use of *t*-BuOOH, we see that the rate of adduct formation is greatly accelerated for not only WT KatG, but for KatG(S315T) as well, with a modest 2-fold difference in overall rate between them, also consistent with our proposed mechanism.

Other plausible explanations for the reduced reactivity of KatG-(S315T) exist, including mutation-induced (1) disruption of the active site hydrogen-bonding network,[19] (2) differences in heme or side-chain redox potential,[20] or (3) reduced affinity for INH,[10] although the latter possibility is not supported by the [INH]-dependent data presented (Table 1). In conclusion, our work here demonstrates the first directly observed difference in the reactivity of the oxyferrous intermediate with INH between WT KatG and KatG(S315T), thus providing the basis for a mechanistic divergence between these two enzymes that correlates to INH resistance.

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Supporting Information Available: UV−visible spectra of the intermediates obtained after PR of KatG(S315T) (±INH), as well as the overexpression and purification protocol for KatG (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Reaction conditions were as follows: 6.25−75 mM KatG in 50 mM sodium phosphate (pH 7.5), 10 mM sodium formate, 5 mM EDTA at room temperature. Radiolysis pulse widths varied between 80 and 200 ns.
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(16) HPLC was performed using an Alltech Nucleosil (C18) column (4.6 × 250 mm, 100 Å, 10 mm) employing a linear gradient from 0 to 37.5% (v/v) acetonitrile in 70 mM sodium acetate (pH 4.5) buffer. Products were monitored at 260 and 328 nm, and initial velocities were determined from integration of the peaks identified as INH−NADH adducts (A328/A260 = 3.3−3.55).
(17) Two possibilities exist for the formation of oxyferrous KatG from INH and air, which are consistent with the mechanism proposed in Scheme 1. (i) Reduction of KatG by contaminating N_2H_4 present in aged INH solutions followed by O_2 binding, 5 or (ii) trace metals have been previously shown to catalyze the autoxidation of INH (or hydrazines in general), yielding superoxide.
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