Isoniazid Oxidation by *Mycobacterium tuberculosis* KatG: A Role for Superoxide Which Correlates with Isoniazid Susceptibility

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Received July 21, 1999

Mycobacterium tuberculosis (Mtb) is one of the most prolific killers among the infectious diseases causing 2−3 million deaths annually.[1] Isoniazid (INH), an antibiotic used in the treatment of tuberculosis, is a prodrug requiring activation by the Mtb enzyme KatG.[2,3] The active form of INH and activation mechanism(s) are not conclusively known. Increasing INH resistance[4−6] underscores the need to understand its mechanism in order to design more effective antibiotics. KatG is a heme protein which possesses catalase-peroxidase and Mn^{2+}-dependent peroxidase activities[3,7−9] and which can utilize peroxides to oxidize INH, presumably via a compound I/II pathway.[10] KatG also oxidizes INH in the absence of peroxides[3,10,11] suggesting oxidation can proceed by an alternative mechanism. In addition, a Ser315 to Thr point mutation in KatG confers INH resistance to Mtb without significantly affecting catalase-peroxidase activity,[11,12] arguing against the physiologic relevance of peroxidative activity for INH activation. Others have suggested INH oxidation occurs via an oxyferrous form of KatG reminiscent of the cytochrome P_{450} oxygenase intermediate.[10] In this work, we define a role for superoxide in KatG-mediated INH oxidation, a hypothesis predicated on reports of superoxide generation during INH oxidation by horseradish peroxidase, myeloperoxidase, and extracts of Mtb H37Ra.[13−16] We also demonstrate that KatG(S315T) exhibits reduced superoxide-dependent INH oxidation rates. In contrast, INH oxidation occurring via peroxidative routes does not correlate with INH resistance conferred by the S315T mutation.

INH oxidation was monitored by HPLC,[17] and products were identified with the use of external standards and mass spectrometry.[18] INH oxidation is KatG-dependent (Table 1). A catalytic quantity of superoxide dismutase (SOD) abolished INH turnover providing evidence of superoxide involvement in KatG-mediated INH oxidation. Addition of a superoxide-generating system consisting of xanthine and xanthine oxidase[19] to KatG plus INH increased the oxidation rate 5-fold, further supporting a role for superoxide. Controls lacking KatG showed little INH turnover, demonstrating a requirement for KatG in this superoxide-dependent reaction. Addition of 1 equiv of the superoxide scavenger cytochrome *c* (Fe^{3+}) decreased INH oxidation, suggesting a competition between cytochrome *c* and KatG for superoxide. Addition of 5 equiv of cytochrome *c* completely prevented INH oxidation (data not shown).

KatG-dependent INH oxidation requires dioxygen both in vitro[10,20] and in vivo.[21,22] A role for dioxygen was described previously when oxyferrous KatG was hypothesized to oxidize

Table 1. INH and NH Oxidation by KatG and KatG(S315T)

	INH ($v \pm$ S.D.) $\times 10^4$ (s^{-1})	**NH** ($v \pm$ S.D.) $\times 10^4$ (s^{-1})
INH only (nonenzymatic control)	2 ± 1	−
+ xanthine/xanthine oxidase (no enzyme)	5 ± 2	−
+ KatG	22 ± 2	19 ± 4
+ KatG + SOD	1 ± 1	1 ± 1
+ KatG + xanthine/xanthine oxidase	113 ± 10	128 ± 19
+ KatG + EDTA	5 ± 1	−
+ KatG + EDTA + xanthine/xanthine oxidase	112 ± 15	124 ± 15
+ KatG + *t*-bOOH[a]	90 ± 11	123 ± 11
+ KatG + *t*-bOOH + SOD	103 ± 9	118 ± 3
+ KatG + cytochrome *c*	8 ± 1	−
+ KatG, anaerobic	3 ± 2	−
+ KatG(S315T)	8 ± 1	5 ± 1
+ KatG(S315T) + SOD	1 ± 1	1 ± 1
+ KatG(S315T) + xanthine/xanthine oxidase	10 ± 1	17 ± 2
+ KatG(S315T) + EDTA	2 ± 1	−
+ KatG(S315T) + EDTA + xanthine/xanthine oxidase	13 ± 2	5 ± 1
+ KatG(S315T) + *t*-bOOH	82 ± 9	94 ± 6
+ KatG(S315T) + *t*-bOOH + SOD	67 ± 6	73 ± 6
+ KatG(S315T), anaerobic	2 ± 1	−

[a] *t*-bOOH is *tert*-butylhydroperoxide; velocities are reported as mol of substrate oxidized·mol of enzyme^{-1}·sec^{-1}, and values are the mean ± standard deviation of three experiments. Dashes (−) represent conditions not examined. **INH** and **NH** initial concentrations were 200 μM in 50 mM sodium phosphate buffer, pH 7.5. KatG and KatG(S315T) concentrations were 6 μM. Other components were as follows: 0.1 μM SOD, 0.5 mM xanthine, 0.012 units/ml xanthine oxidase, 5 mM EDTA, 400 μM *t*-bOOH, 6 μM cytochrome *c*.

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(17) Recombinant KatG and KatG(S315T) were purified as described previously.[11] Reactions were conducted at 37 °C in 50 mM sodium phosphate buffer (pH 7.5) and samples removed at selected times. HPLC was done using a Rainin Dynamax C-18 column (4.6 × 150 mm, 100 Å, 5 μm, Varian Instruments) with a linear gradient of 5−80% acetonitrile (v/v) in 50 mM ammonium acetate (pH 7.0) over 30 min. Products were monitored at 260 nm. Initial velocities were determined by integration of isoniazid peak areas.

(18) The mass spectrometer (MAT95Q tandem, Finnigan MAT, Breman, Germany) was operated in the positive ion electrospray mode and scanned over a range of 40−400 Da. Consistent with previous reports,[3,10] KatG- and KatG(S315T)-catalyzed INH oxidation gave isonicotinic acid (major) and isonicotinamide (minor) as products. NH oxidation by both enzymes also produced the corresponding acid and amide.

(19) Superoxide undergoes spontaneous nonenzymatic dismutation forming H_2O_2 with a second order rate constant of approximately 2×10^7 M^{-1} sec^{-1} near neutral pH. Therefore, a xanthine/xanthine oxidase superoxide-generating system was utilized to provide a constant, low superoxide level. McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049−6055.

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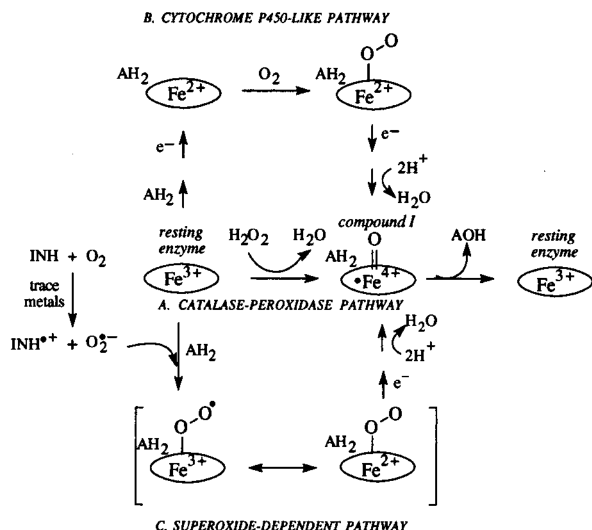
10.1021/ja992590a CCC: $18.00 © 1999 American Chemical Society
Published on Web 10/01/1999

Scheme 1. Pathways for Iron Activation in KatG and Subsequent Substrate (AH_2) Oxidation



INH.[10] The proposed mechanism involved KatG reduction by trace N_2H_4 present in aged INH solutions followed by O_2 binding to form the oxyferrous enzyme. We also find a dioxygen requirement (Table 1) and postulate it is necessary for superoxide formation. A question arises as to the origin of superoxide in solutions containing only KatG and INH. Trace metals can catalyze oxidation of hydrazines by dioxygen generating superoxide, and superoxide is formed following trace metal-catalyzed autoxidation of INH at alkaline pH.[15,23,24] Addition of EDTA to KatG plus INH substantially reduced the INH oxidation rate. Alternatively, addition of both EDTA and xanthine/xanthine oxidase to KatG plus INH restored oxidation. These results suggest a role for trace metals in producing superoxide and indicate INH oxidation proceeds in the absence of trace metals if provided with an alternate source of superoxide. Therefore, the data suggest oxyferrous KatG can be formed by binding of superoxide to the ferric enzyme (Scheme 1), bypassing the requirement for KatG reduction prior to dioxygen binding. While our results support a role for oxyferrous KatG in INH oxidation, they do not preclude INH oxidation by compound I since peroxide increased the oxidation rate 4-fold. Predictably, SOD had no effect on the rate of INH oxidation using *tert*-butylhydroperoxide as the oxidant, eliminating the possibility that peroxide disproportionation to superoxide was responsible for the rate increase. Therefore, KatG-dependent INH oxidation can proceed via at least two routes, oxyferrous or peroxidative compound I/II, depending on oxidative conditions and substrates present within the cell. In addition, oxyferrous KatG can be generated by either dioxygen binding to ferrous KatG or superoxide binding to ferric KatG.

To decipher which enzymatic activity is responsible for INH activation, we made use of KatG(S315T), a mutant found in >50% of isoniazid-resistant Mtb clinical isolates which oxidizes INH more slowly than wild-type KatG in vitro.[4−6,11,25−27] The rate of INH oxidation by KatG(S315T) was reduced compared to that by wild-type KatG (Table 1), while addition of SOD or EDTA abolished INH oxidation, equivalent to their effects with wild-type enzyme. INH oxidation by KatG(S315T) therefore can also proceed via trace metal-catalyzed superoxide generation. In contrast to wild-type KatG, xanthine/xanthine oxidase did not cause a significant increase in the INH oxidation rate, suggesting that the mutation confers an INH-resistance phenotype through reduced reactivity toward superoxide. Addition of peroxide to KatG(S315T) plus INH resulted in an oxidation rate equivalent to the wild-type enzyme, indicating a lack of correlation between KatG peroxidative activity and INH susceptibility. To further explore the role of peroxidative pathways in INH activation, the Mn^{2+}-dependent peroxidase activities of KatG and KatG(S315T) were examined (Table 2). The catalytic efficiencies for Mn^{2+} oxidation by both enzymes are nearly identical, confirming no difference in the peroxidative capabilities of the two enzymes. Taken together, these results suggest INH oxidation does not proceed via peroxidative pathways in vivo. Rather, a superoxide-dependent mechanism appears critical for KatG-mediated INH oxidation.

Table 2. Mn^{2+}-peroxidase Activity of KatG and KatG(S315T)

	k_{cat} (s^{-1})	$K_{M\,Mn^{2+}}$ (μM)	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
KatG	$(9.5 \pm 0.3) \times 10^{-2}$	4.1 ± 0.4	2.32×10^4
KatG(S315T)	$(13.3 \pm 0.5) \times 10^{-2}$	5.8 ± 0.5	2.29×10^4

Another compound useful in INH oxidation studies is nicotinic acid hydrazide (NH), which differs structurally from INH only in the position of the acylhydrazine group on the pyridine ring. Despite structural similarities, NH has a 30-fold higher minimal inhibitory concentration (MIC) than INH making it a poor antitubercular agent.[28] The KatG-dependent NH oxidation rate was nearly identical to that of INH. SOD inhibited NH oxidation, and xanthine/xanthine oxidase increased the rate by > 6-fold, suggesting a role for superoxide in NH oxidation. In light of NH's similar oxidation rate by KatG as compared with INH, the increased MIC must result from other factors. A possible explanation is provided by a report[3] demonstrating more rapid time-dependent inactivation of KatG by NH relative to INH. KatG(S315T)-dependent NH oxidation was slower than that by wild-type KatG, and xanthine/xanthine oxidase did not affect the rates, consistent with the previous trends in INH oxidation rates. Finally, NH and INH oxidation rates by KatG and KatG(S315T) plus peroxide were similar, supporting the finding that peroxidative activity does not correlate with INH susceptibility.

In conclusion, we provide evidence of a role for superoxide in KatG-dependent INH oxidation and correlate increased INH resistance conferred by the S315T mutation to a decreased rate of superoxide-dependent INH oxidation. An unresolved question involves the physiologic role of superoxide in INH activation. Mtb contains both ferric[29] and Cu,Zn[30] SOD and it is curious that superoxide participates in KatG-mediated INH oxidation in the presence of endogenous SOD. One possible explanation is that the Cu,Zn SOD and 76% of the ferric SOD are exported from the cell, presumably to act as extracellular defenses against the host respiratory burst.[30,31] Remaining intracellular SOD may therefore be overwhelmed by superoxide generated during INH oxidation. Subsequent studies are required to confirm this. Nevertheless, recent work in *M. smegmatis* suggested an in vivo role for superoxide in KatG-mediated INH activation.[32] *M. smegmatis* transformed with Mtb *katG* demonstrated increased INH susceptibility upon addition of plumbagin, a superoxide generator. Moreover, SOD effectively blocked the effect of plumbagin, further implicating superoxide in in vivo potentiation of INH toxicity.

Acknowledgment. We are grateful to Ken Johnson and Steve Naylor of the Mayo Biomedical Mass Spectrometry Facility for assistance with the mass spectrometry.

JA992590A

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