The Requirement for Manganese and Oxygen in the Isoniazid-Dependent Inactivation of *Mycobacterium tuberculosis* Enoyl Reductase

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Received November 18, 1996

Isoniazid (INH) continues to be the drug of choice for the treatment and prophylaxis of tuberculosis, and resistance to isoniazid[1,2] poses a significant clinical problem. Despite use of this drug in the treatment of tuberculosis for almost 45 years,[3] the mechanism of the drug is still not well understood. A genetic approach to identifying the target(s) of INH in *Mycobacteria smegmatis* resistant to INH and its structural analog, ethionamide, demonstrated that the target was an NADH-dependent fatty acyl enoyl ACP reductase, termed InhA.[4−6] This enzyme is involved in the biosynthesis of long-chain fatty acids, including the mycolic acids found in the cell wall of mycobacteria, consistent with the observation that mycolic acid biosynthesis is inhibited during INH treatment.[7]

In addition to the enzyme target of isoniazid, much attention has focused on the role of the mycobacterial *katG*-encoded catalase−peroxidase in the activation of isoniazid. Resistance to INH has been correlated with the loss of catalase−peroxidase activity, and the transformation of catalase−peroxidase deficient strains of mycobacteria resistant to isoniazid with a functional *katG* gene confers sensitivity to the drug.[8−10] The involvement of KatG in the antitubercular action of isoniazid suggests that isoniazid is a prodrug and that an oxidized derivative of it interacts with, and inactivates, the enoyl reductase.[4,9,11] Recent studies have shown that incubation of InhA with isoniazid and KatG results in the loss of most of its activity within 2 h.[12]

The addition of manganese chloride to inactivation mixtures has been reported in studies of InhA inactivation,[12−15] but without explanation. When the isoniazid and KatG-dependent inactivation of *M. tuberculosis* enoyl reductase was tested in the absence of $MnCl_2$ in the incubation reaction solution, loss of InhA activity did not occur and $MnCl_2$ could not be replaced by other metals (Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Al^{3+}; Figure 1).[16] When InhA was incubated[16] with $MnCl_2$ and INH, in the absence of KatG, the activity decreased to undetectable levels within 6 h (Figure 1). Inactivation did not occur when either of these two reagents were omitted, and it is clear that manganese ion and isoniazid are both necessary, and sufficient, to inactivate InhA,[17] but that the inactivation of InhA occurs 2.7 times faster when KatG is included. This is consistent with observations that the catalase−peroxidase is important for isoniazid susceptibility of mycobacteria and shows that it plays a role in the *in vivo* inactivation of InhA.[8,9] The demonstration that InhA inactivation requires manganese and can occur in the absence of KatG is consistent with the observations that loss of KatG accounts for less than 24% of clinical isoniazid resistance and that other mechanisms must account for the remaining resistance.[2] It has been shown that manganese and

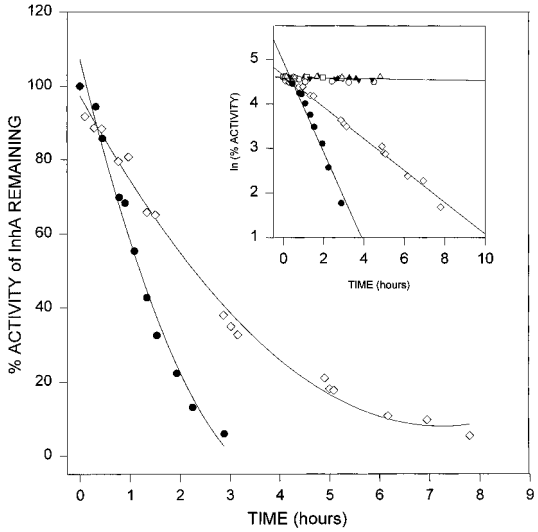

Figure 1. Inactivation of wild type InhA (3 μM) incubated in 100 mM phosphate buffer, pH 7.5 at 25 °C, and including, as stated NADH (100 μM) and INH (100 μM), KatG (1 μM), $MnCl_2$ or other metals as described (1 μM), and Mn-SOD (0.2 μM).[16] (A) ●, NADH, INH, KatG, $MnCl_2$; ◇, NADH, INH, $MnCl_2$. The data is fitted with a curve as a visual aid. (B) The inset shows a plot of natural log of percentage activity of InhA remaining for the respective experiments with the slope indicated in parentheses: ●, same as above (1.013); ◇, same as above (0.367); ▲, NADH, $MnCl_2$, no INH; □, NADH, INH; ○, NADH, INH, KatG, no Mn^{2+}; ▼, NADH, INH, $MnCl_2$, MnSOD; △, NADH, INH, KatG, $CuCl_2$ (similar results when other metals were added, Mg^{2+}, Ca^{2+}, Co^{2+}, Al^{3+}; data not shown).

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(14) The KatG catalase−peroxidase used was purified from *M. smegmatis* as reported.[10] The KatG in the report by Schultz *et al.* was from *M. tuberculosis*.[12] However, the catalatic and peroxidatic activities of these two KatG proteins are comparable.[10] The InhA used was from *M. tuberculosis* and was purified as previously reported.[6]
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(16) (a) The standard incubation reaction conditions consisted of 3 μM InhA, 100 μM INH, and 100 μM NADH, in 100 mM Na_2PO_4, pH 7.5 at 25 °C. Additions included, (±) as indicated (b) 1 μM KatG and (c) 1 μM $MnCl_2$ or other metal as indicated. The reaction solutions were incubated at 25 °C for 5 h or more, as indicated in Figures 1 and 2. (d) Aliquots were taken at times specified on *x*-axes of Figures 1 and 2, and steady-state enzyme activities were determined from rates of decrease in absorbance at 340 nm using NADH (100 μM) and 2-*trans*-hexadecenoyl CoA (15 μM) in 100 mM Na_2PO_4, pH 7.5 at 25 °C. (e) In specified reactions, due to availability of substrate, the activity was measured using 2-*trans*-dodecenoyl CoA (100 μM), at $2K_m$ because substrate inhibition occurs at higher concentrations. The synthesis and kinetic parameters of the enoyl CoA substrates is described elsewhere.[6]
(17) The activity of InhA is stable in the presence of buffer alone.[13, 16] Additionally, the InhA activity was stable in the presence of the following:[16] (i) in buffer, NADH, and INH; (ii) in buffer, NADH, and KatG; (iii) in buffer, NADH, INH, and KatG (Figure 1). Contrary to the previous reports in which glycerol (8% v/v) and acetylated BSA (0.1 mg/ml) were added to maintain protein stability,[12] these reagents were found unnecessary and resulted in a decreased level of activity of InhA by 15−20% over 6 h, when included in a reaction like one described above (1-iii)[16a,b] with no added metals (data not shown).

S0002-7863(96)03973-X CCC: $14.00

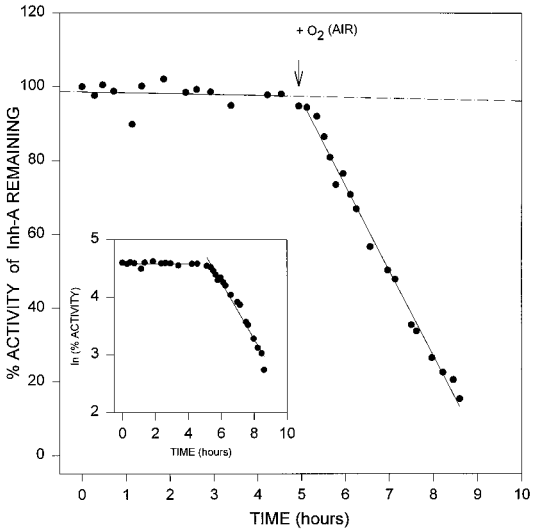

Figure 2. Incubation of InhA (3 μM) as described[22,16] for (●) Figure 1, except under anaerobic conditions for first 5 h and under aerobic conditions after 5 h, as indicated by an arrow.

isoniazid can function in the oxidative damage of DNA.[18] The manganese-dependent inactivation of InhA may be physiologically relevant[19,20] and suggests that manganese coordination by isoniazid, and redox chemistry involving O_2, could account for the reactivity observed.[21]

Anaerobic incubation of InhA, isoniazid, KatG, and $MnCl_2$ for 5 h under argon resulted in no detectable loss of enoyl reductase activity, but subsequent exposure to air resulted in rapid inactivation (Figure 2).[22] These results demonstrate that oxygen is required for both the catalase−peroxidase-dependent and -independent inactivation of InhA. These results are also consistent with reports that O_2 uptake in *M. tuberculosis* is necessary for the bactericidal effects of isoniazid[4] and may support previously proposed peracid metabolites of isoniazid as being inactivating species.[11] The KatG-independent inactivation[16a,c,e] of InhA by isoniazid can be prevented by the addition of 0.2 μM superoxide dismutase, suggesting that the KatG-independent reaction is superoxide-mediated.[23] These results are reminiscent of the results reported previously for the isoniazid and manganese-dependent oxidation of DNA.[18] To demonstrate whether a metabolite of INH generated during the manganese-dependent inactivation reaction was covalently bound to InhA, aliquots were taken over an 8 h period, desalted by HPLC, and analyzed by SDS-PAGE and mass spectrometry. No protein fragmentation could be observed by SDS-PAGE, and an increase in the molecular mass of the InhA monomer could not be detected after LC/MS (data not shown). These results are consistent with previously reported attempts to identify the covalent modification of the enoyl reductase by a metabolite of isoniazid.[12]

Although much remains to be learned of the *in vivo* mechanism of inactivation of InhA by isoniazid, it is clear that manganese, isoniazid, and O_2 are necessary and sufficient to catalyze the inactivation of InhA and that catalase−peroxidase accelerates this process. The KatG-dependent and -independent mechanisms of isoniazid activation are likely to proceed *via* different mechanisms, although both reactions are oxygen- and manganese-dependent. The ability of superoxide dismutase to prevent InhA inactivation by the KatG-independent mechanism suggests that overexpression of superoxide dismutase may influence the effectiveness of isoniazid, as has been discussed in other reports of drug resistance in mycobacteria.[24]

Acknowledgment. The authors acknowledge Dr. Jovita A. Marcinkeviciene and Dr. Luiz A. Basso for their generous gifts of InhA and substrates, respectively, and for intellectual contributions to this work. This work was supported by the National Institutes of Health (AI33696 and AI36849).

JA9639731

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(22) The incubation reaction[16a,b] without $MnCl_2$, in a sealed glass vessel with dual-valve attachment and side arm, was prepared anaerobically at 0 °C by evacuation for 15 min followed by replacement with argon, repeated four times. The $MnCl_2$ solution,[16c] purged separately with argon, was added *via* air-tight syringe. The incubation mixture was maintained at 25 °C under argon. Aliquots were removed *via* syringe and analyzed for activity anaerobically.[16d] After 5 h, the argon atmosphere was evacuated and replaced by air. The activity measurements were continued in air at 25 °C (Figure 2).

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