Binding of Catalase-Peroxidase-Activated Isoniazid to Wild-Type and Mutant *Mycobacterium tuberculosis* Enoyl-ACP Reductases

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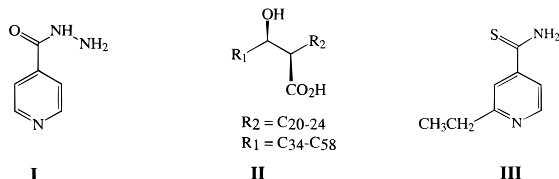
In 1952, isoniazid (INH, **I**) was found to exhibit powerful bactericidal activity against *Mycobacterium tuberculosis*,[1] and it has since been one of the principal agents in both therapeutic and prophylactic treatments of tuberculosis.[2] Resistance to INH was reported shortly after the introduction of INH therapy[3] and currently appears in >20% of drug-resistant tuberculosis cases.[4] In the first report of the isolation of laboratory INH-resistant strains of *M. tuberculosis*, a high correlation between INH resistance and attenuated catalase activity was observed.[5] This has subsequently been confirmed with the cloning of the *katG* gene encoding the mycobacterial catalase-peroxidase[6] and the demonstration that mutations in, or deletion of, the gene results in the acquisition of isoniazid resistance.[7,8] The observation that *katG* was not essential to mycobacterial growth, and that transfer of the gene into INH-resistant cells resulted in the increased susceptibility to INH,[6] suggested that the mycobacterial catalase-peroxidase is an activator of INH.



Isoniazid had been known for many years to inhibit the synthesis of mycolic acids, α-branched, β-hydroxy $C_{60}-C_{80}$ fatty acids (**II**), found in the cell wall of mycobacteria species.[9] The recent identification of mutations in the *inhA* gene, which conferred resistance to both INH and the structurally related antitubercular drug ethionamide (**III**),[10] provided a novel candidate target for INH action in mycobacteria. The point mutation (G→T) results in the conversion of serine-94 to an alanine residue (S94A) in the InhA protein. Point mutations have also been identified in the *inhA* locus of clinical isolates of *M. tuberculosis* displaying resistance to both INH and ethionamide.[7,8] The *inhA* gene product has been expressed, purified, crystallized, and shown to be a NADH-specific enoyl-ACP (acyl carrier protein) reductase.[11] The three-dimensional structures of the wild-type (WT) and S94A mutants have been determined,[12] and the enzyme has been shown to exhibit specificity for long-chain ($C_{16} > C_8$) enoyl thioester substrates.[11] This activity is consistent with the enzyme's function in fatty acid elongation and with the reported effect of INH on mycolic acid biosynthesis.[9,10] These data strongly suggest that the *inhA*-encoded enoyl-ACP reductase is the intracellular target of INH action.

The inability to demonstrate the direct binding of isoniazid or ethionamide to the homogeneous enzyme at pharmacologically attainable concentrations[11] argues that INH is a prodrug. The recent report that the *M. tuberculosis katG*-encoded catalase-peroxidase reacted with INH to form the stable products isonicotinic acid, pyridine-4-carboxaldehyde, and isonicotinamide, via highly reactive intermediates[13] that inhibit the enoyl-ACP reductase,[14] led us to consider possible mechanisms of resistance to INH.

The binding of the stable products of catalase-peroxidase oxidation of isoniazid to wild-type *M. tuberculosis* $H37R_v$ enoyl-ACP reductase could not be detected by titration calorimetry.[15] To evaluate possible differences in the binding of activated isoniazid to the WT and S94A mutants forms of the enoyl-ACP reductase, [*carbonyl*-^{14}C]isoniazid[16] in the presence or absence of the *M. tuberculosis katG*-encoded catalase-peroxidase was incubated with the enzymes. Following incubation for up to 20 h, the reaction mixtures were applied to a Pharmacia PD-10 column, eluted, and analyzed by liquid scintillation counting. As shown in Figure 1, only when catalase-peroxidase was present were significant levels of radioactivity observed to coelute with the WT enoyl-ACP reductase. To ensure that binding was to the enoyl reductase, we repeated this experiment using a gel filtration matrix which would resolve the reductase from catalase-peroxidase and could demonstrate that all of the radioactivity coeluted with the reductase (data not shown). The binding of radiolabeled INH was negligible in the absence of NADH or in the presence of NAD^+ but increased to a stoichiometry of ~30% of the active sites in the presence of 10 μM NADH (Table 1). The stoichiometry of labeling could be increased to 50% of the active sites in the presence of higher concentrations of catalase-peroxidase, resulting in nearly com-

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(15) In 20 mM Hepes, pH 7.3, either in the presence or in the absence of 100 μM NADH, 86 μM InhA was titrated with 25 successive injections of 4 μL of 5.6 mM ligand (isonicotinic acid, isonicotinamide, or pyridine-4-carboxaldehyde) in the same buffer in an Omega microcalorimeter (Microcal, Inc.) at 25 °C.
(16) [*carbonyl*-^{14}C]Isoniazid had a specific radioactivity of 13 mCi/mmol.

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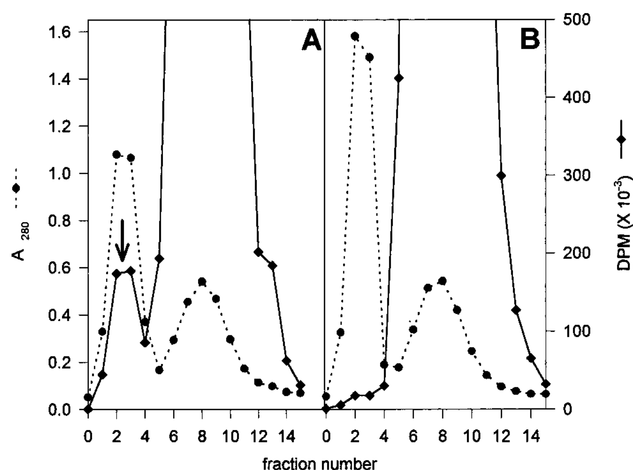


Figure 1. Determination of [^{14}C]isoniazid binding to wild-type *M. tuberculosis* enoyl-ACP reductase. Chromatographic profile of 280 nm absorbance and radioactivity eluting from a PD-10 (Pharmacia) gel filtration column after incubation of the reductase with 100 μM NADH and in the presence (A) or in the absence of catalase-peroxidase (B). The first and second peaks of 280 nm absorbance correspond to the protein and isoniazid peaks, by comparison with standards. See the footnote to Table 1 for complete experimental details and conditions.

Table 1. Binding of [^{14}C]Isoniazid to *M. tuberculosis* Enoyl-ACP Reductase[a]

enoyl reductase	KatG (nM)	nucleotide	concn (μM)	protein-bound DPM ($\times 10^4$)	labeling, % of active sites[b]
WT	0	NADH	100	3.8	1.7
WT	58			4.9	2.2
WT	58	NAD$^+$	100	8.7	3.9
WT	58	NADH	100	27.8	12.4
S94A	58	NADH	100	23.3	10.4
WT	35	NADH	10	17.6	26.5
S94A	35	NADH	10	10.0	15.0
WT	35	NADH	1	8.9	13.1

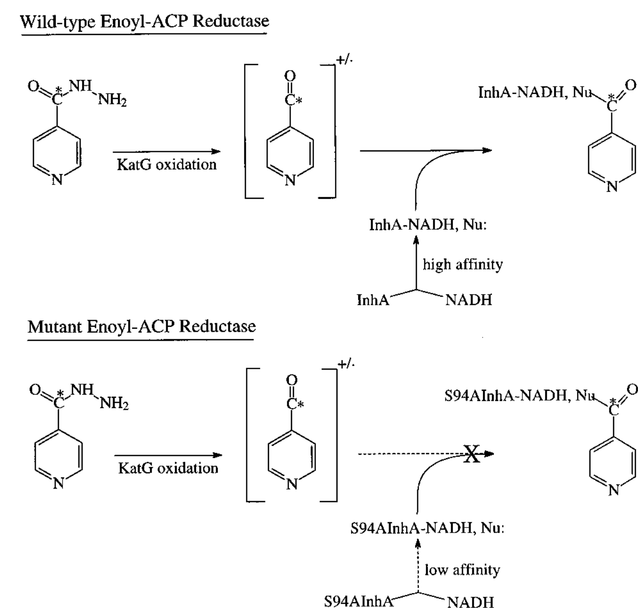
[a] Reactions were performed in 50 mM phosphate buffer, pH 7.4, containing 2 μM $MnCl_2$, 6% glycerol, 100 μM [*carbonyl*-^{14}C]isoniazid, and 31 μM (9 μM at 1 and 10 μM NADH) *M. tuberculosis* enoyl-ACP reductase in a total volume of 2.5 mL. [b] Calculated on the basis of a monomer molecular mass of 28 500 D.

plete inactivation of enzymatic activity.[17] These data confirm the observation[14] of the binding of the catalase-peroxidase-activated INH to the WT *M. tuberculosis* enoyl-ACP reductase and further suggest that activated INH binds exclusively to the enzyme−NADH complex and that one molecule of activated INH per dimer is sufficient for inactivation.

To probe the molecular basis for the resistant phenotype resulting from the single mutation at position 94 of the enoyl-ACP reductase, we examined the ability of the WT and S94A mutant enzymes to bind activated INH as a function of NADH concentration. At saturating concentrations of NADH, no significant differences were observed between the extents of binding of *katG*-activated INH to the WT and S94A enzymes. In contrast, at lower concentrations of NADH, closer to the K_m value for the WT enzyme ($K_m \approx 8$ μM[11]) but well below the K_m value for the S94A mutant ($K_m \approx 37$ μM[11]), the mutant enzyme was labeled to approximately half the extent observed for the WT enzyme. These data are consistent with the hypothesis that the *inhA*-encoded long-chain enoyl reductase is the *bona fide* target for INH action,[11,12] resulting in inhibition of long-chain fatty acid, and subsequently mycolic acid, biosynthesis.

(17) In the presence of 2 μM $MgCl_2$, 6% glycerol, 10 μM NADH, 100 μM [^{14}C]isoniazid, and 1.9 μM KatG, 9 μM InhA was incubated. The InhA activity was reduced by 82% after incubation.

Scheme 1. Proposed Mechanism of Resistance of *M. tuberculosis* to Isoniazid



The binding of activated drug exclusively to the enzyme−NADH complex is particularly relevant to the mechanism of resistance, since the S94A mutation in the *inhA* structural gene is located in the nucleotide binding region[11] and has specific effects on the steady-state K_m value and binding of NADH to the reductase.[11,12] The demonstration that the S94A mutant is labeled to approximately half the extent as the WT enzyme at low NADH concentrations supports a novel type of resistance in which the reduced binding of pyridine nucleotides to the enoyl-ACP reductase correlates with the reduced binding of drug (Scheme 1).[18] At the low concentrations of NADH found intracellularly in *M. tuberculosis* (estimated at <10 μM[19]), the perturbation in the nucleotide binding affinity caused by mutations in the nucleotide binding region will decrease the proportion of the enzyme in the binary nucleotide complex, potentially accounting for the attenuation in drug binding and resulting resistance to INH.[10] The inhibition of enoyl reductase activity and disruption of long-chain fatty acid elongation and mycolic acid biosynthesis would likely prevent the proper assembly of the cell wall and would both inhibit growth and enhance the osmotic sensitivity of mycobacteria. The identification of the activated form of isoniazid bound to the enoyl reductase and its site of binding to the enoyl reductase will finally provide a firm molecular basis for the therapeutic action of this 40 year old drug. Ultimately, these studies may provide insights into the development of new, more powerful antitubercular compounds.

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