Kinetics of Inactivation of WT and C243S Mutant of *Mycobacterium tuberculosis* Enoyl Reductase by Activated Isoniazid

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Isoniazid (isonicotinic acid hydrazide, INH) was first reported to be effective in the treatment of tuberculosis (TB) in 1952.[1] Strains of *Mycobacterium tuberculosis* resistant to INH were reported shortly after the introduction of this drug,[2] and isoniazid resistance was correlated with absent or lowered catalase activity.[3] The latter finding was corroborated by the recent demonstration that high-level resistance to INH is associated with mutations in, or deletion of, the catalase−peroxidase gene, *katG*.[4]

The synthesis of mycolic acids, which are found in the cell wall of mycobacteria species, is known to be inhibited by INH.[5] The product of the *inhA* gene has been suggested as the enzymatic target for INH[6] and identified as an NADH-dependent enoyl-ACP (acyl carrier protein) reductase, which exhibits specificity for long-chain (C_{16} > C_8) enoyl thioester substrates.[7] The three-dimensional structure has been determined and refined to 2.2 and 2.7 Å for wild-type (WT) and S94A mutant reductases, respectively.[8] The resistance of mycobacteria expressing the S94A mutant to isoniazid has been shown to be due to an increase in the steady-state K_m value for NADH as compared to the wild-type reductase[7] and results from a disruption of a hydrogen-bonding network between the enzyme and NADH.[8]

Peroxidases have been shown to react with isoniazid to generate a number of oxidized products similar to those observed *in vivo*.[9] A mechanism for the action of isoniazid has been proposed that involves the conversion of isoniazid, by the mycobacterial *katG*-encoded catalase−peroxidase, into a number of electrophilic intermediates capable of either oxidizing or acylating amino acid residues in proteins.[10] The amino acid residue(s) of the enoyl reductase modified by the electrophilic drug metabolite has yet to be identified, but on the basis of the reaction of the enzyme with ω-bromoacetophenone, Cys243 was suggested as a candidate residue.[11] In order to test this suggestion that activated derivatives of isoniazid, produced by the *katG*-encoded mycobacterial catalase−peroxidase, alkylate the Cys243 residue of the enoyl reductase, we prepared the C243S mutant by site-directed mutagenesis.[12]

Isoniazid oxidation catalyzed by the *M. smegmatis* catalase−peroxidase, in the presence of NADH,[14] resulted in the time-dependent inactivation of WT enoyl reductase (Figure 1A) with an apparent first-order rate constant value of (8.9 ± 0.2) × 10^{-3} min^{-1} (Figure 1A, inset). The inactivation of the C243S mutant enzyme (Figure 1B), under identical conditions, proceeded with an apparent first-order rate constant value of (8.5 ± 0.2) × 10^{-3} min^{-1} (Figure 1B, inset). These results clearly demonstrate that Cys243 is not the residue modified by electrophilic intermediates produced by the catalytic action of catalase−peroxidase on the prodrug. These results are also in agreement with the position of Cys-243 in the three-dimensional structure of WT *inhA* (Figure 2). This residue is located on α-helix 8, and the sulfur atom is 16.6 Å from C4 of the nicotinamide ring of NADH. The statistically indistinguishable rate constants for inactivation of the WT and C243S enzymes suggest that the same residue is modified. Neither the WT or C243S mutant enzymes were inactivated in the absence of INH or NADH, and catalase−peroxidase-activated isoniazid has been shown to bind exclusively to the enzyme−NADH binary complex.[15]

The inactivation of *inhA* by the catalase−peroxidase-activated isoniazid has been shown to be inhibited by the addition of

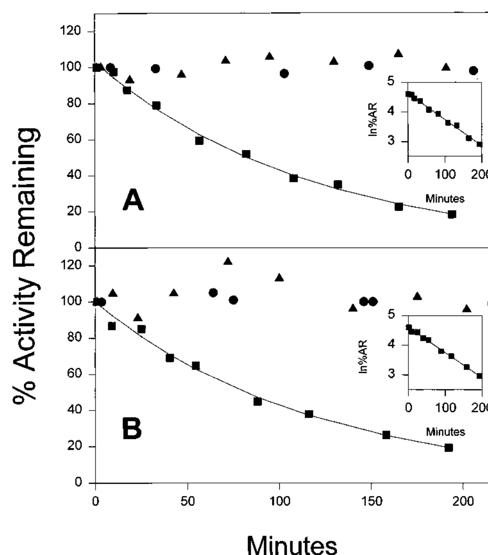

Figure 1. (A) Inactivation of WT *inhA* (3 μM) by oxidized INH (100 μM) derivatives produced by KatG (1 μM):[14] ●, no NADH, no KatG, no INH; ▲, no NADH; ■, 100 μM NADH. The inset shows a plot of the natural log of the percentage of WT *inhA* activity remaining (%AR) versus time. (B) Inactivation of C243S enzyme under the same experimental conditions described in part A (except that WT *inhA* was replaced for C243S): ●, no NADH, no KatG, no INH; ▲, no NADH; ■, 100 μM NADH. The inset shows a plot of the natural log of the percentage of C243S activity remaining (%AR) versus time.

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(12) The mutant gene of C243S *inhA* was constructed by site-directed mutagenesis according to the manufacture's (Promega) instructions using single-stranded template pET23d(+)-*inhA* and expressed in *Escherichia coli* BL21(DE3). The dideoxy chain termination method[13] was used to screen the colonies harboring the mutated gene. Liquid chromatography−electrospray mass spectrometry (LC/MS) was performed on both wild-type and C243S enzymes, which were purified according to the protocol described elsewhere,[7] in order to confirm that no unexpected mutations were introduced and the identity of the mutated enzyme. The deconvoluted data for the subunit molecular mass yielded values of 28 371 and 28 355 Da for wild-type and mutant enzymes, respectively, confirming the substitution of a sulfur for an oxygen atom.

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(14) Reactions were carried out in 100 mM Na_2HPO_4, pH 7.5 at 25 °C, either 3 μM WT *inhA* or 3 μM C243S, and 1 μM $MnCl_2$. Aliquots were taken at times specified on *x*-axes of Figures 1 and 3, and steady-state enzyme activities were determined from rates of decrease in absorbance at 340 nm using 2-*trans*-hexadecenoyl-CoA (15 μM) and NADH (100 μM). 2-*trans*-Hexadecenoyl-CoA was synthesized and purified according to the procedure described elsewhere.[7] The specific activity of WT *inhA* and C243S with 2-*trans*-hexadecenoyl-CoA as substrate at V_{max} were 4.7 ± 0.9 and 4.5 ± 0.8 units mg^{-1}, respectively.

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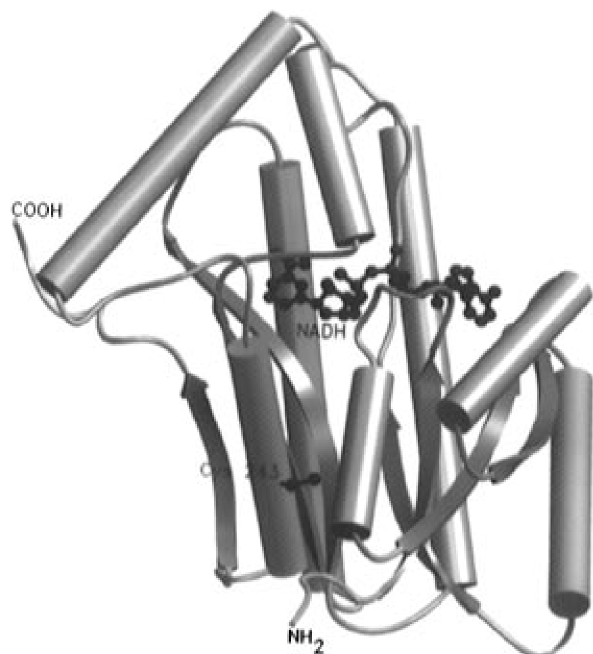

Figure 2. Ribbon diagram of WT *inhA*−NADH binary complex[8] showing the side chain of Cys243 (PDB 1INH).

2-*trans*-octenoyl-ACP to a preincubation mixture containing oxidized coenzyme.[11] In order to probe the location of the residue modified by the catalase−peroxidase-activated isoniazid in the presence of NADH, we investigated the inhibitory activity of saturated fatty acid CoA esters. Palmitoyl-CoA was a linear, competitive inhibitor[16] vs 2-*trans*-hexadecenoyl-CoA, exhibiting an inhibition constant of 8 ± 2 μM. The inactivation of WT enoyl reductase could be substantially protected by the addition of palmitoyl-CoA (Figure 3).[17] The apparent first-order rate constants for inactivation were determined to be $(8.9 \pm 0.2) \times 10^{-3}$, $(5.4 \pm 0.1) \times 10^{-3}$, and $(3.7 \pm 0.1) \times 10^{-3}$ min^{-1} at 0, 10, and 150 μM concentrations of added palmitoyl-CoA,

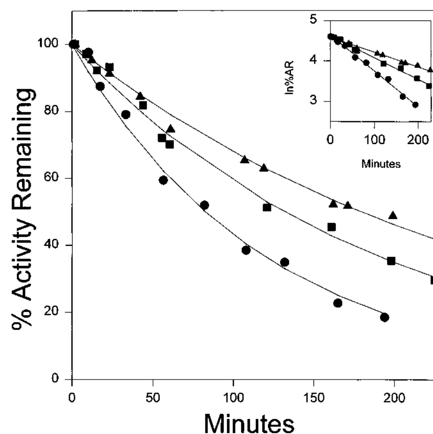

Figure 3. Protection against inactivation of the enoyl reductase by palmitoyl-CoA:[14] ●, 0 μM; ■, 10 μM; ▲, 150 μM.

respectively (Figure 3, inset). These data suggest that the residue modified by the catalase−peroxidase-activated isoniazid is located in the fatty acyl binding pocket of the enoyl reductase. Moreover, the ability of both C_8 and C_{16} chain length thioester to protect against inactivation suggests that the enzyme residue modified is near the thioester function. Although palmitoyl-CoA afforded protection against inactivation of WT *inhA*, this protection was not complete, presumably due to the formation of micelles at high palmitoyl-CoA concentration.[18]

The results here presented argue that Cys243 is not the residue modified by the intermediate produced by the action of catalase−peroxidase on the prodrug, but rather suggest that the drug interacts with residues which make up the fatty acyl binding site. This site has been proposed[8] to be the large 580 Å^3 cavity adjacent to bound NADH, which is lined with hydrophobic and aromatic amino acid residues. The identification of the activated form of isoniazid bound to the enoyl reductase and the enzyme residue modified will provide a complete molecular description of the mode of action of this drug. Eventually, these studies will help in the development of more powerful drugs for the treatment of drug-resistant strains of *M. tuberculosis*.

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(16) Inhibition studies were performed by varying the concentration of 2-*trans*-hexadecenoyl-CoA at different fixed concentrations of palmitoyl-CoA. Initial velocities were determined under steady-state conditions in 100 mM Na_2HPO_4, pH 7.5 at 25 °C, with NADH at 100 μM. The kinetic data were fitted to the equation which describes competitive inhibition ($v = VA/(K(1 + I/K_i) + A)$ using the Fortran programs of Cleland (Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103).

(17) Reactions of protection studies were carried out in 100 mM Na_2HPO_4, pH 7.5 at 25 °C, 3 μM WT *inhA*, 1 μM KatG, 100 μM NADH, 100 μM INH, 1 μM $MnCl_2$, and varying fixed concentrations of palmitoyl-CoA. Initial rates were determined as described in ref 14.

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