Mechanisms of Isoniazid Resistance in *Mycobacterium tuberculosis*: Enzymatic Characterization of Enoyl Reductase Mutants Identified in Isoniazid-Resistant Clinical Isolates

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Mutants in the structural gene of the *inhA*-encoded NADH-dependent 2-trans enoyl–acyl carrier protein reductase were identified from isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. Recombinant InhA proteins with defined single amino acid replacements were expressed in *Escherichia coli* and purified to homogeneity. Steady-state kinetic parameters for wild type (WT) and 116T, I21V, I47T, and I95P mutants of the enoyl reductase were measured spectrophotometrically. NADH binding to WT and 116T, I21V, I47T, S94A, and I95P mutant reductases were determined by fluorescence spectroscopy and demonstrated that all mutant enzymes had reduced NADH affinity and that NADH binding to all mutants was cooperative as compared with the hyperbolic binding of NADH to the WT enzyme. Since KatG-produced electrophilic derivatives of isoniazid have been suggested to inactivate the enoyl reductase–NADH complex, the kinetics of inactivation for the WT and I21V and I95P mutants was determined. Both mutations resulted in significantly increased values for the apparent first-order rate constant of inactivation.

Tuberculosis is the leading cause of death in the world from a single infectious disease [1]. The resurgence of tuberculosis caused by the emergence of multidrug-resistant strains throughout the world [2–5] has led to an increased need for understanding the molecular mechanisms of drug resistance, which should assist in the discovery and development of new drugs. The primary mechanism of multiple drug resistance in *Mycobacterium tuberculosis* is the accumulation of mutations in individual drug target genes [6, 7].

Isoniazid (isonicotinic acid hydrazide, INH) was first reported to be effective in the treatment of tuberculosis in 1952 [8, 9], and strains of *M. tuberculosis* resistant to INH were reported shortly after the introduction of this drug [9]. Biochemical studies provided compelling evidence that INH inhibited mycolic acid biosynthesis and that this inhibition correlated with mycobacterial cell death [10]. More recent genetic studies led to the discovery of the *inhA* gene [11], subsequently identified as an NADH-dependent enoyl-acyl carrier protein reductase that exhibits specificity for long-chain (C₁₆ > C₇) enoyl thioester substrates, consistent with its involvement in mycolic acid biosynthesis [12]. Mutations in the *inhA* structural gene [4, 13] and the *inhA* promoter region [7, 14, 15] have been identified in INH-resistant clinical isolates of *M. tuberculosis*. Additionally, INH resistance can be conferred on sensitive strains by either multicopy expression of the *inhA* gene or a mutation resulting in the substitution of an alanine residue for serine at position 94 [11]. Cell-free extracts with either resistance phenotype were shown to be resistant to inhibition of mycolic acid biosynthesis by INH [11]. The S94A mutant exhibits a 5- to 8-fold increase in the *Kₘ* value for NADH compared with wild type (WT) enzyme, whereas there was no effect on either the maximum velocity or *Kₘ* values for fatty acyl substrates [12]. The molecular basis for this difference in nucleotide affinity has been documented by the three-dimensional structure determination of the WT and S94A mutant reductases [16].

A recent report has questioned the in vivo significance of the *inhA*-encoded enoyl reductase as a bona fide target for INH in *M. tuberculosis* [17]. In the present work, we identified mutations in the *inhA* structural gene from INH-resistant clinical isolates of *M. tuberculosis*. The mutant enoyl reductase genes were constructed, the mutant proteins were expressed and purified, and their steady-state kinetic parameters and nucleotide-binding properties were examined. The kinetics of inactivation of WT, I21V, and I95P enoyl reductases by KatG-activated INH were measured, and both confirm previous suggestions that the enoyl reductase is a target of INH action in *M. tuberculosis* and allow the first correlation to be developed between clinical INH resistance and NADH affinity to mutant enoyl reductases.

**Material and Methods**

INH-resistant *M. tuberculosis* isolates. The *M. tuberculosis* isolates studied were recovered from patients with tuberculosis living...
in the United States. The organisms were all resistant to INH on the basis of results of the BACTEC (Becton Dickinson, Cockeysville, MD) radioreplosor evaluations exhibiting MICs of 1.0 μg/mL. The inhA coding region missense mutations were identified in the course of an ongoing survey of drug resistance-associated gene mutations in organisms from global sources. All isolates had distinct IS6110 subtypes and were assigned to major group 1 or 2 [18]. Hence, they are epidemiologically unrelated isolates.

Automated DNA sequencing. The katG gene, inhA locus, and oxyR-ahpC region were sequenced in their entirety for all isolates by previously described strategies [13, 14, 19]. All mutations in the inhA structural gene were confirmed by resequencing.

Polymerase chain reaction (PCR) amplification and expression of constructs. PCR amplification of genomic DNA preparations, cloning, and overexpression were performed as previously described [12].

Purification of enoyl reductases. Six liters of 1.5× Luria broth medium containing 50 μg/mL carbenicillin were inoculated with an overnight culture, grown to an A600 of 0.8–2, induced with 1 mM isopropyl-β-d-thiogalactopyranoside, and grown for an additional 2 h. Cells were harvested by centrifugation, and the enoyl reductase was purified as described previously [12]. The concentration of enzyme was calculated from the specific absorbance coefficient at 280 nm (37.3 × 10³ M⁻¹ cm⁻¹). Liquid chromatography-electrospray ionization mass spectrometry was performed on homogeneous WT and mutant enzymes to confirm the nature of the mutation in the enoyl reductase and to confirm that no unexpected mutations were introduced.

Synthesis and purification of 2-trans-dodecenoyl-CoA. 2-trans-dodecenoyl-CoA was synthesized from 2-trans-dodecanedioic acid and CoA by the mixed anhydride method and purified by reverse-phase high-performance liquid chromatography using a 19 × 300 mm C18 μBondapak column (Waters Associates, Milford, MA) as previously described [12]. The ratio of absorbances of purified 2-trans-dodecenoyl-CoA at 232 nm and 260 nm was 0.62, a value that meets the established criterion for pure thioesters (A232/A260 ≥ 0.52) [21].

Steady-state kinetics. Steady-state kinetic parameters (Kₘ and Vₘₐₓ) were determined spectrophotometrically by following NADH oxidation at 340 nm when variable concentrations of NADH were used (10–400 μM) at a fixed, saturating concentration of 2-trans-dodecenoyl-CoA (100 μM). Measurements were made at 370 nm when variable concentrations of the enoyl substrate were used (10–100 μM) at a fixed, saturating concentration of NADH (400 μM). Experiments were performed at 25°C and in 0.1 M PIPES buffer, pH 7.0, using a thermostated spectrophotometer (model 9310; Uvikon). Higher concentrations of 2-trans-dodecenoyl-CoA could not be used because of a decrease in the enzyme activity at concentrations of enoyl-CoA substrate above ~150 μM, possibly due to substrate inhibition or the formation of micelles at high concentrations of enoyl-CoA substrate [21].

Fluorescence spectroscopy. Fluorescence titrations were performed at equilibrium in a luminescence spectrometer (model LS 50B; Perkin Elmer, Norwalk, CT) at 25°C by making microliter additions of NADH stock solutions to 2 mL of 2 μM InhA in 0.1 M PIPES, pH 7.0. Dilution caused by NADH addition was kept <1.5%. Controls were determined, following exactly the same procedure, by omitting the enzyme from the cuvette. The binding of NADH to the enoyl reductase causes an enhancement in nucleotide fluorescence at 440 nm (λexc = 360–370 nm, figure 1). Although nucleotide fluorescence is subject to inner filter effects at high NADH concentrations, this was minimized by using an excitation wavelength on the red edge of the longest wavelength absorption band.

Inactivation of the enoyl reductase. Reactions of the time-dependent inactivation of the enoyl reductases were carried out in 100 mM Na₂HPO₄, pH 7.5, at 25°C. Either WT, I21V, or 195P enzymes (10 μM) were preincubated with NADH (10 μM), M. tuberculosis KatG (2 μM), INH (200 μM), and MnCl₂ (2 μM) and aliquots taken at the times specified on the x axes of figure 2. Enzyme activities were determined from rates of decrease in absorbance at 340 nm using 2-trans-dodecenoyl-CoA (100 μM) and NADH (200 μM).

Data analysis. The steady-state kinetic data were fitted to the Michaelis-Menten equation, yielding estimates of Kₘ and Vₘₐₓ. The fluorescence equilibrium data for NADH binding to WT enoyl reductase were fitted to a quadratic equation for a second-order binding process. The data for NADH binding to InhA mutants were fitted to the Hill equation for sigmoidal curves [22], yielding estimates of Iₘₐₓ, Kₘ,5 (concentration of substrate at half-saturation) and h (the Hill coefficient). Curve fitting was performed with the Sigma Plot (Jandel Scientific, Corte Madera, CA) program, which uses the Marquardt-Levenberg algorithm for nonlinear least-squares.

Results

Identification of inhA missense mutations. In the course of an ongoing project to understand the molecular genetics of INH resistance in M. tuberculosis, we have been routinely sequencing the katG gene, inhA locus, and oxyR-ahpC region of INH-resistant clinical isolates. Five INH-resistant isolates have been identified that had missense mutations in the inhA structural gene but lacked mutations in the other genes studied. The four mutations identified in this study were: Ile16Thr (ATG→ACT), Ile21Val (ATC→GTC), Ile47Thr (ATT→ACT), and Val78Ala (GTG→GCG). Additionally, an Ile95Pro (ATT→CCT) mutation has been identified in clinical isolates of INH-resistant M. tuberculosis [4]. These amino acid substitutions have never been identified in INH-susceptible organisms.

Effects of mutations on steady-state kinetic parameters. In order to study the biochemical effect of mutations in the inhA structural gene of INH-resistant clinical isolates of M. tuberculosis, the steady-state parameters for the enoyl reductase—catalyzed reduction of 2-trans-dodecenoyl-CoA were determined for the WT and mutant enzymes (table 1). There was no marked effect on Vₘₐₓ values exhibited by the mutant enzymes, except for the 195P mutant enzyme, which showed an 85-fold reduction compared with the WT enzyme. Similarly, the steady-state Kₘ values for the fatty acyl-CoA substrate showed no obvious trends when the values of the mutant and WT enzymes were compared. All mutant reductases had increased values for the Kₘ of NADH, ranging from 2- to 12-fold. Since the steady-
state $K_m$ value for NADH is a composite of rate constants related to both binding affinity and binding of enoyl-CoA substrate, we directly measured the thermodynamic dissociation constant of NADH.

Effects of mutations on enzyme-NADH binary complex formation. Earlier reports describing titration microcalorimetric measurements of NADH binding to the WT and S94A enoyl reductases revealed that the dissociation constant ($K_d$) for NADH was higher for the S94A mutant than the corresponding $K_d$ for NADH for WT enzyme [12]. To assess the effect of amino acid substitutions on the dissociation constant for NADH, the formation of the enzyme-NADH binary complex was determined using the WT, I16T, I21V, I47T, S94A, and I95P enzymes by measuring the enhancement in nucleotide fluorescence upon nucleotide binding to the reductase (figure 1). The fluorescence enhancement of NADH observed upon titrating 2 $\mu$M WT InhA with NADH was hyperbolic, whereas the binding curves of NADH to I16T, I21V, I47T, and S94A were sigmoidal. Fits of these binding curves for the WT and mutant reductases are presented in table 2. The I95P mutant could not be saturated in the concentration range of NADH used in the present study, which only permits us to set a lower limit for the dissociation constant of NADH and does not allow the determination of the potential cooperative binding of NADH to this mutant. The I16T, I21V, I47T, and S94A substitutions resulted in a 10- to 150-fold increase in the dissociation constant for the enzyme-NADH binary complex.

In order to test this model, the in vitro kinetics of inactivation of the I21V and I95P mutants by KatG-activated INH were compared with that of the WT enzyme. As predicted by the proposed model for INH resistance, both mutations that resulted in increased values for the NADH dissociation constant correspondingly decreased the values for the apparent first-order rate constant of inactivation (figure 2).

Discussion

The S94A mutation in the inhA structural gene was previously shown to specifically affect the steady-state $K_m$ value for NADH [12]. Steady-state kinetic studies on the mutant enoyl reductases from INH-resistant clinical isolates indicate that substantial changes in the $K_m$ value for NADH are observed with I16T,
Figure 2. Time-dependent inactivation of enoyl reductases. Curves were fitted to first-order equation, yielding values of $4.87 \times 10^{-3}$, $3.31 \times 10^{-3}$, and $0.34 \times 10^{-3}$ for apparent first-order rate constant of inactivation of wild type (●), I21V (■), and I95P (▲) enzymes, respectively.

I21V, I47T, and I95P mutants, compared with the WT InhA. There were only modest differences in either the $K_m$ values for 2-trans-dodecenoyl-CoA or the $V_{\text{max}}$ values for the WT and I16T, I21V, and I47T mutants; however, an 85-fold decrease in the $V_{\text{max}}$ value for the I95P mutant was demonstrated. The metabolic significance of this observation is unknown, but INH-resistant strains harboring the I95P mutation must be able to accommodate or compensate for this lower activity.

The direct measurement of NADH binding to the mutant enoyl reductases from INH-resistant clinical isolates of *M. tuberculosis*, by fluorescence spectrophotometry, demonstrated that the amino acid changes of I16→T, I21→V, I47→T, and S94→A result in both a 10- to 150-fold increase in the dissociation constant for the enzyme-NADH binary complex and the appearance of cooperative NADH binding. The cooperative binding of NADH observed for the structurally characterized S94A mutant is not due to the presence of multiple NADH binding sites on each monomer. Rather small structural changes observed in the monomer of the S94A mutant compared with the WT are likely to result in the observed cooperative binding.
The in vivo concentration of NADH in *M. tuberculosis* H37Rv has been estimated to be <10 μM [23]. At these physiologic concentrations of NADH, a significant proportion of I16T, I21V, I47T, S94A, and I95P mutant enzyme molecules are likely to be present in an NADH-unbound form compared with the WT enzyme. Activated INH binding to, and inactivation of, InhA is dependent on the presence of bound NADH [24–26]. Therefore, the increased overall dissociation constant (Kd) for mutant enzyme-NADH binary complex formation can account for both the resistance phenotype and the in vivo survival of the mutants since fatty acid biosynthesis would not be entirely arrested. The fluorescence studies of the clinically important I95P mutant demonstrate that this substitution results in the extremely poor binding of NADH, and we can only estimate the NADH dissociation constant as being >100 μM. This weak binding of NADH to the I95P mutant enzyme correlates with the observation of INH resistance.

The mycobacterial *inhA*-encoded enoyl reductase shares 40% amino acid sequence identity with the *Escherichia coli* *envM*-encoded enoyl reductase [11], which is a target for diazaborines and INH, respectively, and for the enoyl reductase being a target in *M. tuberculosis* [27]. Agents that block production of lipo-polysaccharide. Replacement of glycine with serine at position 93 of the *E. coli* enoyl reductase results in diazaborine resistance [28]. The serine residue of the enoyl reductase, which is mutated to an alanine residue in INH-resistant mutants of *M. smegmatis* [11], and the glycine 93 residue of the enoyl reductase mutated to a serine residue in diazaborine-resistant mutants of *E. coli* [28] are both located in the nucleotide binding site. Binding to, and inactivation of, both the *E. coli* and *M. tuberculosis* enoyl reductases by diazaborines and INH, respectively, requires the presence of nicotinamide adenine dinucleotides [24–26, 28]. The determination of the three-dimensional structure of the ternary complex of the *E. coli* enoyl reductase, NAD+, and diazaborine revealed the presence of a covalent bond between the 2’ hydroxyl of the nicotinamide ribose and the boron atom of the diazaborine [29]. Inhibition is caused by the tight, but noncovalent, binding of this bisubstrate analogue to the enzyme. More recently, the three dimensional structure has been determined for the ternary complex of the WT enoyl reductase of *M. tuberculosis*, and NADH and activated INH [30]. In this complex, generated by the in vitro reaction of manganese, INH, and oxygen [31] with the enzyme-NADH complex, the acylpyridine fragment of INH is covalently attached to the C4 position of NADH. It is likely that the in vivo formation of a similar adduct between the electrophilic intermediate of INH produced by oxidation of the drug by KatG [32] and the enzyme-NADH complex would form this same ternary complex, resulting in enzyme inactivation. The binding and inactivation studies performed here clearly demonstrate that these INH-resistant mutants of clinical isolates of *M. tuberculosis* exhibit an increased dissociation constant for NADH from the binary complex. Reduced affinity of NADH for the enoyl reductase would decrease the amount of enzyme present in an inactivable form, thereby resulting in INH resistance. Consistent with this proposal is the observation that all mutations in the enoyl reductase conferring INH resistance are located in the nucleotide binding site (figure 3).

Based on the results presented in this paper and from genetic selection [11] and biochemical studies [12, 16, 24–26], it is clear that the *inhA*-encoded enoyl reductase is a bona fide target for INH in both *M. smegmatis* and *M. tuberculosis*. Although there has been a recent report that the pattern of accumulation of saturated and unsaturated fatty acids after INH treatment of *M. tuberculosis* is more consistent with the target being the C24 fatty acid desaturase [17], other recent results obtained with *Mycobacterium aurum* [33] are consistent with the identification of the *inhA*-encoded enoyl reductase being a target in

<table>
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<tr>
<th>Table 1.</th>
<th>Steady-state kinetic parameters of <em>M. tuberculosis</em> wild type (WT) and mutants of InhA.</th>
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<tbody>
<tr>
<td>Parameter</td>
<td>WT</td>
</tr>
<tr>
<td>Vmax (U/mg)</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>NADH Kcat (μM)</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>trans-Δ^2-12:1-CoA Kcat (μM)</td>
<td>75 ± 5</td>
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NOTE. Experimental conditions and data analysis are described in Materials and Methods.

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<tr>
<th>Table 2.</th>
<th>Equilibrium measurement of NADH binding to <em>M. tuberculosis</em> wild type (WT) and mutants of InhA.</th>
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<tbody>
<tr>
<td>Sample</td>
<td>Curve type</td>
</tr>
<tr>
<td>WT InhA</td>
<td>Hyperbolic</td>
</tr>
<tr>
<td>I16T</td>
<td>Sigmoidal</td>
</tr>
<tr>
<td>I21V</td>
<td>Sigmoidal</td>
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<td>I47T</td>
<td>Sigmoidal</td>
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<tr>
<td>S94A</td>
<td>Sigmoidal</td>
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<tr>
<td>I95P</td>
<td>No saturation</td>
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NOTE. Experimental conditions and data analysis are described in Materials and Methods.
M. tuberculosis. The present studies of the purified mutant proteins identified in clinical isolates of INH-resistant *M. tuberculosis* suggest that the molecular mechanism of action of, and resistance to, INH is identical to the mechanism previously suggested for *M. smegmatis*. While there may be additional targets affected by INH treatment, the overwhelming weight of evidence supports the enoyl reductase being an important target, potentially responsible for the bactericidal effects of INH. The correlation presented here between the NADH binding properties and resistance to INH inactivation of *M. tuberculosis* enoyl reductase mutants strongly suggests a mechanism of resistance for this clinically important drug.

**Acknowledgments**

We thank Giovanna Scapin for assistance in preparing figure 2, Denise A. Rozwarski and James C. Sacchettini (Texas A & M University) for providing us with results prior to publication [30], and Richard S. Magliozzo for the generous gift of *M. tuberculosis* catalase-peroxidase (KatG).
References