

Mycobacterium tuberculosis Dihydrofolate Reductase Is Not a Target Relevant to the Antitubercular Activity of Isoniazid[∇]

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Mycobacterium tuberculosis enoyl-acyl-ACP reductase (InhA) has been demonstrated to be the primary target of isoniazid (INH). Recently, it was postulated that *M. tuberculosis* dihydrofolate reductase (DHFR) is also a target of INH, based on the findings that a 4R-INH-NADP adduct synthesized from INH by a nonenzymatic approach showed strong inhibition of DHFR *in vitro*, and overexpression of *M. tuberculosis* *dfrA* in *M. smegmatis* conferred a 2-fold increase of resistance to INH. In the present study, a plasmid expressing *M. tuberculosis* *dfrA* was transformed into *M. smegmatis* and *M. tuberculosis* strains, respectively. The transformant strains were tested for their resistance to INH. Compared to the wild-type strains, overexpression of *dfrA* in *M. smegmatis* and *M. tuberculosis* did not confer any resistance to INH based on the MIC values. Similar negative results were obtained with 14 other overexpressed proteins that have been proposed to bind some form of INH-NAD(P) adduct. An *Escherichia coli* cell-based system was designed that allowed coexpression of both *M. tuberculosis* *katG* and *dfrA* genes in the presence of INH. The DHFR protein isolated from the experimental sample was not found bound with any INH-NADP adduct by enzyme inhibition assay and mass spectroscopic analysis. We also used whole-genome sequencing to determine whether polymorphisms in *dfrA* could be detected in six INH-resistant clinical isolates known to lack mutations in *inhA* and *katG*, but no such mutations were found. The *dfrA* overexpression experiments, together with the biochemical and sequencing studies, conclusively demonstrate that DHFR is not a target relevant to the antitubercular activity of INH.

In 1952, isoniazid (INH) was discovered to have bactericidal activity against *Mycobacterium tuberculosis* (4). Since then, it has been used as a potent front-line drug against tuberculosis (5). The mechanism of action of INH has been studied for more than 50 years. Through lipid profiling, INH was found to inhibit mycolic acid biosynthesis in *M. tuberculosis* (28). In addition, the INH-induced inhibition of mycolic acid biosynthesis was demonstrated to correlate with the bactericidal activity of INH (21). Further analysis of the lipids of INH-treated *M. tuberculosis* indicated that the elongation of fatty acids beyond C26 was inhibited, which suggested that the target of INH is an enzyme in fatty acid elongation (20).

INH is a prodrug that must first be activated by KatG, an endogenous catalase/peroxidase (29). The mode of INH action remained unclear until an INH-NAD adduct was identified as the bound inhibitor in the active site of InhA, the enoyl-acyl ACP reductase involved in long-chain fatty acid biosynthesis, by protein crystallography (18). It was hypothesized that KatG cleaves the hydrazide on INH to an isonicotinoyl radical, which then reacts with NAD to form an adduct that binds to and

inhibits InhA (23). The crystal structure of InhA bound with the adduct indicates that an isonicotinoyl moiety was covalently attached to the 4-position of the nicotinamide ring of NAD cofactor in an S configuration. The chemical structure of the INH-NAD adduct was found to be consistent with the molecular weight obtained by the mass analysis (18). Later studies demonstrated that INH-NAD adduct could be generated by a KatG-catalyzed oxidation in the presence of NAD⁺ (13, 27), which strongly inhibits InhA ($K_i = 5$ nM) to block mycolic acid biosynthesis (18, 25, 17).

Mutations within the protein-coding and promoter regions of *inhA* are frequently observed in clinical isolates resistant to INH (7, 22). An S94A mutation in InhA, which was originally identified in an INH-resistant *M. smegmatis* strain, was later found in three *M. tuberculosis* clinical isolates that conferred resistance to both INH and ethionamide (ETH) (16, 3). The S94A allele of *inhA* has been transferred into *M. tuberculosis* by a specialized linkage transduction, which was sufficient to confer 5-fold resistance to INH (25). Moreover, overexpression of *inhA* in *M. tuberculosis* was found to confer >10-fold resistance to INH (12). These genetic observations support that InhA is the primary target of INH.

Although genetic and biochemical studies have provided convincing evidence that InhA is the primary target of INH, other putative targets of INH have also been proposed (15, 24). Recently, 17 proteins other than InhA were identified

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from *M. tuberculosis* lysate that could tightly bind to an affinity matrix derived from INH-NADP or INH-NAD adducts by proteomic analysis (1). Among these proteins, *M. tuberculosis* dihydrofolate reductase (DHFR) was shown to be strongly inhibited by an INH-NADP adduct *in vitro* ($K_{i\text{ app}} = 1\text{ nM}$) in a separate study (2). This INH-NADP adduct was synthesized by incubating INH and NADP⁺ in the presence of Mn(III) as a catalyst. The crystal structure of the complex indicated that an acyclic 4R INH-NADP adduct was selectively bound in the active site of DHFR. In addition, overexpression of *dfrA* in *M. smegmatis* caused a 2-fold increase of resistance to INH compared to the wild-type (2). These observations were taken to suggest that *M. tuberculosis* DHFR is also a target of INH (1, 2).

There are several observations which conflict with the proposal that DHFR might be a target of INH. First, the MIC difference (2-fold) between the *dfrA* overexpressed *M. smegmatis* strain and the wild-type strain is not significant compared to the error of this type of experiment. Second, *dfrA* has not been clearly shown to be essential to *M. tuberculosis*. Although it is an essential gene for nucleotide biosynthesis in many other organisms (10, 6), disruption of *dfrA* by transposon mutagenesis was found not to attenuate *M. tuberculosis* infection in mice (19). Third, the endogenous formation of the 4R INH-NADP adduct by KatG catalysis has not been demonstrated. To determine whether *M. tuberculosis* DHFR is a molecular target of INH, we investigated (i) whether overexpression of *dfrA* under relevant conditions confers resistance to INH in *M. smegmatis* and *M. tuberculosis*, (ii) whether the INH-NADP adduct is an activated INH product generated by KatG catalysis inside the cell, and (iii) whether mutations in *dfrA* could be observed in INH-resistant clinical isolates.

MATERIALS AND METHODS

Cloning, expression, and purification. The *M. tuberculosis dfrA* and *katG* genes were cloned as previously described (2). The plasmids of *M. tuberculosis katG* and *dfrA* were singly and doubly transformed into *E. coli* BL21(DE3) (EMD Bioscience, catalog no. 69387-3). The strain containing plasmids of *katG* and *dfrA* was cultured in LB-Miller medium containing 50 µg of kanamycin/ml and 50 µg of carbenicillin/ml at 37°C until the optical density (OD) at 600 nm reached 0.5. Expression of both genes was carried out by induction for 20 h at 18°C by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). At the same time of induction, 100 µg of INH/ml was also added to the culture. The same protocol was used for the strain containing just the *dfrA* plasmid. Recombinant *M. tuberculosis* DHFR was purified according to a previously described method (2).

DHFR and KatG enzymatic activity assay. All assays were carried out on a Cary 100 Bio Spectrophotometer at 25°C. DHFR assays are performed by monitoring the oxidation of NADPH and reduction of dihydrofolate (DHF) at 340 nm. Reactions were initiated by adding DHFR (10 nM) to assay mixtures containing NADPH (10 µM), DHF (4.5 µM), and phosphate buffer (pH 7.5, 50 mM). The KatG activity was assayed as previously described (31).

Mass spectroscopy analysis. Purified DHFR was heated for 60 s at 100°C. After the heat treatment, denatured enzyme was separated by filtration, using a Centricon (cutoff, 3 kDa). The filtrate was used for mass analysis. The matrix-assisted laser desorption/ionization (MALDI) mass spectrometry experiment was carried out on an ABI Voyager-DE STR.

Crystallization of InhA in complex with INH-NAD adduct. Crystallization was accomplished by the hanging-drop vapor diffusion method. *M. tuberculosis* InhA in complex with INH-NAD was cocrystallized in hanging droplets containing 2 µl of protein solution at 10 mg/ml and with 2 µl of buffer (12% 2-methyl-2,4-pentanediol, 4% dimethyl sulfoxide, 0.1 M HEPES, and 0.025 M sodium citrate) at 16°C in Linbro plates against 1 ml of the same buffer. Diamond shaped protein crystals formed ~4 days later.

T7-based expression system for mycobacteria. (i) **Construction of *M. smegmatis* expression strain, mc²4517.** pT7Pol26 was digested with BamHI to excise the RNA polymerase gene of bacteriophage T7. The 2.7-kb BamHI DNA fragment was ligated to BamHI-digested pSD26 plasmid, and the orientation was confirmed by digestion with the appropriate restriction enzymes. The DNA fragment of an acetamidase promoter fused to T7 polymerase was then cloned into pMV306, to generate pYUB1232. The expression strain mc²4517 was obtained after electroporating pYUB1232 into *M. smegmatis* mc²155.

(ii) **Construction of expression plasmids.** pYUB1062, the parental expression plasmid, was constructed by combining the NarI/SspI DNA fragment (948 bp) from pET30a plasmid and the NarI/EcoRV DNA fragment (4,026 bp) of pMV206. Shuttle vector pYUB1062 contains an *oriE* for replication in *E. coli*, *oriM* for replication in mycobacteria, a hygromycin cassette for antibiotic selection, a T7 promoter/terminator, and a histidine tag region for the purification of overproduced protein. The various *M. tuberculosis* genes were cloned into pYUB1062 using NdeI or NcoI as the upstream site and an appropriate downstream site.

(iii) **Expression of INH-NAD- or INH-NADP-binding proteins from *M. tuberculosis*.** *M. smegmatis* mc²4517 cells were transformed with plasmids expressing INH-NAD- or INH-NADP-binding protein of *M. tuberculosis*. A single bacterial colony was grown in all cases at 37°C to an A_{600} of 0.6 to 0.8 in Middlebrook 7H9 broth medium supplemented with 0.2% Tween 80, 0.05% glycerol, kanamycin (20 µg/ml), and hygromycin B (100 µg/ml). Cell cultures were diluted 1:100 in 50 ml of the same liquid medium and cells were grown to mid-exponential phase ($A_{600} = 0.5$ to 0.6) at 37°C at 120 rpm before induction with acetamide. The bacterial cultures were divided into equal volumes, and 0.2% (wt/vol) acetamide was added to the one of the aliquots (the other aliquot was used as a control). Expression of protein was determined after 6 h of induction by analyzing the cell-free protein extract by 10 to 20% SDS-PAGE.

MIC determination. *M. smegmatis* cells expressing the *M. tuberculosis* proteins were grown to an OD of 0.6 and then induced with 0.2% acetamide for ~6 h until the OD reached ~1.0. The cells were then diluted by 10⁵-fold and incubated in 200 µl of 7H9 medium containing INH (0, 0.6, 1.2, 4.7, 9.4, 18.6, 37.5, 75, 150, and 300 µg/ml) in a 96-well plate. The plates were incubated at 37°C and observed after 72 h. The MIC was determined as the concentration of drug at which no visible growth was observed. MICs for *M. tuberculosis* strains were determined by using an MTT assay (14). The concentrations of INH tested were 0, 0.015, 0.03, 0.06, 0.12, 0.24, 0.48, 0.96, 1.92, and 3.84 µg/ml.

Sequencing of clinical isolates. Six INH-resistant clinical isolates (strains 5071, 5072, 5297, 5324, 5358, and 5400) were obtained for whole-genome sequencing. The clinical isolates were collected from Mexico between 1991 and 1996 by the Laboratory of Clinical Microbiology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, under an IRB-approved protocol. Five of the six strains were genotyped as belonging to SNP cluster group SCG_3b, whereas strain 5358 belongs to the *M. bovis* family based on spoligotyping. MICs for INH ranged from 0.2 to 8.0 µg/ml compared to 0.03 µg/ml for H37Rv.

Genomic DNA was extracted by using a CTAB (cetyltrimethylammonium bromide) protocol as previously described (11). The DNA library was constructed by using a genomic DNA sample preparation kit (Illumina). The sample was first fragmented by using a nebulization technique. Then, the double-stranded DNA fragments comprised of 3' or 5' overhangs were converted into blunt ends, using T4 DNA polymerase and Klenow enzyme. Klenow 3'-to-5' exonuclease minus was used to add an "A" base to the phosphorylated 3' blunt end of the DNA fragments so that the fragments could be ligated to the adaptors, which have a single "T" base overhang at their 3' end. The ligated DNA was then size selected on a 2% agarose gel. DNA fragments of ~300 bp were excised from the preparative portion of the gel. DNA was then recovered by using a Qiagen gel extraction kit and PCR amplified to produce the final DNA library. A total of 5 pmol of DNA from each strain was loaded onto a different lane of the sequencing chip (eight lanes total), and the clusters were generated on the cluster generation station of a Genome Analyzer II using the Illumina Cluster generation kit. Bacteriophage φX174 DNA was used as a control.

The sequencing reaction was run for 36 cycles (tagging, imaging, and cleavage of one terminal base at a time), and four images of each tile on the chip were taken in different wavelengths for exciting each base-specific fluorophore. Image analysis and base-calling were done by using v0.3 of the Illumina GA Pipeline software.

The 36 bp reads that were generated for each strain were mapped (aligned) against H37Rv as a reference sequence (or *M. bovis* in the case of strain 5358, which was the closest reference strain by spoligotyping). Apparent differences (at sites where the consensus base from overlapping reads differed from the expected base in the reference sequence), along with sites where coverage was low or observed bases were heterogeneous, were identified, and local contig-building

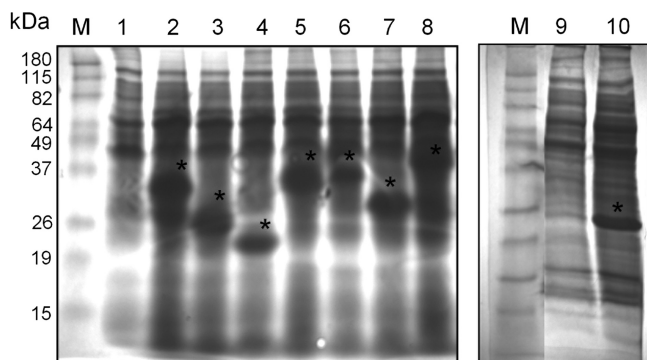


FIG. 1. Overexpression of the proteins in *M. smegmatis* confirmed by SDS-PAGE analysis. Lanes: M, marker; 1, vector; 2, Rv0155 (PntAA, 40 kDa); 3, Rv1484 (InhA, 29.5 kDa); 4, Rv2763c (DfrA, 17.4 kDa); 5, Rv1996 (34.9 kDa); 6, Rv2623 (32.7 kDa); 7, Rv2766c (FabG5, 30.8 kDa); 8, Rv2858 (AldC, 50 kDa); 9, uninduced Rv2763c (DfrA); 10, induced Rv2763c (DfrA).

was used to resolve them into SNPs versus indels, based on alignment to the corresponding region in the reference genome.

RESULTS

Overexpression of *dfrA* does not confer resistance to INH.

The most direct evidence to support that DHFR is a target of INH comes from the observation that overexpression of *dfrA* in *M. smegmatis* conferred a 2-fold increase in resistance to INH (2). Therefore, we first reexamined whether overexpression of this protein using a strong promoter in *M. smegmatis* would lead to increased resistance to INH. Use of cosmids harboring a gene on a multicopy plasmid along with its native promoter might be a desired approach to overexpress putative targets. However, this can result in a failure to observe overexpression whether the expression of the protein is from a tightly regulated promoter. To eliminate the promoter-specific effect, a novel T7-based expression system was developed for the efficient expression of proteins in mycobacteria. In brief, an *M. smegmatis* strain (mc²4517) was generated by integrating a plasmid expressing the T7 polymerase under the acetamidase-inducible promoter into the genome at the mycobacteriophage L5 attachment site. *M. tuberculosis* genes were cloned under the T7 promoter on a separate episomal plasmid pYUB1062. Protein expression was induced by the addition of 0.2% acetamide. To ensure overexpression of *dfrA* and *inhA*, protein lysates were analyzed on SDS gels, and a high level of expression was observed within 6 h of induction as a distinct band corresponding to the expected size compared to the uninduced control (Fig. 1). The strains overexpressing *dfrA* (mc²5097) and *inhA* (mc²5089) were then tested for their susceptibility to INH and ETH and the results obtained are summarized in Table 1. The MIC for the *inhA* overexpression strain mc²5089 increased more than 60-fold to 300 μg/ml for INH, compared to the wild-type strain. In contrast to InhA, we did not observe any increase in MIC for mc²5097, the strain overexpressing DHFR, which had been reported to confer 2-fold resistance to INH when induced in *M. smegmatis* with an acetamidase promoter (2). Other than DHFR and InhA, 16 additional proteins from *M. tuberculosis* were identified in a previous study (1) that bound to an INH-NAD or INH-NADP adduct *in vitro*. We

TABLE 1. MICs determined in 7H9 broth by serial dilution method after overexpressing the INH-NAD binding proteins from *M. tuberculosis* H37Rv in *M. smegmatis*

Overexpressed protein	MIC (μg/ml) ^a	
	INH	ETH
None (mc ² 4517 parental strain)	4.7	9.4
Rv1484 (InhA)	300	150
Rv2763c (DfrA)	4.7	9.4
Rv3248c (SahH)	4.7	9.4
Rv0753c (MmsA)	4.7	9.4
Rv1187 (RocA)	4.7	9.4
Rv0155 (PntAA)	4.7	9.4
Rv2623 (universal stress protein)	4.7	9.4
Rv1996 (universal stress protein)	4.7	9.4
Rv0468 (FadB2)	4.7	9.4
Rv2691 (CeoB/TrkA)	4.7	9.4
Rv0091 (Mtn/Sah)	NA	NA
Rv2858c (AldC)	4.7	9.4
Rv1059 (unknown)	4.7	9.4
Rv0926c (unknown)	4.7	9.4
Rv3777 (probable oxidoreductase)	4.7	9.4
Rv2971 (probable oxidoreductase)	NA	NA
Rv2766 (FabG5)	4.7	9.4
Rv2671 (RibD)	4.7	9.4

^a NA, not available (overexpression was not successful).

applied the same T7 promoter system to successfully overexpress 14 of these genes in *M. smegmatis*, but none of them could confer resistance to INH or ETH (Table 1). Since this analysis was done in liquid culture, we also tested the ability of the strains to grow on plates with two times the MIC for INH. As shown in Fig. 2, the *M. smegmatis* strain containing the overexpressed DHFR (mc²5072) failed to grow on an INH-containing plate, while the strain overexpressing InhA (mc²5071) showed complete resistance. To find out whether DHFR was indeed expressed, the strain containing the over-

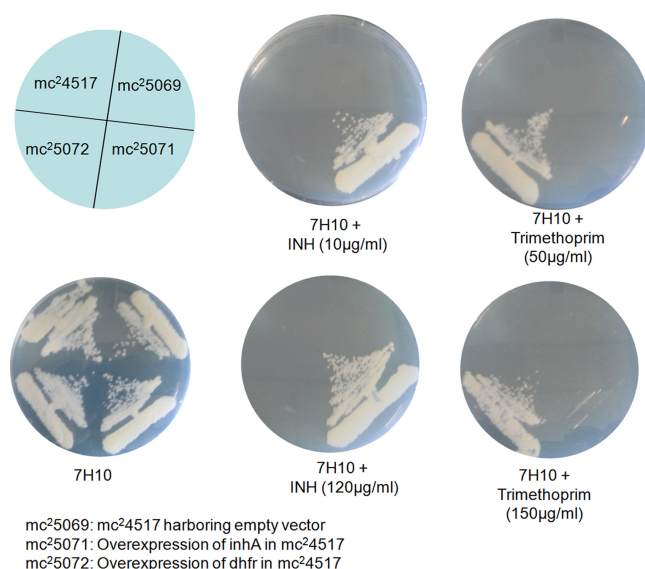


FIG. 2. Growth of the wild-type *M. smegmatis* strain and transformed strains overexpressing DHFR or InhA on media containing the known DHFR inhibitor trimethoprim (50 or 150 μg/ml) or INH (10 or 120 μg/ml).

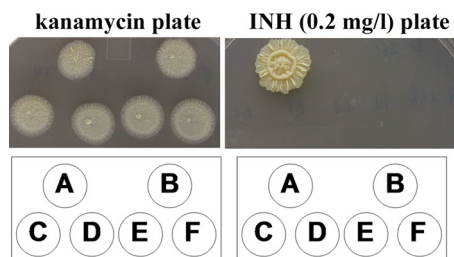


FIG. 3. Growth of *M. tuberculosis* H37Rv strains overexpressing DHFR or *InhA* on media containing INH (0.2 $\mu\text{g/ml}$) or kanamycin (100 $\mu\text{g/ml}$). Spots: A, pMV261::*inhA* (*InhA* overexpresser); B, pMV261 (plasmid only); C to F, four independent transformants of pMV261::*dfrA* (DHFR overexpresser).

expressed DHFR was plated on medium containing the known DHFR inhibitor trimethoprim. Strain mc²5072 (DHFR overexpresser) was able to grow on medium containing 50 or 150 μg of trimethoprim/ml. In contrast, the parental strain mc²5069 or the strain overexpressing *InhA* (mc²5071) failed to grow on trimethoprim-containing plates. One possible explanation for the difference between our findings and those reported previously (2) might be that the growth inhibition experiment in the previous study was carried out at 30°C, which is not optimal for growth, whereas the growth inhibition experiments in our study were performed at 37°C. Our observation that the overexpression of *M. tuberculosis* *InhA* but not DHFR confers resistance to INH and ETH in *M. smegmatis* using this new expression system is consistent with *InhA* being the primary target of INH and ETH. Even though DHFR has been shown to bind an INH-NADP adduct *in vitro*, this phenomenon is not sufficient to confer INH or ETH resistance in mycobacterial cells.

A second overexpression experiment was conducted using the H37Rv strain of *M. tuberculosis*, which is more disease relevant, compared to *M. smegmatis*. The *M. tuberculosis* H37Rv strain was transformed with the pMV261::*dfrA* plasmid. The MIC of INH for the *M. tuberculosis* strain overexpressing *dfrA* is identical to that of the wild-type H37Rv strain (0.06 $\mu\text{g/ml}$). On plates with three times the MIC for INH, no growth of the *M. tuberculosis* strain overexpressing *dfrA* was observed. In contrast, the *M. tuberculosis* strain overexpressing *inhA* survived (Fig. 3), which is consistent with the previous report that the MIC for *M. tuberculosis* overexpressing *inhA* (1.0 $\mu\text{g/ml}$) increased ~20-fold compared to the wild-type H37Rv strain (MIC = 0.06 $\mu\text{g/ml}$) (12). Therefore, similar to what we observed in our *M. smegmatis* experiment, overexpression of *dfrA* did not prevent growth inhibition of *M. tuberculosis* by INH, which genetically demonstrates that *M. tuberculosis* DHFR is not a relevant target of INH.

INH-NADP adduct formation was not observed in an *E. coli*-based activation system coexpressing *katG* and *dfrA*. In the previous study, a synthetic INH-NADP adduct derived from INH demonstrated strong inhibition of *M. tuberculosis* DHFR *in vitro* (K_i app = 1 nM) (2). However, the INH-NADP adduct was synthesized by using an inorganic catalyst, Mn(III). Thus, the yield of adduct generated from this approach might not truly reflect an enzyme-mediated process inside the cell. To better mimic the *in vivo* activation of INH, a cell-based acti-

vation system was designed to examine the KatG-catalyzed adduct formation and the inhibition of DHFR by the adduct. This *E. coli*-based activation system is similar to the one used previously to activate the prodrugs ETH and protonamide (PTH) (26). In this system, *katG* and *dfrA* were coexpressed in *E. coli* in the presence of INH to investigate whether the activated drug would inhibit DHFR. To construct this system, *katG* and *dfrA* were cotransformed into the *E. coli* BL21(DE3) strain and selected on 50 μg of kanamycin and carbenicillin/ml. The *E. coli* strain containing *katG* and *dfrA* genes was grown and induced in the presence and absence of INH, respectively. After the coexpression of both genes was confirmed by SDS-PAGE, recombinant KatG and DHFR proteins were readily purified.

Mass spectroscopy was used to determine whether DHFR purified from the experimental sample was bound with any inhibitor. We have previously applied mass spectroscopy to identify the INH-NAD, ETH-NAD, and PTH-NAD adducts bound to *InhA* (18, 26). Before the mass spectroscopic analysis, the purified DHFR was concentrated and then denatured, followed by filtration to separate small molecules from the denatured protein. The MALDI mass spectrum of the filtrate, which ranged between 200 and 1,200 Da, was carefully analyzed. We were not able to identify any compound that has a molecular mass corresponding to an INH-NADP adduct. The peaks shown on the spectrum most likely resulted from the cofactor NADP and some small fragments of the protein.

An enzyme assay was performed to determine the activity of purified DHFR. DHFR isolated from the experimental sample was found to be fully active (specific activity of 12 $\mu\text{mol mg}^{-1} \text{min}^{-1}$) compared to the enzyme purified from expression in the absence of INH. Since the acyclic 4R INH-NADP adduct is extremely potent against DHFR *in vitro*, it would tightly bind to DHFR if the adduct is indeed generated by KatG catalysis inside the cell. However, both the activity assay result and the mass analysis indicated that no detectable amount of the INH-NADP adduct had bound to DHFR. The coexpressed KatG from the same experimental sample was purified and assayed for its activity *in vitro*. The specific catalase activity of isolated KatG was 17 $\text{mol mg}^{-1} \text{min}^{-1}$, a finding comparable to published data (21 $\text{mol mg}^{-1} \text{min}^{-1}$) (13), which confirmed that the lack of the INH-NADP adduct did not result from the absence of KatG activity. Therefore, the acyclic 4R INH-NADP adduct is not an activated INH product generated by KatG catalysis inside the *E. coli* cell-based system.

INH-NAD adduct was detected in the *E. coli* based activation system coexpressing *katG* and *inhA*. It has been shown that KatG activates INH and catalyzes the formation of an INH-NAD adduct *in vitro* (23, 27). In order to demonstrate that this KatG-catalyzed INH-NAD adduct formation and its inhibition of *InhA* can be reproduced in the *E. coli*-based system, both *katG* and *inhA* were transformed into *E. coli* and coexpressed in the presence of INH. *InhA* was rapidly purified by a Ni-NTA affinity column, and an *in vitro* enzyme assay was performed. *InhA* isolated from the experimental sample had <15% of the specific activity of *InhA* purified without the addition of INH under the same assay condition. The bound inhibitor was isolated by denaturing the *InhA* purified from the experimental sample, and a 1 μM concentration of the inhibitor led to complete inhibition of native *InhA*. The crystal

TABLE 2. Mutations in genes identified as potential INH-NAD(P) binders^a

Protein	Mutation in strain:							<i>M. bovis</i> BCG
	5071	5072	5297	5324	5358	5400		
Rv2763c/ <i>dfrA</i>	None	None	None	None	None	None	None	None
Rv3248c/ <i>sahH</i>	None	None	None	None	None	None	None	None
Rv0753c/ <i>mmsA</i>	None	None	None	None	None	None	None	None
Rv1187/ <i>rocA</i>	None	None	None	None	None	None	None	None
Rv0155/ <i>pntAa</i>	A274A	None	None	None	None	None	None	None
Rv2623	None	None	None	None	None	None	None	None
Rv1996	None	None	None	None	None	None	None	None
Rv0468/ <i>fadB2</i>	None	None	None	None	L87L	None	None	L87L
Rv2691/ <i>ceoB</i>	T117A	T117A	T117A	T117A	T117A	T117A	T117A	T117A
Rv0091/ <i>mtn</i>	None	A155P	None	Q174Q	None	931-bp deletion	None	None
Rv2858c/ <i>aldC</i>	None	None	T21A	None	P77P	None	None	P77P
Rv1059	a-64c ^b	1-bp deletion in P99	None	None	None	None	None	None
Rv3777	None	None	P101A	None	None	None	None	None
Rv3777	V160A	V160A	V160A	V160A	V160A	V160A	V160A	V160A
Rv3777	L63L	L63L	L63L	L63L	L63L	L63L	L63L	L63L
Rv0926c	None	None	None	None	None	None	None	None
Rv2766c/ <i>fabG5</i>	None	None	None	None	None	None	None	None
Rv2671/ <i>ribD</i>	None	None	None	None	None	None	None	None
Rv2971	None	None	None	None	N152H	None	None	N152H

^a Argyrou et al. (1). Mutations are indicated relative to the amino acid in the H37Rv reference sequence. Mutations that are shared with *M. bovis* BCG are assumed not to cause isoniazid resistance.

^b Single nucleotide polymorphism in promoter region.

structure of InhA in complex with the inhibitor was solved to 2.4-Å resolution. In the active site of InhA, an unbiased electron density map clearly indicated the presence of a modified NAD with an isonicotinic-acyl group covalently attached to the 4-position of the nicotinamide ring in a 4S configuration, which is consistent with the previously identified INH-NAD adduct (18). This is the first time that the activation of INH by KatG and the formation of the INH-NAD adduct has been demonstrated in a whole-cell environment. This confirms that our *E. coli* cell-based system is capable of activating the prodrug INH.

Absence of polymorphisms in *dfrA* in INH-resistant clinical isolates. As an alternative approach to determining whether DHFR might be a relevant target of INH, we used whole-genome sequencing to determine whether mutations in *dfrA* could be observed in INH-resistant clinical isolates. We sequenced the genomes of six clinical isolates that were INH resistant but known not to have relevant mutations in *inhA*, *katG*, or their promoters. The six strains were sequenced by using an Illumina Genome Analyzer II (see Materials and Methods). The depth of coverage (i.e., the number of reads overlapping each site, averaged over the whole genome) was 36.9x to 49.4x for each strain, providing high confidence in the final base calls. This method has been used successfully to identify known and novel mutations associated with resistance to INH, rifampin, pyrazinamide, kanamycin, ofloxacin, streptomycin, etc., in many other strains sequenced in our lab.

Coding regions for the 17 putative INH-NADH-binding proteins identified earlier (1) were searched for polymorphisms. The 100-bp regions upstream of the operon containing each gene were also searched for possible SNPs in the promoter region that might affect expression level. None of the six clinical isolates harbored any mutations in *dfrA* or its promoter (Table 2). Furthermore, few nonsynonymous mutations were found in any of the other 16 genes identified as potential proteins that can bind an INH-NAD(P)H adduct. Strain 5358

showed the most mutations, due to its evolutionary distance from the others (5358 belongs to the *M. bovis* family), but most of the mutations in strain 5358 were also observed in the genome for INH-sensitive *M. bovis* BCG, implying that they are not responsible for INH resistance. The only sites where there are potentially relevant differences (i.e., nonsynonymous and not in BCG) are a 931-bp deletion of *mtn* in 5400, *aldC*(T21A) in 5297, the a-64c promoter mutation upstream of Rv1059, the 1-bp frameshift mutation in Rv1059 in 5297, and Rv3777:P101A in 5297.

For completeness, other genes traditionally associated with INH resistance were also searched, including *katG*, *iniC*, *ndh*, *ahpC*, etc. (8), but no known mutations responsible for INH resistance could be identified (Table 3). Two strains showed mutations in *katG* (F129S in strain 5400 and G285V in strain 5324), and one strain has a mutation in *efpA* (Q513R in strain 5324). None of these mutations has been previously reported in INH-resistant strains. The *katG* F129S mutation is located at the dimer interface, and the *katG* G285V mutation is located at the mouth of the active site entrance, so their effect on INH activation is unclear. Mutations in *mshA*, which catalyzes the first step in mycothiol biosynthesis, have also been linked with INH and ETH resistance, but the N111S allele appears in the Erdman strain, which is as susceptible to these drugs as H37Rv (25a). Although the true cause of INH resistance in these six strains remains undetermined, the lack of mutations in *dfrA* and the 16 other putative INH-NAD(P)-binding proteins suggests that they are unlikely to be targets of INH. We also have sequenced the *dfrA* gene in six additional *M. tuberculosis* strains known to be INH-resistant but with no known relevant mutations. None of these had a mutation in the *dfrA* gene. Consistent with our findings, a recent large-scale screening of 127 INH-resistant *M. tuberculosis* isolates from Singapore by PCR sequencing also revealed no mutations within *dfrA* (9).

TABLE 3. Mutations in genes traditionally associated with INH resistance^a

Protein	Mutation(s) in strain:						
	5071	5072	5297	5324	5358	5400	<i>M. bovis</i> BCG
Rv1484/ <i>inhA</i>	None	None	None	None	None	None	None
Rv1908c/ <i>katG</i>	None	None	None	G285V	R463L	F129S	R463L
Rv1909c/ <i>furA</i>	None	None	None	None	A46V	None	A46V
Rv1854c/ <i>ndh</i>	None	None	None	None	G313R	None	G313R
Rv0342/ <i>iniA</i>	None	None	None	None	N88S, H481Q	None	N88S, H481Q
Rv0341/ <i>iniB</i>	None	None	None	None	None	None	None
Rv0343/ <i>iniC</i>	None	None	None	None	None	None	None
Rv3139/ <i>fadE24</i>	None	None	None	None	None	None	None
Rv2245/ <i>kasA</i>	None	None	None	None	None	None	None
Rv2246/ <i>kasB</i>	None	None	None	None	None	None	None
Rv2428/ <i>ahpC</i>	None	None	None	None	None	None	None
Rv2242	None	None	None	None	A363T	None	A363T
Rv0340	None	None	None	None	None	None	None
Rv1592c	I322V	I322V	I322V	I322V	I322V	I322V	I322V
Rv1772	None	None	None	None	Deletion	None	Deletion
Rv2846c/ <i>efpA</i>	None	None	None	Q513R	T15R	None	T15R
Rv0486/ <i>mshA</i>	N111S	N111S	None	N111S	None	N111S	None

^a Mutations are indicated relative to the amino acid in the H37Rv reference sequence. Synonymous mutations are excluded.

DISCUSSION

To validate a target of a bactericidal drug, it is necessary to show that binding of the drug to the putative target causes inhibition of biological activity, which leads to the death of the cell. In the case of INH, it is more complicated to elucidate its mechanism of action since INH is a prodrug. It is necessary to not only demonstrate that the putative target is relevant to the bactericidal activity of INH but also show how the active form of INH inhibits the target protein. It has recently been postulated that *M. tuberculosis* DHFR might be a target of INH, mainly based on two independent observations: (i) a 4R INH-NADP adduct, synthesized by a nonenzymatic approach, showed strong inhibition of DHFR *in vitro*; and (ii) overexpression of *dfrA* in *M. smegmatis* conferred resistance to INH (2). However, the 4R INH-NADP adduct has not been demonstrated to be generated as a product of INH inside the cell by KatG activation. By coexpressing *katG* and *dfrA* genes in *E. coli* cells in the presence of INH, we showed that the DHFR protein isolated from the experimental sample was not bound with the INH-NADP adduct. This demonstrates that the 4R INH-NADP adduct is not generated by KatG catalysis. In contrast, KatG has been shown to generate the 4S isomer of INH-NAD endogenously, which has been shown to bind to the active site of InhA (18). Thus far, KatG is the only identified activator of INH, and it is very unlikely that INH could be activated by another unknown protein to form the INH-NADP adduct in *M. tuberculosis*. Therefore, we conclude that the synthetic INH-NADP adduct is not biologically relevant to INH inhibition. We also examined the ability of overexpressed *dfrA* to confer resistance to INH in *M. smegmatis* and *M. tuberculosis*. We found that at 37°C, overexpression of *dfrA* in both *M. smegmatis* and *M. tuberculosis* did not confer any resistance to INH. Although *dfrA* overexpression was previously found to confer low-level resistance in *M. smegmatis* (2), that experiment was carried out at a temperature (30°C) which is not optimal for cell growth. If DHFR were a target of INH, then it might be expected that mutations might occur in *dfrA* in INH-resistant clinical isolates lacking mutations in *inhA* or

katG. Although mutations in *inhA* or *katG* and their promoters account for most cases of resistance to INH, the remaining 10 to 25% of cases must have mutations in other genes relevant to INH toxicity (30). In the present study, we sequenced clinical isolates selected from this subpopulation but did not observe any mutations in *dfrA*. Taken together, these pieces of evidence refute the hypothesis that DHFR or any of the other INH-NAD(P)-binding proteins is a biologically relevant target for INH in *M. tuberculosis*.

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