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Proteome-wide Profiling of Isoniazid Targets in *Mycobacterium tuberculosis*

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Abstract

Isoniazid (INH) is an essential drug used to treat tuberculosis. The mycobactericidal agents are INH adducts (INH-NAD(P)) of the pyridine nucleotide coenzymes, which are generated *in vivo* after INH activation, and which bind to, and inhibit, essential enzymes. The NADH-dependent enoyl-ACP reductase (InhA) and the NADPH-dependent dihydrofolate reductase (DfrA) have both been shown to be inhibited by INH-NAD(P) adducts with nanomolar affinity. In this paper, we profiled the *M. tuberculosis* proteome using both the INH-NAD and INH-NADP adducts coupled to solid supports and identified, in addition to InhA and DfrA, sixteen other proteins that bind these adducts with high affinity. The majority of these are predicted to be pyridine nucleotide-dependent dehydrogenases/reductases. They are involved in many cellular processes including *S*-adenosylmethionine-dependent methyl transfer reactions, pyrimidine and valine catabolism, the arginine degradative pathway, proton and potassium transport, stress response, lipid metabolism, and riboflavin biosynthesis. The targeting of multiple enzymes could, thus, account for the pleiotropic effects of, and powerful mycobactericidal properties of, INH.

The powerful and specific antitubercular effects of isoniazid (isonicotinic acid hydrazide, INH) were discovered in 1952, and revolutionized the treatment of tuberculosis (1-3). INH continues to be extensively used in the treatment of the disease to this day; singly in prophylaxis or in a multi-drug combination with rifampicin, pyrazinamide and ethambutol for active infections.

The mechanism by which this simple compound exerts its powerful effect on *Mycobacterium tuberculosis* (minimum inhibitory concentration, MIC = 0.02–0.05 µg ml⁻¹) began to be unraveled in the early 1990's shortly after genetic tools for mycobacteria were being developed. Strains of *M. tuberculosis* and *Mycobacterium smegmatis* that were resistant to high levels of INH were first shown to have deletions or point mutations in the *katG* gene, which led to the proposal that INH was a pro-drug that was oxidatively activated by the *katG*-encoded mycobacterial catalase-peroxidase (4). In a second genetic study, a spontaneous *M. smegmatis* mc²155 mutant and a *Mycobacterium bovis* BCG mutant that were co-resistant to

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SUPPORTING INFORMATION AVAILABLE

Proteomic analysis of INH-NAD(P)-binding proteins (Supplementary Table 1). This information is available free of charge via the Internet at <http://pubs.acs.org>.

INH and ethionamide, a structural analog of INH, were both shown to have a point mutation (S94A) in the *inhA*-encoded enoyl-ACP reductase (5). Furthermore, resistance was observed through overexpression of wild-type *InhA* or the S94A mutant in *M. smegmatis* mc²155, which led to the identification of *InhA* as a target for INH (5).

A consensus on the mechanism of action of, and resistance to, INH has emerged over the past few years. The oxidation of INH by KatG generates an isonicotinoyl radical that reacts non-enzymatically with cellular pyridine nucleotides to generate an ensemble of isonicotinoyl-NAD(P) adducts (INH-NAD(P); Figure 1) (6). Of the twelve possible adducts of INH, only two have thus far been shown to inhibit essential enzymes. The acyclic 4*S* isomer of INH-NAD (compound 1, Figure 1) is known to be a slow-onset, tight-binding inhibitor of *InhA* ($K_i^* = 1$ nM) (7,8). Inhibition of *InhA* prevents the elongation of C₂₆ fatty acids by the fatty acid synthase II complex (9,10), and prevents the formation of the mycolic acids that are an important defense against the host immune surveillance and defense system (11,12). We have recently demonstrated that the acyclic 4*R* isomer of INH-NADP (compound 4, Figure 1) inhibits the *dfrA*-encoded dihydrofolate reductase (DHFR) with sub-nanomolar affinity, and acts as a bisubstrate analog (13). DHFR maintains the cellular pool of folic acid in the four-electron reduced form by catalyzing the NADPH-dependent reduction of dihydrofolate (and folate, albeit less efficiently) to tetrahydrofolate (14). By acquiring single-carbon units at the methyl, methylene, and formyl oxidation levels, tetrahydrofolate is an important single-carbon coenzyme donor involved in many important enzymatic reactions, which are necessary for the biosynthesis of nucleic acids, purines, pyrimidines, and several amino acids (14).

Clinical resistance to isoniazid has been increasing rapidly leading to treatment failure and diminished therapeutic outcomes. The appearance of multi-drug resistant tuberculosis (resistance to the two first line drugs, isoniazid and rifampicin) requires the lengthy use of second line drugs that are less effective, more toxic, and more costly than the first line drugs (15). Thus, new anti-tubercular drugs are urgently needed. Effective drug development requires good drug target selection. A powerful way to identify such targets is to identify the targets of existing effective drugs, such as INH against tuberculosis.

Are there other INH targets? The majority of INH-resistant *M. tuberculosis* clinical isolates have mutations in *katG* or *inhA*, however, 10–25 % of these have unknown genotypes (15) suggesting that there may be additional targets. In addition, the complete genome sequence of *M. tuberculosis* (16) suggests that, like other organisms, there are a large number of pyridine nucleotide-dependent reductases and dehydrogenases with substrates that could be mimicked by INH-NAD(P) adducts acting as bisubstrate analogs of these enzymes.

Although mycobacterial genetics has continued to provide insight into the mechanisms of resistance to INH (17-19), it has not yet provided any additional INH targets. Genomic (20-24) and proteomic (25) studies have also been employed to identify genes that show altered levels of expression when mycobacteria are treated with INH. However, the RNA and protein levels of KatG, *InhA*, and DHFR are unaltered after INH treatment and, to date, these studies have failed to provide any new targets for INH. In this paper we employed the old, but extraordinarily powerful, method of affinity chromatography on solid supports to which the INH-NAD and INH-NADP adducts were coupled to effect the selective purification of those enzymes that bind the INH adducts tightly. In addition to *InhA* and DHFR, we identified sixteen other proteins that bind these adducts tightly. These newly identified proteins are potential candidates for drug development that require further validation including gene knock-out studies to assess essentiality, overexpression of these proteins in *M. tuberculosis* to determine if they can confer resistance to isoniazid by drug sequestration, recombinant protein expression and purification, assay development to confirm or assign function and determine the extent of

inhibition of enzyme activity by the INH adducts *in vitro*, and X-ray crystallography structural studies.

MATERIALS AND METHODS

Determination of Protein Concentration

Protein concentration was determined using the bicinchoninic acid assay kit (PIERCE) and bovine serum albumin as standard (26).

Synthesis and Purification of INH-NAD(P)

The mixture of INH-NAD(P) adducts were synthesized and purified as described previously (6,27). The $A_{260\text{ nm}}$ to $A_{324\text{ nm}}$ ratio (an indicator of purity) of the purified products was 3.95–4.00, which is close to the value of 3.94 reported previously for INH-NAD (28). The concentration of INH-NAD(P) was determined from $\epsilon_{324\text{ nm}} = 6900\text{ M}^{-1}\text{ cm}^{-1}$ reported previously for INH-NAD (28).

Preparation of INH-NAD(P)-Sephacrose

Three milliliters of *N*-hydroxysuccinamideactivated Sepharose 4 fast flow resin (Amersham Biosciences) was washed with 10 ml of icecold 1 mM hydrochloric acid. NAD(P)⁺ was first covalently attached via the exocyclic adenine amine to the resin by suspending the beads in 10 ml of 100 mM NAD(P)⁺ dissolved in 100 mM Pipes buffer, pH 7.5. This reaction was allowed to proceed for 20 h at room temperature by end-over-end mixing. Unreacted sites were then blocked by washing the resin twice with 10 ml of 100 mM Tris, pH 8.0 and stored at 4 °C for 24 h. After washing thoroughly with 100 mM sodium phosphate, pH 7.5, INH (2 mM) and Mn(III) pyrophosphate (4 mM) were added to the NAD(P)⁺-Sephacrose resin with gentle magnetic stirring to generate the INH-NAD(P) inhibitor. Mn(III) pyrophosphate (6) was added in one-tenth portions every 2 min (the final resin suspension volume was 15 ml). The INH-NAD(P)-sephacrose suspension was then poured into a 50 ml disposable column (BIO-RAD) and washed extensively with 50 mM sodium phosphate, pH 7.0, containing 100 mM NaCl, and 1 mM EDTA (buffer A). The final bed volume of the columns was 2 ml.

Isolation of INH-NAD(P)-binding Proteins

M. tuberculosis H37Rv whole cell lysate (10 ml of 20 mg ml⁻¹ protein, generously provided by the Tuberculosis Vaccine Testing and Research Materials Contract of the Colorado State University) was supplemented with 2 tablets of complete protease inhibitor cocktail (Roche) and 1 mM EDTA and centrifuged at 20,000g for 30 min at 4 °C. The supernatant was then dialyzed for 2 h against 4 L of buffer A at 4 °C. Half of the dialysate was applied to the INH-NAD(P)-sephacrose column while the other half was applied to the NAD(P)⁺-sephacrose control column without flow. The columns were capped, the resin suspended, and binding of proteins to the resin was allowed to proceed for 2 h at 4 °C by end-over-end mixing. The resin was then allowed to settle and the chromatography procedure initiated at room temperature. After discarding the unbound proteins, the columns were washed with 50 ml of buffer A followed by 20 ml of 1 mM NAD(P)⁺ in buffer A to remove weakly bound dehydrogenases. Two ml of 3.5 mM INH-NAD(P) inhibitor was then applied to the columns and the inhibitor allowed to enter the resin. Flow was stopped and equilibration was allowed to proceed for 1 h. The eluted proteins were collected and combined with the eluate after applying 4 ml of buffer A to the columns. These combined fractions were then concentrated to 50 μ l (~50 μ g total protein) using a centrifugal filter device through a 10 kDa cut-off membrane (Millipore) and the proteins were analyzed by SDS-PAGE followed by silver-staining.

Identification of INH-NAD(P)-binding Proteins

Protein bands were excised from the gels, reduced, alkylated with iodoacetamide, and digested with proteomics-grade trypsin (Promega). The resulting tryptic peptide mixture was then analyzed by liquid chromatography combined with on-line electrospray mass spectrometry. Peptides were separated on a C18 PepMap 100 column (75 $\mu\text{m} \times 15\text{ cm}$; Dionex) at a constant flow-rate of 250 nl min^{-1} as follows: 20 min at 2 % B, 15 min linear gradient from 2 % to 52 % B, and 5 min at 90 % B. Solvent A consists of 2 % (v/v) acetonitrile and 0.08 % (v/v) formic acid while solvent B consists of 80 % (v/v) acetonitrile and 0.1 % (v/v) formic acid. The eluent was directly introduced into the electrospray port of a Finnigan LTQ Linear Ion Trap mass spectrometer (Thermo Electron). The mass spectrometry data were acquired in data dependent mode. The MS survey scan was recorded between m/z 300-2000, which was then followed by MS/MS scans of the three most intense ions. The normalized collision energy was set at 35 %, the repeat count of dynamic exclusion was set at 2 and the exclusion duration at 180 s.

Database Search and Data Analysis

The raw data file was converted into a peaklist using an in-house script (RawFileExtractor), and was then searched against the non-redundant NCBI database for proteins from the *M. tuberculosis* complex using the Mascot search engine. The search criteria were as follows: 2 missed cleavages by trypsin were allowed; carboxamidomethylation of cysteine residues was fixed; modification of methionine residues by oxidation was allowed to vary; the mass tolerance for the peptide was set at 2 Da while the MS/MS tolerance was set at 0.8 Da. Positive protein identification requires a minimum of two unique peptides with peptide ion scores $\geq$ MOWSE score.

RESULTS

Purification of INH-NAD(P)-binding proteins

To isolate proteins from *M. tuberculosis* that bind INH-NAD(P) with high affinity, we covalently linked INH-NAD(P) onto column supports and affinity-purified those proteins from *M. tuberculosis* crude soluble cell extracts that bind these adducts with high affinity (Methods). After extensive washing with buffer and either NAD^+ or NADP^+ , specific elution of INH-NAD(P)-binding proteins was accomplished using the same INH-NAD(P) adduct pool that was covalently attached to the columns. From 100 mg of *M. tuberculosis* crude soluble protein cell extract, approximately 50 μg of total protein was recovered from solid supports containing the covalently coupled INH-NAD(P) adducts, representing an approximately 2000-fold enrichment of proteins that were bound to the coupled adducts. Figure 2 shows silver-stained SDS-PAGE gels of the final INH-NAD(P) protein eluates from the NAD(P)^+ -Sephacrose and INH-NAD(P)-Sephacrose columns.

Identification of INH-NAD(P)-binding proteins

Protein bands from the INH-NAD(P) protein eluates of the INH-NAD(P)-Sephacrose columns were excised for in-gel trypsin digestion followed by analysis of the peptides by liquid chromatography coupled to mass spectrometry (Methods). Table 1 lists the identity of the proteins, their molecular weight, their annotated function, whether they are predicted to bind pyridine nucleotides, and whether they are essential for mycobacterial growth in liquid culture. Supplementary Table 1 gives additional information including the accession numbers, overall protein score, number of unique peptides detected for each protein, the sequence and score for each unique peptide, and the sequence coverage for each protein in Table 1. We note that bands 1, 3, 5, 8, and 10 contain more than one protein species with similar molecular weights that were not resolved by SDS-PAGE (see Table 1).

DISCUSSION

The Power of INH-NAD(P) Affinity Chromatography

Large amounts of *M. tuberculosis* cell extract are difficult to obtain because of the organism's highly infective nature, requiring high-level safety containment, and its unusually slow doubling time of 18–24 h. These realities present significant challenges to performing biochemical characterizations in this organism, and in the present study, we were restricted to a total of approximately 400 mg of *M. tuberculosis* crude soluble protein cell extract to perform all the experiments presented in this paper. We developed a one-step INH-NAD(P)-affinity chromatography purification method (see Methods section) that permitted the highly selective purification and identification of proteins that bind INH-NAD(P) with high affinity (see below), which is similar to a general procedure that has recently been introduced (29). The known three-dimensional structures of the 4*S*-isonicotinoyl- NAD adduct bound to InhA and the 4*R*-isonicotinoyl-NADP adduct bound to DHFR revealed that in these two known targets, there are essentially no interactions between the exocyclic adenine amine of the adducts and the enzyme (8,13). We thus elected to covalently couple the adducts via this position using *N*-hydroxysuccinamide-activated Sepharose (Methods). From 100 mg of *M. tuberculosis* crude soluble protein cell extract, approximately 50 μ g of total protein was recovered from solid supports containing the covalently coupled INH-NAD(P) adducts. This amount of protein, specifically eluted by the identical adduct pool that was covalently bound to the columns, was sufficient to obtain the identity of all the protein bands in the two silver-stained gels shown in Figure 2. We note that because of the stereochemically-uncontrolled nature of the non-enzymatic reaction between activated isoniazid and pyridine nucleotides, as well as the subsequent spontaneous, but reversible, cyclization of the acyclic forms (compounds **1** and **4**; Figure 1) to generate the corresponding cyclic forms (compounds **2**, **3**, **5**, and **6**; Figure 1), the proteins we isolated could, in principle, bind any of the six isomers of INH-NAD(P) shown in Figure 1.

INH-NAD(P) Affinity Chromatography is Highly Selective

To determine the selectivity of our approach, we analyzed the final INH-NAD(P) protein eluates from the INH-NAD(P)-Sepharose as well as from the NAD(P)⁺-Sepharose columns by SDS-PAGE followed by silverstaining (Figure 2). Most of the proteins that bind to the INH-NAD(P) columns are unique when compared to the corresponding proteins that bind to the NAD(P)⁺-Sepharose control columns (lane 2 versus 1, and lane 4 versus 3, Figure 2) demonstrating the high selectivity of this method for the proteins of interest. In addition, the profile of proteins bound to the columns containing covalently bound INH-NAD is completely different from that of the INH-NADP column (lane 2 versus 4, Figure 2) as expected, since most dehydrogenases are specific for either the nonphosphorylated (NADH) or phosphorylated (NADPH) forms of the pyridine nucleotides. Finally, band 4, which is the most intense band in lane 2, was unambiguously identified as InhA, a known and validated target of INH. Moreover, even though no silver-stained band is observed within the box marked 11 in lane 4, probably reflecting its low cellular abundance, we excised this gel-slice because it corresponds to the position in the gel where DHFR was expected to migrate, and we detected a single DHFR tryptic peptide with a high score from this region after in-gel digestion. The isolation of InhA and DHFR, the only two *M. tuberculosis* proteins known to bind INH-NAD(P) adducts with sub- to low-nanomolar affinity (7,13), demonstrates that the method is successful at isolating the known high-affinity INH-NAD(P)-binding proteins. We note that identification of the proteins in the final INH-NAD(P) eluates from the NAD(P)⁺- Sepharose control columns was not attempted as these proteins appear to have a high affinity for NAD(P)⁺ since the prior 1 mM NAD(P)⁺ wash was not sufficient to remove them from the columns. Thus, endogenous NAD(P)⁺ is expected to protect these proteins from INH-NAD(P) inhibition *in vivo*.

INH has also been proposed to target the *kasA*-encoded β -ketoacyl ACP synthase (30). This enzyme is also a component of the fatty acid synthase II complex and it catalyzes the condensation of malonyl-ACP with acyl-ACP during each elongation cycle of fatty acid biosynthesis. The observation of a covalent complex between KasA, AcpM, and INH in isoniazid-treated *M. tuberculosis* led to the proposal that KasA was the primary target for INH (30). We note that since the KasA-catalyzed reaction is not pyridine nucleotide-dependent, it is not surprising that we did not isolate this protein using our method.

High-Affinity INH-NAD(P)-binding Proteins

Of the eighteen proteins listed in Table 1, only InhA and DHFR have previously been demonstrated to be inhibited by INH-NAD(P). Nine others are predicted to be pyridine nucleotide dependent dehydrogenases/reductases. SahH (Rv3248c), which codes for *S*-adenosylhomocysteine hydrolase, is not a dehydrogenase/reductase but uses a tightly-bound pyridine nucleotide coenzyme, which cycles between the NAD⁺ and NADH oxidation states, to catalyze the cleavage of the homocysteine moiety of *S*-adenosylhomocysteine (31-33). CeoB/TrkA (Rv2691) shares 24 % sequence identity with the *Escherichia coli* TrkA protein, which codes for the NAD⁺-binding subunit of the potassium transport system. It is interesting to note that the *M. tuberculosis* CeoB/TrkA gene had previously been identified in a novel selection for INH resistance in an *E. coli oxyR* strain, which is more susceptible to INH and hydrogen peroxide than the parent strain (34). The open reading frames, Rv0926c and Rv1059, which share 32 % sequence identity with one another, have unknown function, but examination of the two aligned amino acid sequences shows that they have a GXGXXG amino acid motif (35) near their N-termini, an indicator that they could bind pyridine nucleotides.

As with any method, it is likely that there will be some false positives. Rv2623 and Rv1996, for example, which share 40 % sequence identity with one another, are predicted to be universal stress proteins, which bind and hydrolyze ATP (36). A large body of evidence suggests that Rv2623 is important for the non-replicating persistence state of *M. tuberculosis*: Rv2623 has been shown to be induced under hypoxic conditions (37,38), in standing cultures (39), upon macrophage phagocytosis (40), and during lung infection (41). *M. tuberculosis* has been termed “the world’s most successful pathogen” in part because of its ability to persist inside the human host for years or decades without causing fulminant disease, resulting in the infection of one-third of the human population (42). It is possible that Rv2623 and Rv1996 were identified using our method because they bind to the ADP portion of the INH-NAD inhibitor. However, their absence in lane 1 (Figure 2), and the probability that they would have been removed in the 1 mM NAD⁺ wash suggests that this is unlikely. Expression and purification of these proteins is required to verify their annotated function as well as their ability to bind INH-NAD. Finally, Mtn/Sah (Rv0091), which codes for a bi-functional 5'-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase does not use pyridine nucleotides as substrates. It is possible that it has high affinity for a protein that binds to INH-NAD-Sepharose, for example *S*-adenosylhomocysteine hydrolase (above), resulting in the co-purification of these two proteins as a complex. Again, expression and purification of Mtn/Sah is required to determine if it has the ability to independently bind to, and be inhibited by, INH-NAD.

The Pleiotropic Effects of INH

An important, as yet unanswered, question is which of the twelve INH-NAD(P) adducts is/are the active forms of the drug ? Mutations in the promoter region and within the structural gene of *inhA* have been identified in INH-resistant *M. tuberculosis* clinical isolates that confer low-level resistance to isoniazid (15,43,44). Since InhA binds the acyclic 4*S* isomer of the INH-NAD adduct (compound 1, Figure 1), one might argue that this or one of its two equilibrating cyclic forms (compounds 2 and 3, Figure 1) is/are the bactericidal species and that the remaining nine adducts, although they do form *in vivo*, do not inhibit significantly any other

essential enzyme *in vivo*. However, these mutations confer only low-level resistance to isoniazid (15,43,44). In addition, the purified recombinant InhA structural mutants still bind tightly and are inhibited potently by compound 1 ($K_i^* = 2.3\text{--}5.3\text{ nM}$)(7) suggesting that the mechanism of action of isoniazid is more complex than simply inhibiting a single enzyme target.

INH has previously been shown to inhibit mycolic acid (45-47) and nucleic acid biosynthesis *in vitro* (48). INH has also been proposed to affect NAD^+ and pyridoxal phosphate metabolism by acting as an antimetabolite of these coenzymes (2,47). In addition to InhA and DHFR, we have found sixteen other enzymes that bind tightly to, and could be inhibited by INH-NAD(P). These are involved in an extraordinary variety of cellular processes including *S*-adenosylmethionine-dependent methyl transfer (SahH, see below), pyrimidine and valine catabolism (MmsA), the arginine degradative pathway (RocA), proton and potassium transport (PntAA and CeoB/TrkA, respectively), stress response (Rv2623 and Rv1996), lipid metabolism (FadB2 and FabG5), riboflavin biosynthesis (RibD), and various pyridine nucleotide-dependent redox reactions of unknown biochemical function (AldC, Rv3777, and Rv2971). The high-density mutagenesis study of Sasseti *et al.* (49), which identified candidate genes required for normal growth but which require individual confirmation, suggests that four of these new targets (SahH, Rv2623, Rv2971 and Rv1187) are essential for optimum growth of *M. tuberculosis in vitro* (49). We note that some genes, which are not essential for mycobacterial growth *in vitro* may be essential for growth *in vivo*. Thus, INH has multiple targets, and it may be that the simultaneous inhibition of these multiple enzymatic targets results in the thoroughly documented bactericidal effects of INH treatment, resulting in cell lysis.

Inhibition of a specific cellular process by INH could also be the result of inhibition of multiple enzymes involved in the same biosynthetic process. Inhibition of mycolic acid biosynthesis, for example, has been credited to inhibition of InhA (10,15) or KasA (30), both of which are components of the type II fatty acid synthase complex (9). Mycolic acids are decorated with various functional groups including cyclopropyl, keto, and methoxy groups, which are essential for *M. tuberculosis* survival within macrophages (11,12). The methoxy groups are introduced by *S*-adenosylmethionine-dependent methyltransferases (12). Since most methyltransferases are product-inhibited by *S*-adenosylhomocysteine, cells have evolved *S*-adenosylhomocysteine hydrolase (SahH, Rv3248c), to keep the cellular levels of *S*-adenosylhomocysteine low (32,33). We suggest that by inhibiting SahH, the INH-NAD adduct would indirectly interfere with these essential steps in mycolic acid maturation. We have recently proposed that inhibition of nucleic acid biosynthesis by INH results from inhibition of DHFR by INH-NADP (48). By inhibiting SahH, INH would additionally, though indirectly, inhibit the methyltransferases involved in the methylation of uracil and cytosine residues in tRNA by the accumulation of *S*-adenosylhomocysteine.

CONCLUSIONS

The single-drug/single-target paradigm of most antibiotics may not apply to INH. There are twelve INH-pyridine nucleotide metabolites (6), each one potentially targeting a different enzyme. We demonstrate here that there are at least eighteen proteins that bind INH-NAD(P) adducts with high affinity. There may be even more proteins in the *M. tuberculosis* proteome that we failed to isolate and identify because they may not be sufficiently abundant, they may be expressed only *in vivo*, or they may be sterically-prevented from binding because of the covalent attachment of INH-NAD(P) via the exocyclic adenine amine to the solid support. We believe that this study will encourage gene knock-out studies of the genes we have identified here to determine if they are essential for *in vitro* and *in vivo* growth of *M. tuberculosis*. We also believe that this study may assist in guiding the identification of mutations, within the

genes we identified, in *M. tuberculosis* INH-resistant clinical isolates for which the genotypes are presently unknown (15).

The treatment of tuberculosis involves the co-administration of four drugs, most of which are synthetic compounds specific to the treatment of this human disease. Many of these, like isoniazid, are pro-drugs (15), including ethionamide (50,51), pyrazinamide (52) and the nitroimidazole, PA-824 (53), which is being tested for its activity against the persisting population of organisms (54). In many cases, scant evidence exists for the active form of the drug, or its intracellular mycobacterial target. The emergence of multidrug resistant (isoniazid and rifampicin resistant) clinical isolates is threatening the effectiveness of these few compounds, forcing clinicians to resort to less effective, more toxic and more expensive treatment options. The proteins and enzymes that we have identified in this study appear to provide promising new candidates for biochemical- and structure-based inhibitor design that could lead to new therapeutic approaches for the control and eradication of tuberculosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACP, acyl carrier protein
DHFR, dihydrofolate reductase
InhA, enoyl-ACP reductase
EDTA, ethylenediaminetetraacetic acid
INH, isonicotinic acid hydrazide (isoniazid)
INH-NAD(P), isonicotinoylated nicotinamide adenine dinucleotides
NAD(P)⁺, oxidized nicotinamide adenine dinucleotide (phosphate)
NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate)
Pipes, piperazine-1,4-bis(2-ethanesulfonic acid)
SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis
Tris, tris(hydroxymethyl)aminomethane

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FIGURE 1.

Chemical structures of the INH-NAD and INH-NADP adducts. The adenosinediphospho-ribose (for INH-NAD) and 2'-phospho-adenosine-diphospho-ribose (for INH-NADP) moiety of these molecules is abbreviated as ADPR(P). Each of the *R* and *S* acyclic forms is in equilibrium with the corresponding pair of diastereomeric, cyclic, hemiaminal forms. Cyclization occurs by intramolecular attack of the amide nitrogen of the nicotinamide ring on the carbonyl group of the isonicotinoyl moiety (6).

FIGURE 2.

SDS-PAGE of the INH-NAD(P) protein eluates from the NAD⁺-sepharose (lane 1), INH-NAD-sepharose (lane 2), NADP⁺-sepharose (lane 3), and INH-NADP-sepharose (lane 4) columns. The gels were silver-stained. The two lanes marked *M* represent molecular weight markers. The identities of the protein bands marked with numbers are shown in Table 1. The two bands marked with an asterisk (lane 2) correspond to degradation products of band 4 (InhA).

Table 1
High Affinity INH-NAD(P)-binding proteins from *Mycobacterium tuberculosis* H37Rv.

Band no.	Rv no.	Gene	M.W.	Annotated Function ¹	Pyridine Nucleotide Binding?	Essential? ²
1	Rv3248c Rv0753c Rv1187 Rv0155 Rv2623 Rv1996 Rv0468 Rv1484 Rv2691	SahH MmsA RocA PntAA FadB2 InhA CeoB/TrkA	54324 54454 58841 37694 31652 33880 30728 28528 24240	S-Adenosylhomocysteine hydrolase Methylmalonic acid semialdehyde dehydrogenase Pyroline-5-carboxylate dehydrogenase Proton translocating transhydrogenase α -subunit Universal stress protein, similar to Rv1996 Universal stress protein, similar to Rv2623 3-Hydroxybutyryl-CoA dehydrogenase Enoyl-ACP reductase NAD ⁺ -binding subunit of potassium uptake protein complex Bi-functional 5-methylthioadenosine/ S-Adenosylhomocysteine nucleosidase Aldehyde dehydrogenase Unknown; similar to Rv0926c Probable oxidoreductase Probable oxidoreductase 3-Oxoacyl-ACP reductase Bi-functional deaminase/reductase involved in riboflavin biosynthesis Dihydrofolate reductase	Yes Yes Yes Yes Unknown Unknown Yes Yes Yes Yes No Yes Yes Yes Yes Yes Yes Yes Yes	Yes No Yes Unknown Yes No No Yes/No ^{3,2} No No Unknown Unknown No Yes No No Yes/Unknown ^{4,2}
6	Rv2858c	AldC	48259			
7	Rv1059 Rv3777 Rv2971 Rv2766c Rv2671		36910 33963 30364 27140 27694			
11	Rv2763c	DfrA	17640			

¹ Tuberculist (<http://genolist.pasteur.fr/Tuberculist/>).

² Sasseti, C. M. *et al.* (2003); Ref. (49).

³ Vitcheze, C. *et al.* (2000); Ref. (10).

⁴ Suling, W. J. *et al.* (1998); Ref. (55).