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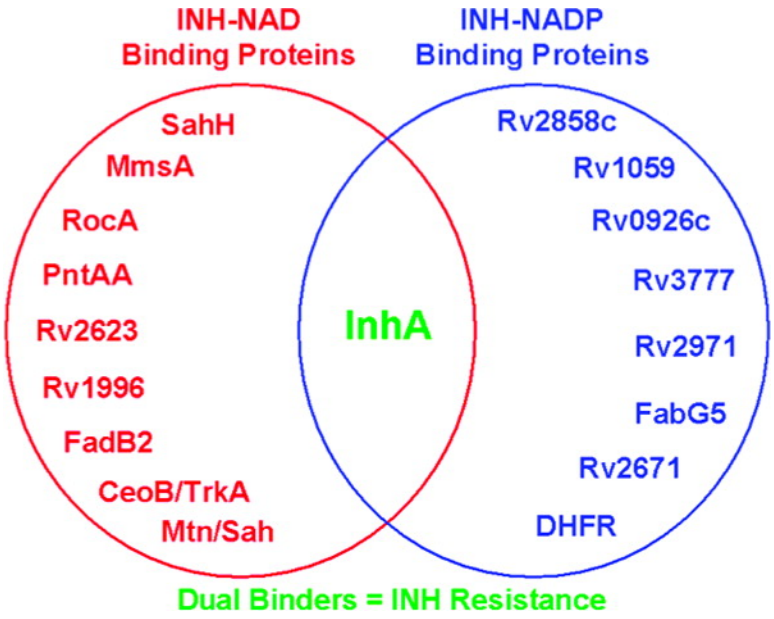

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New Insight into the Mechanism of Action of and Resistance to Isoniazid: Interaction of *Mycobacterium tuberculosis* enoyl-ACP Reductase with INH-NADP

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Isoniazid (isonicotinic acid hydrazid, INH) is an essential drug used in the treatment of tuberculosis.[1] INH is a pro-drug, which is oxidatively activated in vivo by the *katG*-encoded mycobacterial catalase peroxidase[2] to generate an isonicotinoyl radical. This highly reactive species then reacts nonenzymatically with the cellular pyridine nucleotide coenzymes, NAD^+ and $NADP^+$,[3] to generate 12 isonicotinoyl-NAD(P) adducts [INH-NAD(P), Figure 1].[4] Of these, the acyclic 4*S* isomer of INH-NAD (compound **1**, Figure 1) and the acyclic 4*R* isomer of INH-NADP (compound **4**, Figure 1) inhibit the *inhA*-encoded enoyl-ACP reductase[5−7] and the *dfrA*-encoded dihydrofolate reductase (DHFR),[8] respectively, with sub- to low-nanomolar affinity. We have recently identified 16 additional proteins from *Mycobacterium tuberculosis* cell extracts that bind INH-NAD(P) adducts with high affinity.[9]

An important question is which of the 12 INH-NAD(P) adducts is the mycobactericidal species. Overexpression of InhA in mycobacteria results in a 20-fold increase in the minimum inhibitory concentration (MIC) of INH.[10] This 20-fold increase in resistance to INH, which results from drug sequestration by the overexpressed InhA protein, suggests that the primary mycobactericidal species must, therefore, be that form(s) (or any equilibrating forms) that InhA binds to with high affinity. Even though binding to and inhibition of InhA by INH-NAD (compound **1**) has been thoroughly characterized, both enzymologically[6] and structurally,[5] the interaction of InhA with INH-NADP has, to date, not been examined. The likely reason for this is that InhA has been reported to be NADH-specific.[11] This result was confirmed with concentrations of NADPH and InhA up to 0.5 mM and 1 μM, respectively. These concentrations represent 100 times more enzyme than was required to observe activity with low micromolar concentration of NADH as substrate (see Figure 2). Yet, as we will show next, INH-NADP is a nanomolar inhibitor of InhA.

The double reciprocal plot of Figure 2 shows that one of the species that comprises the INH-NADP mixture is a competitive inhibitor (with respect to NADH) of InhA with an apparent K_i of 265 nM. The time courses were linear, suggesting that INH-NADP is not a slow-onset inhibitor of InhA. Minimally, the apparent K_i should be corrected for the fact that INH-NADP is a mixture of the *R* and *S* isomers. Assuming an equimolar *R* and *S* mixture, the true K_i is, thus, 130 nM. This value is similar to the K_i value of 100−150 nM measured previously for the initial formation of the InhA:INH-NAD inhibitory complex prior to the slow isomerization step.[6] We suggest that inhibition of InhA by INH-NADP would be particularly important when the in vivo level of $NADP^+$ is higher than that of NAD^+, the two species that react nonenzymatically with activated INH to generate the INH-NAD(P) inhibitors.[3] Dual inhibition of InhA by INH-NAD and INH-NADP would ensure that InhA and, consequently, fatty acid and mycolic acid biosynthesis are inhibited under physiological states where concentrations of either NAD^+ or $NADP^+$ predominate.

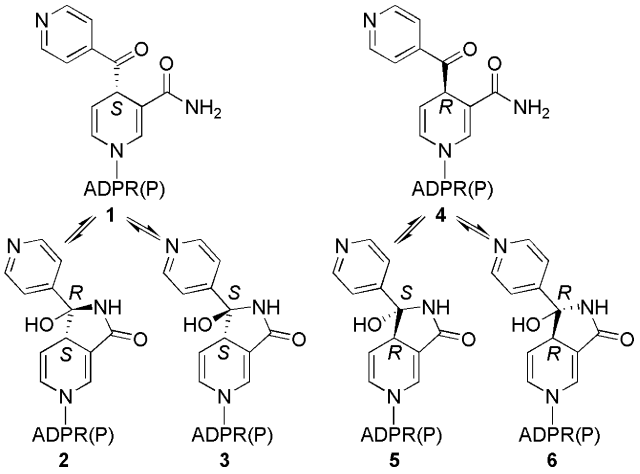

Figure 1. Chemical structures of the INH-NAD and INH-NADP adducts. The adenosine diphosphoribose (for INH-NAD) and 2′-phosphoadenosine diphosphoribose (for INH-NADP) moieties of these molecules are abbreviated as ADPR(P). The acyclic forms are in equilibrium with the corresponding pair of diastereomeric, cyclic, hemiaminal forms.

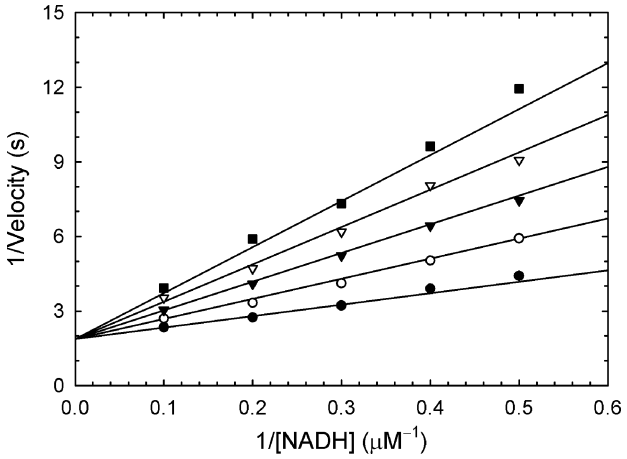

Figure 2. Inhibition of *M. tuberculosis* InhA by INH-NADP. The solid lines represent the fit to $v = VA/[K_mA(1 + I/K_i^{app}) + A]$, giving $K_i^{app} = 265 \pm 15$ nM. Assays contained 100 mM Pipes, pH 7.0, 10% (v/v) glycerol, 10 nM *M. tuberculosis* InhA, 100 μM 2-*trans*-octenoyl-CoA, varied levels of NADH (2, 2.5, 3.3, 5, and 10 μM) at five fixed levels (0, 200, 400, 600, and 800 nM) of INH-NADP.

We crystallized InhA with INH-NADP bound and solved the structure of the complex. Clear electron density arising from the

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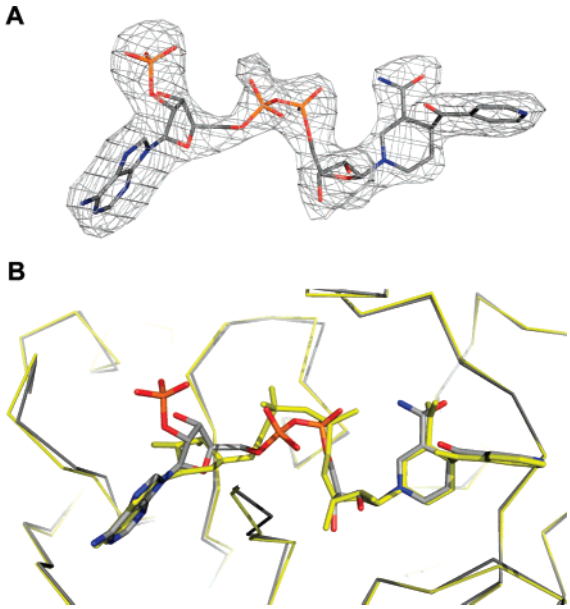

Figure 3. Structure of the InhA:INH-NADP complex. (A) *Fo*−*Fc* omit map for INH-NADP contoured at 3σ. (B) Superposition of the InhA:INH-NAD structure (PDBID: 1ZID; colored yellow) and the InhA:INH-NADP structure (colored by atom type, gray carbons).

isonicotinoyl moiety as well as from the 2′-phosphate of INH-NADP was observed (Figure 3A). As expected, the structure demonstrates that it is the acyclic 4*S* isomer of INH-NADP that binds to the enzyme. The overall structures of the InhA:INH-NAD and the InhA:INH-NADP complexes are very similar (rmsd of 0.39 Å, 267 Cα, Figure 3B). The majority of the inhibitor including the isonicotinoyl and adenine moieties bind similarly, however, the ribose moiety of the adenosine is rotated approximately 90° and the hydrogen bond between its 5′-phosphate and $Ser20^{OG}$ is disrupted. This conformation projects the 2′-phosphate out into solvent minimizing interactions with the protein. This disruption in hydrogen bonding may explain the lack of activity of InhA with NADPH.

Even though both DHFR[8] and InhA[6] are inhibited potently by the activated form of INH in vitro, the in vivo resistance profiles exhibited by DHFR and InhA when they are overexpressed in mycobacteria are different; expression of DHFR causes only a modest 2-fold increase in resistance to INH,[8] while expression of InhA uniquely causes substantially higher levels of resistance to INH.[10] It is clear now why these two results have been observed and how they can be reconciled. DHFR binds only to the phosphorylated adduct pool (INH-NADP), allowing the INH-NAD adducts to bind and inhibit InhA, a demonstrated bactericidal target.[12] However, the results presented here demonstrate that the previously unrecognized dual capability of InhA to bind both nonphosphorylated and phosphorylated adduct pools, thus sequestering all forms of the drug, allows both InhA and DHFR to function. We note that sequestration of all bactericidal forms of the drug by InhA requires equilibration of the *R* and *S* enantiomers of INH-NAD(P). This could occur by nonenzymatic enolization/reprotonation of the acidic C_4-proton.

In conclusion, genetic selection for resistance to antibiotics is a time-honored and sensitive method for the identification of molecular targets for antibiotics. The currently understood mechanism of action of INH has, in fact, been largely deciphered using these methods.[2,13] However, there are numerous examples where antibiotic resistance is the result of the expression of proteins that are not themselves targets. Conspicuous among these nontarget resistance determinants are enzymes that can no longer activate the prodrug, covalently modify the antibiotic or modify the target, or actively efflux the drug from the cell.[14] Thus, resistance alone is insufficient evidence for defining molecular targets. In the particular case of INH, the activation by KatG and subsequent formation of 12 pyridine nucleotide adducts that are bisubstrate analogues complicates the situation even further. However, the data presented here demonstrate how expression of InhA, a molecular target itself, can generate high level resistance to INH.

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Supporting Information Available: Synthesis of INH-NAD(P) and 2-*trans*-octenoyl-CoA. Cloning, expression, purification, enzymatic assay, crystallization, X-ray data collection, structure determination and refinement, and crystallographic statistics for InhA (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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