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Letter

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Covalent Modification of the *Mycobacterium tuberculosis* FAS-II Dehydratase by Isoxyl and Thiacetazone

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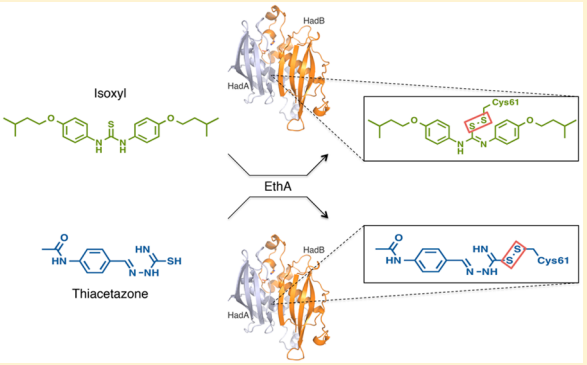
Supporting Information

ABSTRACT: Isoxyl (ISO) and thiacetazone (TAC) are two antitubercular prodrugs formerly used in the clinical treatment of tuberculosis. Although both prodrugs have recently been shown to kill *Mycobacterium tuberculosis* through the inhibition of the dehydration step of the type II fatty acid synthase pathway, their detailed mechanism of inhibition, the precise number of enzymes involved in their activation, and the nature of their activated forms remained unknown. This paper demonstrates that both ISO and TAC specifically and covalently react with a cysteine residue (Cys61) of the HadA subunit of the dehydratase, thereby inhibiting HadAB activity. The results unveil for the first time the nature of the active forms of ISO and TAC and explain the basis for the structure−activity relationship of and resistance to these thiourea prodrugs. The results further indicate that the flavin-containing monooxygenase EthA is most likely the only enzyme required for the activation of ISO and TAC in mycobacteria.



KEYWORDS: *Mycobacterium, tuberculosis, isoxyl, thiacetazone, FAS-II, dehydratase*

The continuing rise of multidrug-resistant tuberculosis (TB) throughout the world places a high priority on the development of new anti-TB drugs with bactericidal mechanisms different from those of the presently available agents. In this context, elucidating the mechanism of action of drugs formerly deemed efficient in the treatment of TB may prove useful in identifying validated targets of therapeutic interest and developing new anti-TB agents with greater potency, improved pharmacokinetics, and reduced toxicity. Two such drugs, isoxyl (ISO) and thiacetazone (TAC), display minimal inhibitory concentrations (MIC) against clinical isolates of *Mycobacterium tuberculosis* (*Mtb*), including multidrug-resistant ones, in the ranges of 1−10 and 0.1−0.5 μg/mL, respectively.[1,2] Both are thiocarbamide-containing prodrugs (Figure 1A) that require activation of their thiocarbonyl moiety by the flavin-dependent monooxygenase EthA for bactericidal activity.[3−6] Although the oxidation of ISO and TAC by purified EthA in vitro has led to the identification of a number of metabolites of those drugs,[4,6] the nature of their reactive intermediate(s) is still unknown. Moreover, while the finding in drug-resistant mutants of *Mtb* of missense and frameshift mutations affecting mycolic acid methyltransferases has led to speculations that other enzymes may be required for the activation of ISO and TAC, biochemical proof for this assumption has been lacking.[2,7,8] Following activation, ISO and TAC inhibit mycolic acid biosynthesis, resulting in bacterial death.[1,5,9] ISO and TAC thus target the same critical pathway as the TB drugs isoniazid (INH) and ethionamide (ETH), albeit through a distinct mechanism independent of the enoyl-CoA reductase InhA.[10]

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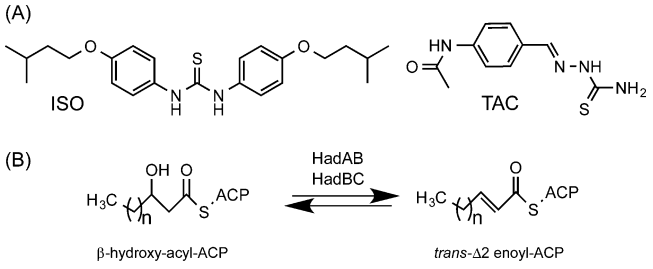

Figure 1. Isoxyl, thiacetazone, and the dehydration step of the FAS-II elongation cycle: (A) structures of ISO and TAC; (B) (3*R*)-hydroxyacyl dehydratases HadAB and HadBC catalyze the (reversible) dehydration of *β*-hydroxyacyl-ACP meromycolate precursors to yield *trans*-Δ2-enoyl-ACP products (the substrates of the enoyl-CoA reductase, InhA) in the FAS-II elongation cycle. The HadAB heterodimer is thought to be involved in the early stages of the elongation cycle, whereas the HadBC heterodimer, which displays a greater affinity for longer fatty acyl substrates than HadAB in vitro, is believed to participate in the late stages of the elongation of the meromycolic acid chain.

Indeed, recent biochemical and genetic evidence established that ISO and TAC inhibit the dehydration step of the type II fatty acid synthase (FAS-II) elongation cycle[2,8,11,12] (Figure 1B). Due to our limited understanding of how the two (3*R*)-hydroxyacyl-ACP-dehydratase heterodimers of FAS-II, HadAB and HadBC,[13] function in whole cells and of the complexity of FAS-II wherein protein interactions govern the activity and substrate specificity of the entire complex,[14−16] details of the molecular mechanism of action of ISO and TAC were lacking. In particular, earlier studies failed to establish whether the two drugs acted as direct inhibitors of one or the two dehydratases or rather as molecules perturbing protein−protein interactions within FAS-II in a way that indirectly abolished the activity of these enzymes.[8] The present studies were undertaken with the goal of resolving these issues.

C61G and C61S point mutations in HadA are the most common amino acid changes associated with high-level ISO and TAC resistance in the FAS-II dehydratases of *Mtb* and *Mycobacterium bovis* BCG and result in >20−80-fold increases in MIC, respectively.[2,8,11,12] This observation raised the possibility that the inhibition of the dehydratase activity of FAS-II by the prodrugs resulted from the formation of a covalent complex between residue Cys61 and the thiocarbonyl moiety of ISO and TAC. To test this hypothesis, *M. bovis* BCG expressing a C-terminal His-tagged recombinant form of HadA (HadA^{WT}-His) was incubated for 15 h with ISO (10 *μ*g/mL; 4 × MIC; the solubility limit of ISO in this culture medium) or TAC (10 *μ*g/mL; 20 × MIC) at 37 °C with shaking and subsequently lysed in Tris-HCl buffer [see the Supporting Information (SI)]. These experiments were performed in situ using whole mycobacterial cells rather than in vitro using purified HadAB to ensure the proper activation of the prodrugs. Previous attempts to incubate purified HadAB with ISO and TAC in a cell-free assay had indeed failed to reveal any inhibition of the dehydratase by the drugs.[8] HadA^{WT}-His from drug-treated and untreated cells was partially purified by affinity chromatography and analyzed by SDS-PAGE. As expected, HadA^{WT}-His copurified with untagged HadB[13] (Figure 2). Changes affecting the migration of HadA^{WT}-His were clearly visible in the ISO- and TAC-treated cells. Although HadA^{WT}-His from untreated cells yielded a single protein band of the expected size (18.4 kDa), two forms of HadA^{WT}-His were

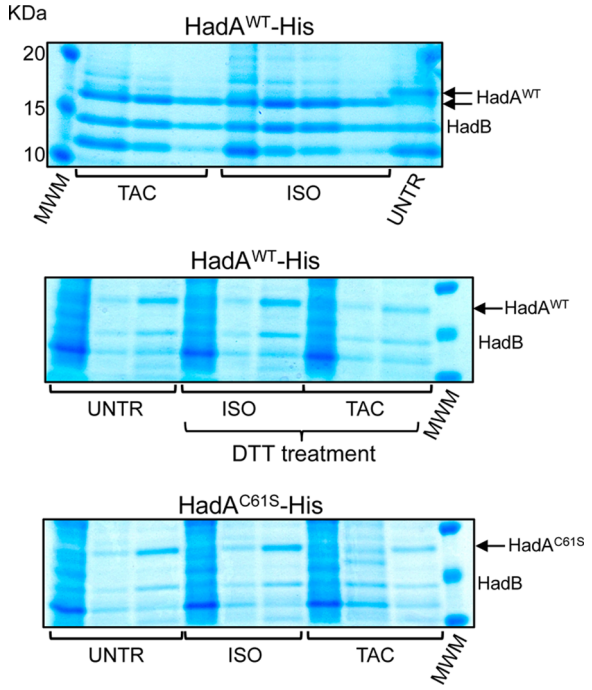

Figure 2. ISO and TAC react with HadA^{WT}-His but not HadA^{C61S}-His in whole *M. bovis* BCG cells. HadA^{WT}-His and HadA^{C61S}-His were partially purified by standard Ni-NTA chromatography from recombinant *M. bovis* BCG cells that were either untreated or treated with ISO or TAC (see the SI for details). Shown on the 15 ± 5 kDa regions of the Coomassie blue-stained SDS-PAGE are the flow-throughs (middle and bottom gels only) and last two to four elution fractions for each drug-treated sample (one representative elution fraction for the untreated sample on the top gel only). The positions of the two HadA-His variants (which were confirmed by immunoblot with anti-His antibodies) are indicated by arrows. Incubation of the HadA^{WT}-His drug-treated samples with DTT prior to separation by SDS-PAGE reverts their migration profile to that of the untreated control. MWM, molecular weight marker; UNTR, untreated.

detected in the drug-treated samples. One of them migrated similarly to HadA^{WT}-His from untreated BCG, and the other migrated with an apparent slightly lower molecular weight. Consistent with the likely reactivity of the drugs with Cys61, this migration shift was not observed when a C61S mutant of HadA (HadA^{C61S}-His), similarly expressed and purified from *M. bovis* BCG, was used (see the SI) (Figure 2), and high-level resistance to ISO and TAC was noted for this recombinant strain (MIC of both drugs >25 *μ*g/mL compared to MICs of 2.5 *μ*g/mL for ISO and 0.5 *μ*g/mL for TAC against WT *M. bovis* BCG or BCG expressing HadA^{WT}-His). Subsequent purification of the treated and untreated forms of HadA^{WT}-His from the gel and analysis of the full-size proteins and corresponding derived tryptic peptides by liquid chromatography tandem mass spectrometry (LC-MS/MS) under denaturing conditions (see the SI) unexpectedly revealed that drug treatment had caused the oxidation of the two cysteines in HadA to form a disulfide bond as demonstrated by the presence in the drug-treated samples of a protein approximately 2 amus lower in mass than the reduced form of HadA^{WT} (Figure S1A-C). Consistent with this finding, the SDS-PAGE migration shift of HadA^{WT}-His in the drug-treated samples was reversed upon treatment with dithiothreitol (DTT) (Figure 2), and only the reduced form of HadA^{WT}-His was present by LC-MS analysis (Figure S1A). Suspecting that the only other

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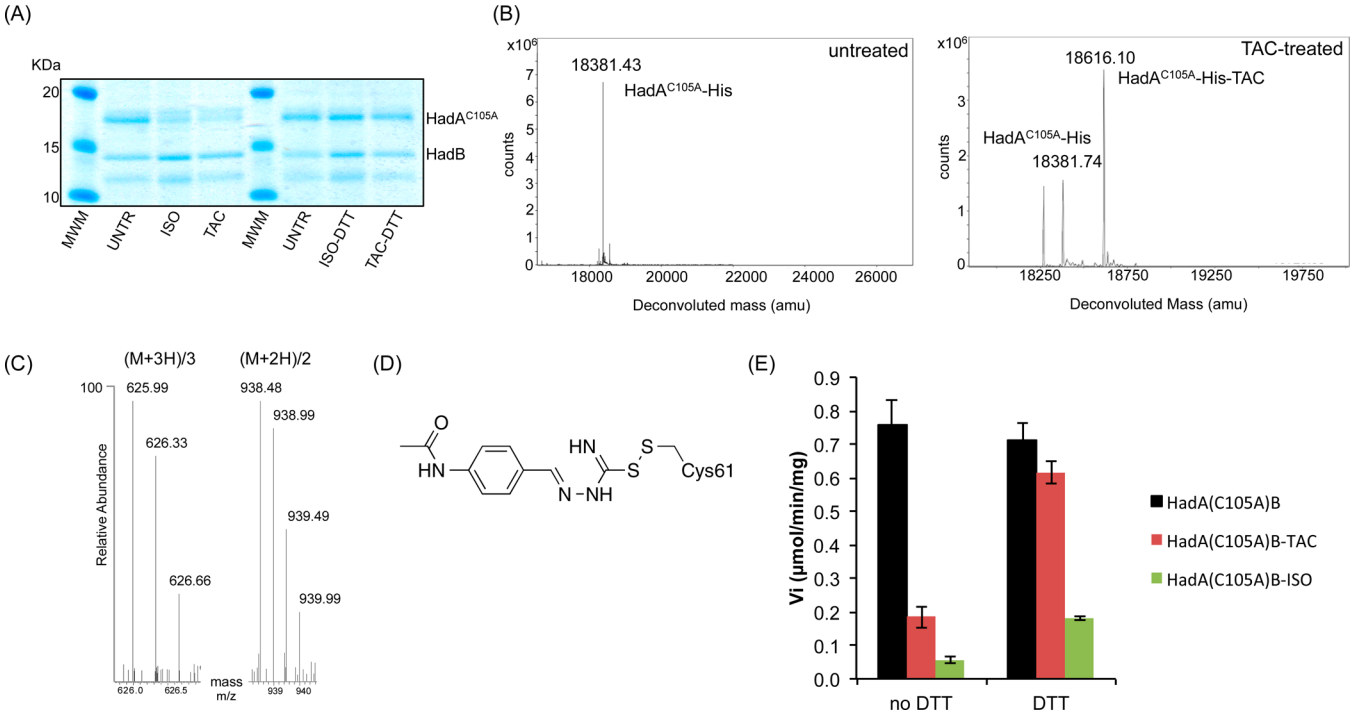

Figure 3. Covalent binding of ISO and TAC to HadA^{C105A}-His in whole *M. bovis* BCG cells. (A) HadA^{C105A}-His was partially purified from recombinant *M. bovis* BCG cells that were either untreated or treated with ISO or TAC as in Figure 2. The samples annotated as ISO-DTT and TAC-DTT were incubated with 500 mM DTT for 5 min at 70 °C prior to SDS-PAGE. (B) LC-MS analysis of the intact HadA^{C105A}-His protein devoid of drug (untreated) and in covalent complex with TAC. The ESI mass spectra show the mass of HadA^{C105A}-His in the untreated control and both the masses of HadA^{C105A}-His and HadA^{C105A}-His in covalent complex with TAC (mass expected from attachment to Cys61 via an S−S bond) in the TAC-treated sample. Calculated masses: HadA^{C105A}-His = 18381.45; HadA^{C105A}-His-TAC = 18,615.15; (∗) an unidentified protein was observed in the TAC-treated sample. (C) HadA^{C105A}-His purified from TAC-treated BCG cells was in-gel trypsin digested, and the peptides were analyzed by LC-MS/MS. Cys61-containing tryptic peptide in complex with TAC at charge states (+2) and (+3) = 938.48 (M + 2H)/2 and 625.99 (M + 3H)/3, respectively. (D) Structure of the proposed HadA^{C105}-TAC adduct purified from *M. bovis* BCG. (E) Activity of the HadA^{C105A}-His proteins purified from drug-treated and untreated *M. bovis* BCG cells shown in (A). Kinetic assays in the presence of C12:1-CoA were performed as described in the SI and initial velocities determined by linear fitting. Shown are the average initial velocities ± standard deviations of at least three independent measurements.

cysteine in HadA (Cys105) reacted with the Cys61 drug complexes to form the oxidized protein, we next sought to repeat these experiments in the absence of thiols by mutating Cys105 to an alanine. That Cys105 is not required for the drugs to inhibit HadAB was supported by the fact that the MICs of ISO and TAC against BCG expressing HadA^{C105A}-His are similar to those against BCG expressing HadA^{WT}-His or wild-type *M. bovis* BCG (2.5 μg/mL for ISO; 0.5 μg/mL for TAC). Whereas HadA^{C105A}-His from untreated BCG cells yielded a single protein band of the expected size (18.3 kDa), HadA^{C105A}-His from the drug-treated samples migrated as two more diffuse bands (Figure 3A). Reduction of the samples with DTT prior to SDS-PAGE reversed the migration shift of HadA^{C105A}-His in the ISO- and TAC-treated samples (Figure 3A). MS analysis of the treated and untreated forms of HadA^{C105A}-His revealed that the full-size protein and derived Cys61-containing peptides exhibited a shift in mass of 234 amu in the TAC-treated sample (Figure 3B,C) which matched that expected for the covalent HadA^{C105A}-His-TAC complex presented in Figure 3D. This shows that a single site on the Cys61-containing peptides was covalently modified with one TAC metabolite. Whether due to the insufficient amount or degree of purity of the material purified from the ISO-treated BCG cells or otherwise, MS analysis failed to reveal the nature of the HadA^{C105A}-His-ISO complex.

To overcome this problem, we followed a similar *Escherichia coli*-based approach as that used previously to study the mechanism of inhibition of the enoyl-CoA reductase InhA by ETH.[17] To this end, *ethA* and *hadA^{C105A}BC* (allowing for the expression of an N-terminal hexahistidine-tagged form of HadA^{C105A}) were coexpressed in *E. coli* BL21(DE3), and HadA^{C105A}B purified from treated and untreated *E. coli* cells was analyzed by LC-MS for covalent modification of HadA^{C105A} by the drugs. MS analyses of the HadA^{C105A}-His-TAC adduct confirmed the data previously obtained in BCG, whereas that of the HadA^{C105A}-His-ISO complex revealed for the first time the nature of the active form of ISO in covalent linkage with its target; the full-size protein exhibited a shift in mass of 398 amu in the ISO-treated sample (Figure 4A) that matched the mass expected for the covalent HadA^{C105A}-His-ISO adduct presented in Figure 4B. The results of these *E. coli*-based experiments further indicated that the coexpression of *ethA* potentiated the formation of HadA^{C105A}-His-TAC and -ISO complexes in the cells, although the presence of this gene is not an absolute requirement for the drugs to react with the dehydratase. We believe this to be due either to the spontaneous oxidation of ISO and TAC inside the cells or to the existence of *E. coli* monooxygenase(s) capable of activating the two prodrugs.

The nature of the complexes formed between HadA and ISO and TAC is in line with the results of preliminary structure−activity relationship studies using a limited number of analogues

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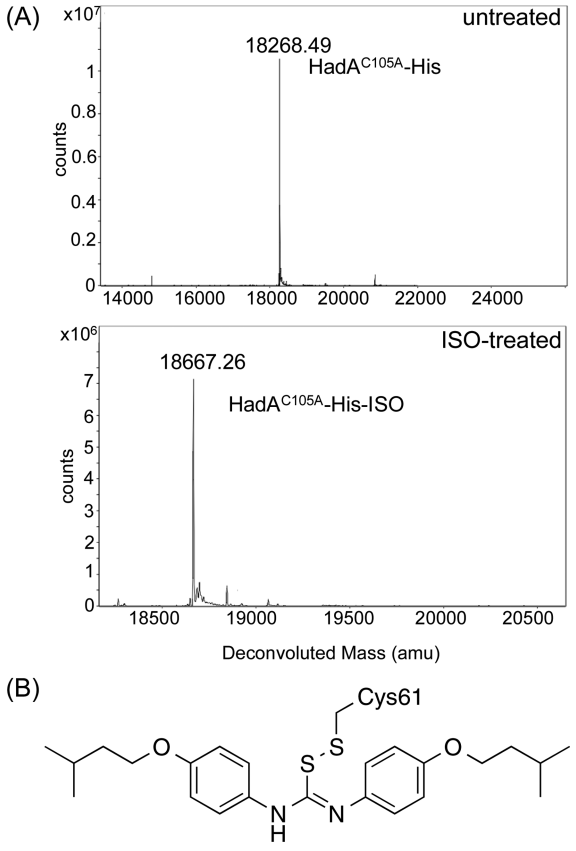

Figure 4. Covalent binding of ISO to HadA^{C105A}-His in the *E. coli* activation system. (A) LC-MS analysis of the intact HadA^{C105A}-His protein devoid of drug (untreated; top panel) and in covalent complex with ISO (bottom panel). The HadA-ISO complex exhibited a longer retention time on the HPLC column and, as expected, was not present in the untreated control (data not shown). Calculated masses: HadA^{C105A}-His = 18268.24; HadA^{C105A}-His-ISO where ISO is attached via an S−S bond = 18666.08. (B) Structure of the proposed HadA^{C105}-ISO adduct purified from *E. coli*.

and, in particular, the fact that the modification of their thiocarbonyl moiety resulted in loss of activity.[12,18] Compound **41**[18] (see the SI and Figure S2A) is the urea analogue and a known metabolite of ISO.[6] Consistent with the lack of thiocarbonyl-reacting moiety on this analogue, this compound did not react with HadA^{C105A}-His in *M. bovis* BCG- or *E. coli*-treated cells (Figure S2B), showed no bactericidal activity on *Mtb* (MIC > 200 μg/mL), and failed to inhibit mycolic acid synthesis in treated cells (Figure S2C).

To determine whether the modification of Cys61 by ISO and TAC affected the activity of HadAB, we next compared the hydratase activity of untreated HadA^{C105A}HadB and the HadA^{C105A}HadB-drug adducts partially purified from BCG on a *trans*-2-dodecenoyl-CoA substrate using the spectrophotometric assay described by Sacco et al.[13] Note that although >50% of the HadA^{C105A}-HadB heterodimers recovered from the drug-treated cells apparently reacted with the drugs under our experimental conditions (Figure 3A), the samples also contained some unbound and presumably active enzyme. Results showed that the HadA^{C105A}-HadB enzymes from TAC- and ISO-treated cells exhibited only 24 and 7%, respectively, of the activity of HadA^{C105A}-HadB partially purified from untreated cells (Figures 3E and S3). Incubation of the protein samples for 4.5 h in a solution containing 10 mM DTT to reverse the covalent modification of Cys61 by the drugs led to recovery of enzymatic activity, albeit to a significantly greater extent in the case of TAC than ISO (which recovered 82 and 24%, respectively, of the activity of the non-drug-treated control) (Figure 3E), most likely reflecting differences in the susceptibility of the HadA^{C105A}-HadB-drug complexes to the reducing agent. Finally, because of our finding that the presence of the second Cys residue (Cys105) in HadA may cause the HadA-drug adducts to be unstable, ultimately causing the formation of a disulfide bond in the protein (as described in Figure 5 for ISO), we tested whether the oxidized form of HadAB generated in vitro upon treatment of the purified enzyme with diamide (see the SI and Figure S4) displayed enzymatic activity. The results showed that HadAB-treated diamide exhibited only 22.5% of the activity of the untreated enzyme.

Different mechanisms may account for the inhibition of the dehydratase activity of FAS-II upon covalent binding of ISO and TAC to the Cys61 residue of HadA. HadA, HadB, and HadC were shown to associate in two functional heterodimers, HadAB and HadBC, where HadB is the common subunit carrying the catalytic site.[13] In vitro, HadAB displays a greater affinity for shorter fatty acyl chains than HadBC, and it is thought that HadAB acts at the early stages of the elongation of meromycolates, whereas HadBC dehydrates longer fatty acyl chains in a mechanism reminiscent of that described for the FAS-II β-ketoacyl-ACP synthases KasA and KasB.[19] HadA and HadC were proposed to play a role in the stabilization of the acyl-ACP substrates by keeping open the active site tunnels in HadAB and HadBC.[13] On the basis of the experiment presented in Figure 3A which shows that comparable amounts of HadB coeluted with HadA^{C105A}-His in the untreated and drug-treated samples, it appears that the Cys61 modification of HadA by the drugs does not result in the dissociation of the HadAB heterodimer. That the drugs covalently bind to the catalytic subunit HadB or to HadC or cause the dissociation of the HadBC heterodimer is also not supported by our data (Figures 2, 3 and S5). Instead, and in light of the structural modeling of HadAB presented in Figure 6, it is likely that the modification of Cys61 by ISO and TAC blocks the acyl-ACPs' access to the acyl-binding channel located at the interface of HadA and HadB (Figure 6A). Indeed, in addition to affording disulfide bond formation between the thiol of HadA-C61 and the tested compounds, this channel is likely to accommodate all of TAC and one of the two phenyl isopentyl ether arms of ISO (Figure 6B,C). Multiple interactions between amino acid side chains forming this pocket appear to promote binding of either drug as outlined in Figure 6 and the SI. The resulting inability of the HadAB-ISO or HadAB-TAC adducts to dehydrate early meromycolate precursors would interrupt their elongation by FAS-II and explain the complete shut-down of mycolic acid biosynthesis that accompanies the buildup of early (C_8−C_{22}) 3-hydroxy meromycolic acids observed in drug-treated cells.[8] Clearly, the structural characterization of the FAS-II dehydratases alone and in complex with ISO and TAC will be crucial to the further understanding of their catalytic activity and inhibition by both drugs.

Altogether, the experiments presented herein unveil for the first time the nature of the active forms of ISO and TAC and support the formation of covalent adducts between Cys61 of HadA and the activated forms of the two drugs thought to be their sulfenic acid derivatives (Figure 5). These reactions proceed with high efficiency in whole mycobacterial cells, and

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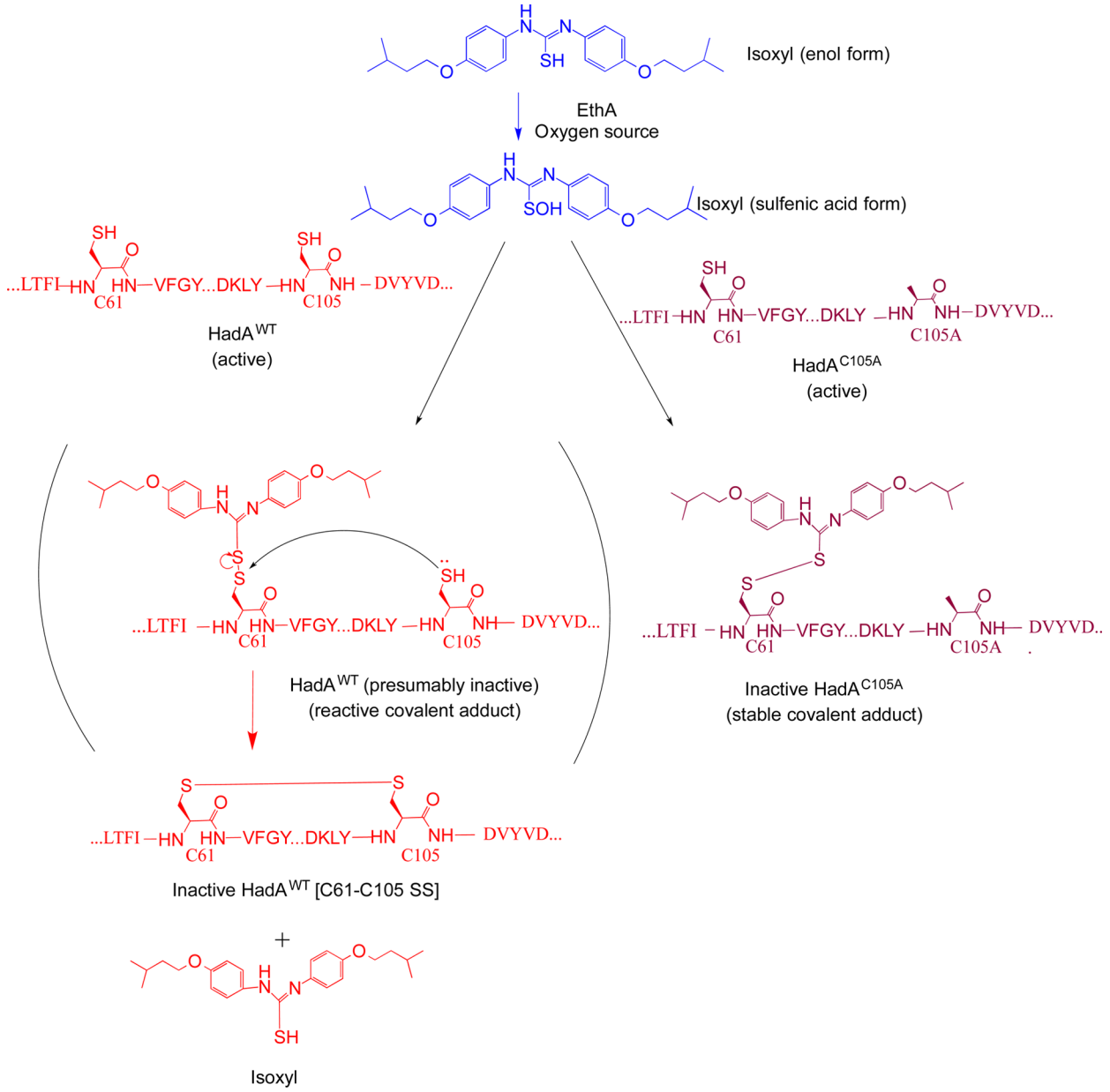

Figure 5. Proposed mechanism of activation of ISO by EthA and inhibition of the FAS-II HadAB dehydratase. ISO in the enol form is oxidized by EthA to the sulfenic acid, which reacts with the sulfhydryl on Cys61 of HadA^{WT} or HadA^{C105A}, thereby inactivating the HadAB dehydratase. In the case of HadA^{WT}, either in the cell itself or during its isolation, the sulfhydryl of Cys105 further displaces the ISO forming, as shown herein, an additional (oxidized) inactive form of the dehydratase. The peptide backbone is shown in part for HadA.

the HadA-HadB/drug complexes that ensue are devoid of dehydratase activity, resulting in the interruption of the FAS-II elongation cycle and the abolition of mycolic acid synthesis.[8] The potentiation of the activity of ISO and TAC upon *ethA* expression in our *E. coli* activation system further suggests that the flavin-containing monooxygenase EthA is most likely the only enzyme required for the activation of ISO and TAC in mycobacteria. It is interesting that ISO and TAC are now with ebselen and the novel TB drug candidates, benzothiazinones and dinitrobenzamides, two additional prodrugs having modes of action against *Mtb* that involve the covalent modification of a Cys residue on their enzymatic target, albeit through slightly different mechanisms.[20−24] Overall, the findings reported herein mark an important step in our understanding of the mechanisms of ISO and TAC sensitivity and resistance and will facilitate the development of improved inhibitors of this critical step of the mycolic acid biosynthetic pathway.

METHODS

Drug Treatment of Whole *E. coli* and *M. bovis* BCG cells and Protein Purification. Details of the constructs used in this study are provided in the SI. Actively growing *M. bovis* BCG ($Abs_{600\ nm}$ = 0.5) overexpressing C-ter His_6-tagged recombinant forms of HadA^{WT}, HadA^{C105A}, and HadA^{C61S} from pVV16-*hadA*^{WT}, pVV16-*hadA*^{C105A}, and pVV16-*hadA*^{C61S}, respectively, were treated with either no drug, 10 μg/mL ISO (4 × MIC), 10 μg/mL TAC (20 × MIC), or 20 μg/mL compound **41** for 15 h at 37 °C with shaking. BL21(DE3) cells coexpressing *ethA* and *hadA*^{C105A}*BC* were treated with either no drug, 50 μg/mL ISO, or 50 μg/mL TAC. The drugs were

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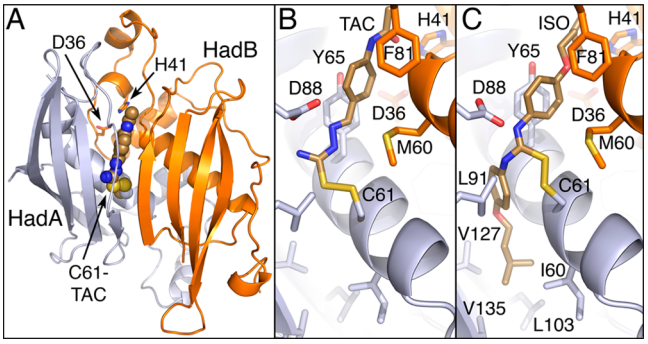

Figure 6. Structural model of *M. tuberculosis* HadAB modified at HadA-C61. (A) The heterodimeric HadAB complex is shown as a ribbon diagram with HadA in gray and HadB in orange. Carbon atoms and bonds of each protein molecule are shown in the respective ribbon color. The carbon atoms of TAC are shown in bronze. All other atoms are colored by CPK. HadB-D36 and HadB-H41, essential active site residues, highlight the HadAB active site. The atoms of HadA-C61 are shown as spheres. HadA-C61 is at the end of an internal channel that extends from the enzyme surface near active site residues HadB-D36 and HadB-H41. (B) The predicted interactions supporting complex formation between TAC and HadAB are shown. This model exhibits significant chemical complementary between the predicted lipid-binding pocket of HadAB and the TAC molecule. (C) The predicted interactions supporting complex formation between ISO and HadAB are shown in the same orientation as in panel B. In addition to the complementary interactions observed in the TAC model, the ISO model exhibits significant van der Waals interactions between the atoms of the second phenyl isopentyl ether arm of ISO and the atoms forming a hydrophobic pocket in HadAB abutting HadA-C61.

added at the same time as 0.2 mM IPTG, and the *E. coli* cells further incubated at 37 °C for 4 h. Treated and untreated *M. bovis* BCG and *E. coli* cells were harvested and resuspended in 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, 10 mM imidazole, 10% glycerol, 10 μg/mL DNAase, and protease inhibitors (SIGMAFAST Protease inhibitor cocktail tablets, EDTA free) prior to breaking with a French press (Sim Aminco) at 1500 psi. Unbroken cells and bacterial debris were removed by centrifugation at 15000 rpm for 45 min. HadA and HadC purifications were performed using Ni-NTA affinity chromatography (Qiagen) as previously described.[13] The recombinant proteins were eluted from the resin with 300 mM imidazole. Protein samples were analyzed by LC-MS and LC-MS/MS (see the SI) and resolved by SDS-PAGE in the presence or absence of reducing agent (DTT) and visualized by Coomassie blue staining. Prior to LC-MS analysis, the buffer was exchange to 10 mM ammonium acetate.

Dehydratase Assay. The enzymatic activity of HadA^{C105A}B partially purified from untreated and ISO- or TAC-treated *M. bovis* BCG and that of the oxidized form of HadAB generated in vitro upon treatment with diamide (see the SI) was measured in the presence of *trans*-2-dodecenoyl-CoA (C12:1-CoA) as the substrate as previously described.[13]

■ ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/id500032q.

Detailed experimental procedures and additional references and analytical data (PDF)

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Author Contributions

Performed the experiments: A.E.G., N.E., E.J.N., A.M., J.J.L., V.J., and J.K. Interpreted the data: A.Q., E.J.N., P.J.B., R.E.L., D.R.R., M.M., and M.J. Wrote the manuscript: A.Q., E.J.N., D.R.R., M.M., and M.J. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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