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Inhibition of *Mycobacterium tuberculosis* Transaminase BioA by Aryl Hydrazines and Hydrazides

Ran Dai^a, Daniel J. Wilson^b, Todd W. Geders^a, Courtney C. Aldrich^a, and Barry C. Finzel^{*,a}

^a Department of Medicinal Chemistry University of Minnesota 308 Harvard St. SE, Minneapolis, MN 55455, United States

^b Center for Drug Design University of Minnesota Academic Health Center, U of Minnesota, MN, 55455, United States

Abstract

7,8-diaminopelargonic acid synthase (BioA) of *Mycobacterium tuberculosis* is a recently validated target for therapeutic intervention in the treatment of tuberculosis (TB). Using biophysical fragment screening and structural characterization of compounds, we have identified a potent aryl hydrazine inhibitor of BioA that reversibly modifies the pyridoxal-5'-phosphate (PLP) cofactor, forming a stable quinonoid. Analogous hydrazides also form covalent adducts that can be observed crystallographically but are incapable of inactivating the enzyme. In the X-ray crystal structures, small molecules induce unexpected conformational remodeling in the substrate binding site. We compare these conformational changes to those induced upon binding of the substrate (7-keto-8-aminopelargonic acid), and characterize the inhibition kinetics and the X-ray crystal structures of BioA with the hydrazine compound and analogs to unveil the mechanism of this reversible covalent modification.

Keywords

tuberculosis; X-ray crystal structures; transaminase; reversible covalent inhibitor; hydrazine

Introduction

Mycobacterium tuberculosis (*Mtb*), the major pathogen causing tuberculosis (TB) in humans is more prevalent in the world than ever before.^[1] One-third of the world's population harbor a latent form of *Mtb* and endure a lifelong risk of reactivation. Immune-compromised individuals, such as those co-infected with human immunodeficiency virus (HIV), are particularly at risk for *Mtb* infection.^[2] The emergence of multi- and extensively-drug-resistant (MDR-TB and XDRTB) strains has complicated treatment of *Mtb* infections worldwide. The failure rate using current combination therapy in the treatment of MDR-TB infections is almost 30%, while therapeutic options for XDR-TB-infected patients are very limited due to the extensive drug resistance.^[3] Since the approval of Rifampicin in 1970, no

* Fax: (+1)612-624-0139 finze007@umn.edu.

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new anti-TB drugs were approved that act through a novel mechanism until Bedaquiline in late 2012.^[4] To control the global TB pandemic, there is an urgent need for additional antitubercular agents with new mechanisms of action that can act in synergy with existing therapy to treat MDR-TB and XDR-TB and shorten the duration of treatment.

The enzymes involved in biotin biosynthesis by *Mtb* represent potential drug targets because the biotin synthetic pathway is required for *Mtb* survival both *in vitro*^[5] and *in vivo*^[6] and unlike bacteria or plants, mammalian hosts do not have enzymes for biotin cofactor synthesis.^[7] *Mtb* utilizes four enzymes to synthesize biotin from pimeloyl-CoA.^[8] The enzyme catalyzing the second step, DAPA synthase (BioA) has been shown by Schnappinger and co-workers to be essential for persistence during chronic infections in a murine model of TB using a conditionally regulated *Mtb* knockdown strain.^[6] Furthermore, biotin deprivation of the *bioA Mtb* mutant leads to cell death in the presence of a carbon source in the growth media (i.e. bactericidal), which is an unusual phenotype since most auxotrophs simply undergo growth arrest when deprived of their nutrient. Collectively, these findings make BioA a promising new TB drug target.^[6]

BioA is a Class I aminotransferase responsible for the conversion of 7-keto-8-aminopelargonic acid (KAPA) to 7,8-diaminopelargonic acid (DAPA) employing S-adenosyl methionine (SAM) as the amino donor via a ping-pong bi-bi mechanism using PLP as a co-factor^[9] that cycles between the reduced pyridoxamine (PMP) and oxidized (PLP) states (Scheme 1). First, SAM reacts with the internal aldimine of PLP and Lys283 to donate an amino group to the PLP, forming pyridoxamine phosphate (PMP). KAPA then reacts with the PMP-bound form of BioA, receiving the amino group to form the product DAPA. With this last step, BioA returns to its PLP bound holo form.

Generally, transaminases can use many endogenous amino acids as amino donors, but BioA has an unusual preference for SAM, which normally serves as a methyl donor.^[10] SAM is structurally very different from the amino acceptor KAPA, and how the enzyme retains specificity in the recognition of these two different substrates is only partially understood. *Mtb* BioA has been structurally characterized with bound PLP co-factor (PDB-id 3BV0, 3TFT).^[7, 11] Based on a comparison of a H315R mutated *Mtb* BioA complex with the unreactive SAM analog sinefungin (PDB-id 3LV2)^[7] and an *E. coli* BioA complex with KAPA (PDB-id 1QJ3),^[12] Sacchettini and co-workers have proposed that *Mtb* BioA catalyzes the transamination by an induced fit mechanism that relies upon active site conformational changes that accommodate structurally different substrates.^[7] This necessary capacity for change in the active site conformation makes it challenging to design *Mtb* BioA inhibitors and predict ligand binding using computational modeling methods, particularly based on homology models derived from structures from other species.

Limited efforts to identify selective inhibitors of *Mtb* BioA have been reported. Amiclenomycin (ACM), a natural product extracted from *Streptomyces* strains, and a simplified derivative were identified many years ago as mechanism-based inhibitors of *Mtb* BioA.^[9, 13] ACM showed good activity against *Mtb* cells, but failed in animal models^[13] likely due to its low chemical stability.^[14] More recently, mechanism-based inhibitors based on ACM with improved chemical stability have been described, but the stability comes at

the expense of lower potency.^[11, 15] A need therefore remains for more diverse chemical scaffolds that can serve as a starting point for inhibitor optimization and the development of potential drugs targeting *Mtb* BioA.

We have employed Fragment-based Drug Discovery (FBDD) methods to identify new inhibitors.^[16] Fragment screening provides a means to empirically identify small molecules that bind to a protein target, and subsequent structural characterization of these compounds can provide information about alternate conformational states that support that binding. Here, we report our discovery and kinetic characterization of a series of reversible covalent inhibitors of *Mtb* BioA selected for study based on their similarity to a fragment hit identified during biophysical fragment screening. Using X-ray crystallography, we also reveal the binding mode of the inactivated complexes, and describe unexpectedly diverse protein conformational changes that occur near the ligand binding site upon binding of a series of hydrazine and hydrazide analogs.

Results

KAPA binding

To provide a more reliable picture of different conformational states promoted by substrate binding, we determined the structure of the KAPA-bound enzyme at 1.8 Å resolution (Table 1). At this resolution we can confirm from the carbon hybridization and stereochemistry that, as expected, KAPA is not catalyzed in the active site to form DAPA (Figure 1C). We had no difficulty preparing this complex by co-crystallization, once the fully PLP-saturated holo enzyme was prepared and crystallized.^[17] KAPA binds to the holo enzyme much as predicted, and induces no significant conformational changes to binding site residues, except for an inward shift of the guanidinium of Arg400, which moves in to pair against the KAPA carboxylate and cap the binding pocket (Figure 1D). Such a shift had been predicted^[7] based on similarity to the *E. coli* enzyme structure.^[12] The KAPA amino group does not displace the lysine bond to the PLP, but instead sits between the Tyr25 OH and the PLP phosphate, making strong hydrogen bonds to both. Other H-bonds to Tyr-157 OH and the carbonyl O of Gly316' complete the tetrahedral H-bonding around the protonated amine (Gly316' is contributed by the alternate chain of the BioA homodimer). The ketone O of KAPA makes no hydrogen bonds, but is positioned just 3.4 Å from the N ζ of Lys283, which remains covalently bound to the PLP. The close packing of the β -methyl group against the aromatic ring of the Trp64 indole adds a strong hydrophobic contact to enhance binding.

Fragment screening and characterization

In order to discover novel BioA inhibitor scaffolds, we screened a fragment library of ~1000 small molecules to identify compounds that bind to BioA. Screening employed biophysical measurements using differential scanning fluorimetry^[18] (also known as DSF or ThermoFluor^[19]) to identify compounds that induce a shift in the denaturation temperature (T_m) upon exposure to small molecules at a concentration of 5 mM. Compounds that effected a T_m shift greater than ± 2 °C were selected for subsequent characterization by macromolecular crystallography. While 12 of the 21 compounds selected for crystallographic study caused a downward (destabilizing) shift in the T_m , only one of these

was ultimately characterized structurally, the 2-(aminomethyl)-benzothiazole hereafter designated Compound **1** (Table 2). The T_m of the holo enzyme under similar conditions (including 2.5% DMSO vehicle) is 85 °C (Supplemental Figure S1). The T_m observed in the presence of 5 mM **1** is 78 °C (Table 2).

To show that binding of **1** occurs in solution, and that binding is not simply a crystallographic artifact, a mixture of Compound **1** and BioA was followed by saturation transfer difference NMR.^[20] In this experiment, the amplitude difference of Compound **1** resonance between on resonance spectrum (in which the magnetization of the protein is saturated) and off resonance spectrum (in which the magnetization of the protein is unsaturated) was measured (Supplemental Figure S2). In the on-resonance spectrum, ligand NMR signals are attenuated by saturation transfer from the target protein to the bound ligand.^[20] Significant STD-NMR signal for compound **1** indicated that it is binding with *Mtb* BioA, which coincides with the DSF result (Table 2).

The binding site of **1** is unambiguously identified by the crystallographic data, which extends to 2.1 Å resolution. The molecule occupies only one of the two BioA active sites in the asymmetric unit. There is no obvious difference between the two active sites that may readily explain this, but we can relate the empirical observation that we have frequently seen differences in the sensitivity toward binding of small molecules in the two “equivalent” positions in this crystal form,^[7] even with respect to the covalent adduct we have previously described.^[11] The two binding environments are not identical, and they do not always behave so.

Compound **1** binds in a pocket adjacent to the PLP cofactor, but does not disrupt the internal aldimine that defines the resting state of the enzyme. Electron density (Figure 2A) clearly confirms that the covalent bond between Lys283 and the PLP remains intact. Compound **1** can be positioned without ambiguity due to higher density of the sulfur density on the thiazole ring. Compound **1** binds in direct contact with the PLP, oriented so that the amino group is hydrogen bonded to the phosphate of PLP, Tyr25 OH, Tyr157 OH and Gly316' O (Figure 2B). The amino group of KAPA in the complex described above makes these same interactions. The binding of **1** is accommodated with little change to the holo enzyme conformational state (Figure 1A). The benzothiazole heterocycle lies aside the Lys283 side chain, and between the side chains of Trp64 and Trp65. The two tryptophan indoles are oriented with a 90° angle between them, and the benzothiazole plane almost perfectly bisects that angle, so that comparable hydrophobic contacts are made with each tryptophan. The benzothiazole is just long enough to stretch across the binding pocket and to pack at right angles against the face of the aromatic ring of Phe402. Hydrophobic contacts also exist with Met174 and Ala226 (not shown).

Structure-guided fragment modification

In an effort to develop Structure-Activity Relationships (SAR) around the benzothiazole scaffold, commercially available analogs were purchased and evaluated for their ability to shift the T_m of BioA (Table 2). When the T_m shift was significant, compounds were further investigated by crystallography. One compound from this set, the 2-

(hydrazinyl)benzothiazole (Compound **2**; Table 2) stood out. Compound **2** was selected because from the Compound **1** structure it appeared that replacing the α carbon with nitrogen would result in an extra hydrogen bonding interaction with Thr318' hydroxyl group. This compound induced a destabilizing shift of nineteen degrees ($T_m = 67^\circ\text{C}$) (Figure S1). It did not escape our attention that this is nearly the T_m assigned to apo BioA.^[17]

As crystal soaking experiments were begun with Compound **2**, it became clear that this analog behaves differently in the presence of BioA. The PLP-bound holo enzyme crystals typically grown under the same conditions are yellowish in color.^[11] Holo BioA crystals changes color from yellow to red within 2 minutes in Compound **2** soaking solution (Figure 3). In co-crystallization experiments, similar phenomenon was observed. Within seconds of the addition of Compound **2** to crystallization drops, the solution develops a deep red color suggestive of chemical reaction. The reaction of **2** with BioA can be followed spectroscopically, with the time-dependent increase in absorbance at 330 and 500 nm and a decrease at 420 nm that suggests the reaction is complete in just a few minutes (Figure 3). Co-crystals of the complex were prepared under the same conditions as holo crystals, but the red color clearly concentrates in the crystals. Diffraction data for Compound **2** co-crystals were collected to 1.90 Å resolution (Table 1).

The crystal structure of the complex with **2** is shown in Figure 4. The hydrazine analog forms a covalent adduct with the PLP aldehyde to form an extended *cis*-azo quinonoid species. The bond between PLP and Lys283 is clearly broken, and the lysine amino group is relocated to H-bond with Thr318'. A planar Schiff base is formed with the hydrazine, and the entire benzothiazole is well-defined in unambiguous electron density (Figure 4A). While the benzothiazole can still be said to occupy the SAM binding subsite, it is rotated $\sim 90^\circ$ from the position occupied by **1**, shifted significantly away from Tyr 25, and out of direct contact with Phe402 (Figure 4A). Two localized enzyme conformational adjustments involving only the reorientation of side chains occur in conjunction with adduct formation. The hydroxyl group of Tyr 407 shifts 2.5 Å from the holo enzyme position to make a new H-bond to the carbonyl O of Arg400. If unchanged, a short contact to benzothiazole C7 would have existed. In the largest conformational change, the side chain of Trp65 rotates from the holo position ($hi1=-60^\circ$; $hi2=-20^\circ$) to a new position ($hi1=-60^\circ$; $hi2=90^\circ$) where the hydrogen on the indole N ϵ 1 is positioned for optimal interaction with the benzothiazole π system. Tyr25 occupies two different positions in this complex with roughly equal occupancy. In one conformation, the OH is H-bonded to Asp160 and the OH of Tyr157 as in the holo structure. In the other, the sidechain is rotated so that the OH makes an H-bond with the alternate carboxylate oxygen of Asp-160 (Figure 4A).

To determine whether or not the inhibition of BioA by Compound **2** is reversible, the BioA–Compound **2** adduct was dialyzed, which resulted in loss of the deep red color and full restoration of the enzyme activity. To further characterize the modality of inhibition, steady-state kinetic studies were performed under initial velocity conditions. Double reciprocal plots of initial reaction velocity under conditions with varying inhibitor and reactant concentration (Figure 5A) show that Compound **2** is a competitive reversible inhibitor of BioA with respect to SAM, ($K_i = 10.4 \pm 0.6 \mu\text{M}$), and an uncompetitive inhibitor with

respect to KAPA ($K_{iu} = 85.4 \pm 3.4 \mu\text{M}$). This is the expected behavior for an inhibitor of one step of a ping pong bi-bi mechanism^[21]. In forming a reversible adduct with the PLP cofactor, Compound **2** inhibits the same BioA enzyme form (PLP-BioA) with which SAM reacts. The inhibition pattern against KAPA is uncompetitive, because KAPA binding is required to regenerate the PLP-bound form of BioA (Scheme 1).

In an effort to explore whether Compound **2** possesses any selectivity, it was evaluated as an inhibitor of alanine transaminase (ALT), and of aspartate transaminase (AST), two other important mammalian PLP-dependent transaminases^[22]. Mode of inhibition studies reveal that Compound **2** is an uncompetitive reversible inhibitor of ALT with respect to α -ketoglutarate with a K_{iu} value of $74.0 \pm 8.2 \mu\text{M}$ and a competitive inhibitor with respect to alanine ($K_i = 18.9 \pm 1.6 \mu\text{M}$) (Figure 5B). As with BioA, the inhibitor competes for binding with the compound that is the amino group donor responsible for converting the PLP bound holo form to the PMP state. We did not observe any detectable inhibition of AST, however (data not shown). While Compound **2** may not be a selective inhibitor of BioA, it is also not entirely non-specific.

Armed with the knowledge that Compound **2** is a reversible competitive inhibitor of BioA, we chose to investigate attributes of the analogous hydrazide (Compound **3**; Table 2). Compound **3** also gave rise to a large destabilizing T_m shift (-15°C ; Figure S1). We were also able to characterize the complex by co-crystallization (Table 1). No visible color change is observed upon addition of **3** to BioA.

The crystal structure of Compound **3** also confirms the formation of a covalent adduct, but again, the bound adduct conformation is unique (Figure 4B). From high resolution diffraction data ($1.7 \text{ \AA}$ resolution) we can assert that conjugation throughout the Schiff base appears complete; each of the atoms is sp^2 -hybridized and planar in its bonding, but there is a subtle curvature imposed throughout the adduct that likely implies some degree of electronic strain. While the side chain of Trp65 remains primarily in the rotated position required to accommodate the adduct with **2**, the benzothiazole in the complex with **3** is positioned well above and out of contact with it, but stacks instead against the indole of Trp64. The hydrazide carbonyl is oriented toward the Trp65 indole nitrogen, but it is $3.9 \text{ \AA}$ away from it – too far to be considered a stabilizing hydrogen bond. A water-mediated interaction is possible, but no well-ordered water molecule is observed. The benzothiazole also does not point directly toward Phe402, but instead reaches past it and into the larger pocket bounded by Arg400, the side chain that interacts directly with the carboxylate of KAPA. Tyr25 is observed in the same two conformations found in the complex with **2**.

The structural similarity between Compound **3** and isoniazid (Compound **4** in Table 2), one of several chemical agents that comprise the cocktail of drugs often used to treat *Mtb* infections, led us to evaluate this aryl hydrazide as a BioA inhibitor. In the same battery of biophysical studies, isoniazid appears to interact with BioA (Table 2). It produces a more modest but still large negative shift in the T_m (-8°C), and crystallographic analysis shows that it forms a covalent adduct with the PLP that is very similar to that formed by compound **3** (Figure 4C). Nevertheless, in the coupled BioA assay used to assess inhibition kinetics^[23], both hydrazides **3** and **4** show no inhibitory activity toward BioA ($K_i > 100 \mu\text{M}$). We are led

to conclude that the adducts seen crystallographically are so easily reversed upon exposure to substrates that no significant amount of competitive inhibition can be observed by these low affinity compounds.

Discussion

The flexibility of the active site

As previously described,^[7] *Mtb* BioA (or DAPA synthase; DAPAS) is a functional homodimer with two catalytic sites that lie about 18 Å apart. Each active site is comprised of loops contributed by both polypeptide chains, including residues Pro24-Ser34, Ser62-Ala67, Arg156-Asp160, His171-Arg181, Gln224-Gly228, Arg400-Arg403, Met87'-His97', and Ala307'-Asn322' (Figure 1A). Residues tagged with a prime originate in the alternate chain. Ser125, Asp254, Lys-283 and Thr-318' all make specific interactions with the PLP, are strictly conserved in all Class I transaminases,^[24] and adopt a conserved conformation in all BioA structures reported to date. While residues 25–33 are disordered in the original BioA structure reported by Dey *et al.* (PDB-id 3BV0),^[7] Tyr25 is well-ordered in the structure we reported as a “pre-reaction” conformation in our study of an irreversible inhibitor of BioA,^[11] and the side chain hydroxyl of the tyrosine lies H-bonded to Asp-160 and poised to interact with substrates. This structure (PDB-id 3TFT) will stand to represent the holo enzyme (PLP-bound resting state) conformation in the remainder of this discussion. In this state, the PLP is covalently bound to the side chain amine of Lys283, a well-known feature of transaminases (Figure 1A).

Structures of *Mtb* BioA with substrates SAM and KAPA have not been previously reported, but detailed homology-based conceptions of substrate binding have been proposed based on studies with substrate surrogates (e.g., SAM analog sinefungin; PDB-id 3LV2), and homologous proteins (e.g., *Bacillus subtilis* BioA co-structure with KAPA, PDB-id 3DU4).^[7] Despite the fact that the two substrates are quite different, both appear likely to occupy the same binding site, comprising a largely hydrophobic pocket between aromatic side chains of Trp64, Trp65, Tyr25 and Phe402. The adenosine of SAM likely extends further (Figure 1B), but apart from the side chain of Arg400, the specific groups that interact with the nucleobase are not convincingly known because of disorder observed in surrounding loops (residues 23-34, and 309-317) in many of the homolog and BioA crystal structures. This flexibility may be an important characteristic of an enzyme that must adapt to diverse substrates during the catalysis of transamination.

Our own attempts to obtain an experimental complex structure of *Mtb* BioA with SAM or sinefungin have been unsuccessful. It can be seen, however, that the benzothiophene compounds each occupy much of the same subsite (Figure 2B). In the sinefungin complex, the amino group makes a cation- π interaction with the Phe402 side chain conserved in both the *B. subtilis* and *Mtb* enzymes. In the complex with **1**, C6 of the benzothiazole sits in exactly the same position. KAPA also binds against this phenylalanine side chain (Figure 1D), providing an explanation for why a phenylalanine at a comparable position is conserved in all homolog structures. Models for the binding of both KAPA and SAM substrates led to assertions of a significant role for Tyr25 as a participant in hydrogen bonding that helps orient substrate heteroatoms so they are poised to displace the lysine and

form adducts with the PLP.^[7] This role is supported by our complex with KAPA. The Tyr25 OH is in direct contact with the KAPA amino group, as well as side chains of Tyr157 and Asp160 – residues that combine to hold the KAPA amino group against the PLP. The exchange of hydrogen bonds between the Tyr25 OH, Asp160 and any potential substrate amino groups is facilitated by the flexibility the Tyr25 sidechain, which can move in and out of contact with the substrate during catalysis, using small conformational changes such as those observed in different complexes with the covalent adducts of this structural study. This role may be sufficient to explain why mutation of Tyr25 in *B. subtilis* BioA leads to catalytic inactivation of the enzyme.^[7]

In the *B. subtilis* BioA-sinefungin complex reported by Dey *et al.*,^[7] Tyr25 is observed in a different conformation with the side chain rotated 180° to contact the adenosine base (Fig 2B). They conclude that π -stacking between the adenosine and this tyrosine or the phenylalanine found in other bacterial species is important feature stabilizing SAM binding in Mtb BioA. While we also have no specific data to contradict this hypothesis, we do not find their arguments compelling. The geometry for effective π -stacking in the sinefungin complex structure is poor, as the phenol ring of Tyr25 is tilted 45° from the plane of the adenosine; the Tyr25 OH makes the closest contact to sinefungin in this complex, and this is not indicative of favorable π -stacking. In all complexes reported here, Tyr25 is oriented as seen in the KAPA or apo complexes, and never as seen in the sinefungin complex (Figure 1). The binding of most of the benzothiophene molecules is enhanced by π -stacking, but none of them precipitate such a dramatic change to involve Tyr25. The *B. subtilis* BioA complex (PDB-id 3LV2) is a co-crystal with a H315R mutant, and the histidine occupies the position taken by the side chain of Tyr25 in the wild type enzyme (Figure S3). It is possible that the shift in Tyr25 observed in the sinefungin BioA complex, is a consequence of this mutation, rather than sinefungin binding.

Stabilization of an azo-quinonoid intermediate

Compounds **2**, **3** and **4** all react with the PLP co-factor reversibly to form stable covalent adducts. The UV-Vis spectroscopy and crystallography results for Compound **2** confirm that the hydrazine analog forms an extensively conjugated *cis*-azo-quinonoid species. While similar quinonoids form transiently as intermediates in the catalytic mechanism of all transaminases, they are rarely captured.^[25-26] We propose a mechanism for the stabilization of this adduct from **2** in Scheme 2A. By analogy to the reaction with substrates, the hydrazine first displaces the lysine to form a PLP-coupled imine. Tautomerization of this imine leads to the formation of an azo-quinonoid; the delocalized bonding is responsible for the beautiful sanguine color. Further reversible tautomerization of the pyridoxal ring is possible through conjugation all the way to the benzothiophene (Scheme 2B). This is a unique chemical feature of Compound **2** not shared with the hydrazides (**3** or **4**) that can only form less extensively delocalized imines (Scheme 2C). While these hydrazide adducts are evidently more stable than the PLP-lysine Schiff base that they supplant, the fact that these compounds show no detectable inhibition in competition with substrates suggests that their stability is low. The hydrazine adduct, however, with a K_i of 10 μ M is unusually stable, which is approximately 80-fold lower than the K_m of SAM.^[9]

The reactivity of hydrazines and hydrazides with PLP dependent transaminases has been known for decades^[27] and the kinetics of inhibition were studied in detail long ago.^[28] More recently, there have been a number of attempts to increase the potency of inhibitors of PLP-dependent enzymes that contain a reactive hydrazide. Ejim *et al.* confirmed the formation of a stable adducts to PLP bound in cystathionine beta-lyase with a series of hydrazinocarbonylmethylbenzamides, and were able to improve inhibitor binding affinity by 50-fold with the preparation of small analog library.^[29] Another hydrazide has recently been identified as an inhibitor of *E.coli* BioA by whole-cell phenotypic screening.^[30] Others have investigated a series of aryl hydrazides as inhibitors of LL-diaminopimelate aminotransferase.^[31] Structure-activity relationships revealed in the study of these systems have identified the reactive hydrazide as a necessary, but not sufficient molecular feature needed for inhibition. Potent inhibition can only occur when other binding site complementarity also exists. These examples do not benefit from the extensive electronic delocalization that occurs upon reaction with the hydrazylbenzothiophene of Compound **2**. For this reason, we suggest that this compound may serve as a particularly effective starting point for further optimization. Whether selective inhibitors of BioA can be generated from the 2-(hydrazinyl)benzothiophene lead remains to be determined, but the complexes with compounds characterized as part of this study have exposed a variety of different conformational states that are accessible to the BioA enzyme that should prove valuable in the structure-based design of more potent inhibitors based on this scaffold.

The value of destabilizing fragment hits

Many of the studies evaluating the use of thermal shift methods for identification of protein ligands focus only on molecules that increase the transition temperature.^[18-19,32] All the compounds that were part of this study shift T_m significantly *downward*. This is true of the initial non-covalent fragment hit **1** ($T_m = -5$ °C), as well as the reversible adducts with δT_m shifts in the range of -6 to -16 °C. In prior DSF studies, we had tentatively ascribed a much lower T_m (67 °C) to the apo (PLP-free) form of BioA,^[17] and were initially drawn to these fragment hits because we expected to find that the molecules bound in place of the PLP. Structural characterization of these compounds clearly shows this is not the case. While fragment hits that shift the BioA T_m to higher temperature may be described in a future report, we can confirm that other molecules have been identified that bind in the same sub-sites and shift the T_m upward (data not shown). Why these compounds are so sharply destabilizing is not known; it may be that they play a greater role in the stabilization of the unfolded state. Empirically, it is useful to note for the benefit of those hoping to conduct similar studies with other proteins, that destabilizing compounds may also be worth structural investigation. Compounds that destabilize a protein are expected to lead to more rapid protein degradation. In *Mtb*, damaged proteins are removed by the Pupproteasome system.^[33] Thus small molecules that lead to protein destabilization could potentially result in protein depletion, which may advantageous under non-replicating conditions where protein synthesis is not occurring to replenish degraded proteins.

Conclusion

Fragment screening has permitted an exploration of BioA conformational space. By coupling a screen of a small library of compounds for those capable of affecting the BioA temperature of unfolding, with structural characterization of resulting compounds, we identified an 2-(aminomethyl)benzothiazole (Compound **1**) that binds in a position where it must compete with substrate KAPA. Commercially available analogs were acquired and characterized, leading to the identification of hydrazine and hydrazide analogs that are capable of forming covalent adducts with the PLP cofactor. The structural characterization of adducts formed with these compounds has provided a glimpse of the different ways that BioA can adapt in response to the binding of small molecules. The side chains of Tyr25, Trp65, Arg400, and Tyr407 are shown to be quite flexible. While the movement of Arg400 and Tyr 25 had been predicted, the flexibility of the other side chains was not anticipated. Reactive analogs including the hydrazines and hydrazides are useful tools for exploration of structure-activity relationships of fragment hits, as they provide a means to explore a broader sampling of conformational changes near the PLP binding site.

Some aryl hydrazines and hydrazides appear to readily form covalent complexes with the PLP co-factor in this enzyme. In many cases, however, this adduct can be readily reversed so that the formation of covalent bond is no guarantee of potent inhibition. While the hydrazides we investigated can form covalent adducts, no inhibition was observed by these compounds. Apparently, the resulting PLP-hydrazones formed upon reaction of these hydrazides are as reactive in the presence of substrates as the Schiff base conjugate of the native protein lysine. Isoniazid is one such compound that can form an easily reversible adduct, but shows no activity as a BioA inhibitor.

The 2-(hydrazinyl)benzothiazole (Compound **2**) does seem to have unusually stable characteristics that distinguish it as a reversible covalent inhibitor of BioA with $K_i = 10 \mu\text{M}$. The compound displays some selectivity with respect to other PLP dependent transaminases (ALT $K_i = 19 \mu\text{M}$; no effect on AST), so the benzothiazole constitutes a viable scaffold for the development of more selective inhibitors of biotin biosynthesis in *Mtb*.

Experimental Section

BioA protein expression and purification

BioA protein was expressed and purified according to published procedures.^[7, 17, 23] *Escherichia coli* Rosetta 2 (DE3) cells (EMD Millipore) transformed with a plasmid (pCDD126) encoding N-terminally His-tagged BioA were initially grown at 37 °C in Luria–Bertani (LB) media (5 mL) containing ampicillin (100 $\mu\text{g/mL}$) and chloramphenicol (50 $\mu\text{g/mL}$) until an OD_{600} of 0.6. The culture was transferred into four 2-liter baffles containing Terrific Broth (400 mL) with ampicillin (100 $\mu\text{g/mL}$) and chloramphenicol (50 $\mu\text{g/mL}$) to grow for 16 hours at 37 °C. The cells were harvested by centrifugation (8000g for 20 min at 4 °C). The cells were re-suspended in lysis buffer (70 mL, 50 mM HEPES, 500 mM NaCl, 1 mM PMSF, 0.4 mM PLP, 1 mM MgCl_2 , pH 7.5) and lysed by sonication. Next, TCEP (0.1 mM) and benzonase (3 units, EMD Millipore) were added to the lysate and clarified lysate was obtained by centrifugation (53,300g for 45 min

at 4 °C). Clarified lysate was filtered and loaded onto a HisTrap HP Ni-NTA column (5 mL, GE Healthcare). The column was washed to baseline with buffer A containing HEPES (50 mM pH 7.5), NaCl (500 mM), PLP (0.1 mM), TCEP (0.1 mM), and imidazole (40 mM) and eluted with a linear gradient to 100% buffer B (buffer A containing 500 mM imidazole). BioA eluted as a single peak 45% buffer B. This BioA fraction was pooled, concentrated by centrifugation (Amicon Ultra), and loaded onto a HiPrep 26/60 Sephacryl S-200 HR column (GE Healthcare) pre-equilibrated with SEC buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 0.1 mM TCEP and 1 mM EDTA). Full PLP occupancy was ensured by adding PLP (1 mM) to the pooled BioA fraction, concentrating by centrifugation to a low volume (2 mL). Unbound PLP was removed by repeated dilution and reconcentration using SEC buffer lacking PLP. The homogeneity of the protein was assessed by SDS-PAGE (4–20% Tris-HCl BioRad). Differential scanning fluorimetry (DSF) was used as previously described to ensure that the holo enzyme was fully saturated with co-enzyme.^[17]

Fragment Library

The Maybridge Ro3 1000 Diversity Fragment Library was obtained from Thermo Fischer Scientific. The library includes ~1000 compounds that are “rule-of-three” compliant (MW 300D; cLogP 3.0; H-bond acceptors 3; H-bond donors 3; rotatable bonds 3; polar surface area 60Å²). Small molecules obtained in solid form were dissolved in 100% DMSO to a final compound concentration of 200 mM and stored at -20 °C.

Differential scanning fluorimetry (DSF) for fragment screening

DSF was used to assess the homogeneity of the BioA protein; it was also used to identify small molecules that could cause a significant shift of the denaturation temperature (T_m) of the protein. To assess the homogeneity of the protein, purified BioA was diluted at 4 °C to give a DSF solution (40 µL) consisting of BioA (0.05 mg/mL), HEPES (25 mM, pH 7.5), NaCl (50 mM) and 5X SYPRO Orange (Life Technologies). To identify small molecule hits, small molecules from the Maybridge Ro3 library were added individually to each DSF solution to a final concentration of 5 mM. The fluorescence response (melting curve) was measured across a temperature range following established procedures.^[18] A T_m was determined from the peak of the first derivative of each melting curve; calculations were made by Bio-Rad CFX Manager software. The plots were generated using R (<http://www.R-project.org>).

Saturation transfer difference NMR spectroscopy (STD NMR)

STD NMR spectra were obtained at 20 °C using a Bruker 700 MHz NMR spectrometer with a TCI cryoprobe, incorporating Z-axis gradients.^[20] Samples contained BioA protein (30 µM) and compound 1 (200 µM). A one-dimensional pulse sequence incorporating a $T_{1\rho}$ filter was used for the acquisition of STD NMR spectra. The on-resonance frequency was set to 0.8 ppm and the off-resonance frequency was set to 30 ppm. Irradiation was performed using 50-Gaussian pulses with a 1% truncation and a 49-ms duration and separated by a delay of 1 ms to give a total saturation time of 2 s. The duration of the $T_{1\rho}$ filter was 15 ms. STD NMR spectrum was acquired with a total of 6144 transients in

addition to 32 scans to allow the sample to come to equilibrium. The spectral width was 8 kHz. Reference spectrum was taken under the same conditions.

Crystallization and X-ray Data Collection

The crystallization conditions used were as described.^[7] BioA was co-crystallized with the compounds respectively by the vapor diffusion method in a hanging drop at 20 °C. Protein solution (10 mg/mL in 25 mM HEPES pH 7.5, 50 mM NaCl, 0.1 mM TCEP) was mixed with reservoir solution (9–14% PEG 8000, 100 mM HEPES pH 7.5, 100 mM MgCl₂, and 5 mM compound) and a seed solution (a reservoir solution containing crushed BioA crystals) in a 4:3:1 ratio. Crystals appear in the drop within 24 hours and grow to their full size in 72 hours. BioA-compound co-crystals were cryo-protected by transferring to a cryo solution (15% PEG400, 15% PEG 8000, 100 mM HEPES pH 7.5, 100 mM MgCl₂, and 5 mM compound) and then flash frozen in liquid nitrogen. The diffraction data for KAPA, and co-crystals with compounds **1**, **2** and **3** were collected at 100 K using synchrotron radiation with a Dectris Pilatus 6M Pixel Detector on beamline 17-ID (IMCA-CAT) at APS, Chicago, United States. The data were processed, integrated, and scaled with XDS^[34] and SCALA^[35] using the autoPROC scripts available at APS-17-ID (IMCAT-CAT). Data for the compound **4** co-crystal were collected at 100 K using Cu *K*α radiation on a Rigaku HighFlux HomeLab rotating-anode system with a Saturn 944+ CDD detector in the Kahlert Structural Biology Laboratory at the University of Minnesota. This data was processed, integrated and scaled with d*TREK.^[36] The data collection and processing statistics are given in Table 1.

Structure determination

The structures were solved by molecular replacement using Phaser^[37] in the CCP4 package^[38] using atomic coordinates from PDB code 3TFT as a search model.^[11] Refinement and model building was done using REFMAC5^[39] and coot.^[40] The figures were prepared with PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.). Structures were superimposed for analysis and display using the shared BioA-PLP overlay method of the DrugSite server.^[41] Atomic coordinates and diffraction data for the five crystals structures presented in this report have been deposited in the Protein Data Bank with accession codes 4MQN, 4MQO, 4MQP, 4MQQ, and 4MQR (Table 1).

UV-Vis spectroscopy

A NanoDrop 1000 UV–Vis spectrophotometer (Thermo Scientific) was used for all UV–Vis spectroscopy. *Mtb* BioA protein (2.0 μL, 0.16 mM) in HEPES (25 mM, pH 7.5), NaCl (50 mM, 1 mM EDTA), TCEP (0.1 mM) was mixed with compound **2** (2.0 μL, 0.4 mM) in HEPES (25 mM, pH 7.5), NaCl (50 mM), EDTA (1 mM), TCEP (0.1 mM) and its UV-Vis spectrum was immediately measured upon mixing. UV-Vis spectra were taken at 0 s, 15 s, 30 s, 60 s and 120 s after mixing.

Biochemical evaluations

Mode of inhibition studies were carried out under initial velocity conditions in a total volume of 50 μL at 25 °C in 384 well black plates (Corning 3575). Reactions were set up in

triplicate and consisted of BioA (114 nM) in reaction buffer (100 mM Bicine pH 8.6, 50 mM NaHCO₃, 1 mM MgCl₂, 0.0025% Igepal CA-630, 5 mM ATP, 0.1 mM PLP, 320 nM *E. coli* BioD, 20 nM Fl-DTB, 184 nM streptavidin, and 1 mM TCEP) with either variable amounts of KAPA (0.94–7.5 μM) with SAM (2.34 mM) or with a variable amount of SAM (0.3–2.5 mM) with a fixed amount of KAPA (1.9 μM). Each substrate concentration was run with 0, 31.25, 62.5, and 125 μM inhibitor. Reactions were monitored on a microplate reader using an excitation of 485 nm, an emission at 535 nm. A standard curve of dethiobiotin (2.7 nM–2 μM) in reaction conditions lacking only BioA was used to convert fluorescence into enzyme velocities as previously described^[23]. The data was fit using the enzyme kinetics module of SigmaPlot to competitive, uncompetitive, and non-competitive models and the model with the highest r^2 value was selected. The PLP utilizing enzymes alanine transaminase (ALT, EC 2.6.1.2) and aspartate transaminase (AST, EC 2.6.1.1) were used to test off target inhibition of compound **2**. Reactions were carried out in 100 μL in 96 well UV clear half-area plates (Corning 3679). For ALT, reactions consisted of enzyme (10 mU) in reaction buffer (60 mM Bicine pH 8.0, 0.1 mM NADH, 1 mM TCEP) containing 100 mU lactate dehydrogenase (LDH) and either fixed α-ketoglutarate (75 μM) with 0.625–10 mM alanine or fixed alanine (10 mM) with 6.25–50 μM α-ketoglutarate. Each concentration of substrate was run with DMSO or 29.6–66.7 μM compound **2**. The reactions (in duplicate) were monitored by the decrease in A₃₄₀ that corresponds to the consumption of NADH by LDH upon the formation of pyruvate from ALT. Initial velocities were calculated using the molar absorptivity of NADH (6220 M⁻¹ cm⁻¹ at 340 nm) and fit as described above using SigmaPlot. To test for inhibition of AST 2 mU of enzyme in reaction buffer containing 100 mU malate dehydrogenase (MDH), 236 μM α-ketoglutarate and 0.156–2.5 mM aspartate was tested against compound **2** (100 μM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 1.
Catalysis of DAPA synthesis by BioA

Scheme 2.

A) Proposed mechanism of Compound 2-PLP adduct formation; B) Tautomerization of Compound 2-PLP adduct; C) Proposed mechanism of Compound 3-PLP adduct formation.

Figure 1.

A comparison of the BioA complex with KAPA to other structures. A) The holo (PLP-bound) *Mtb* BioA structure (PDB-3TFT)^[11]. The enzyme resting state with PLP covalently linked to K283. B) Sinefungin (SFG)-bound structure of *B. subtilis* BioA (PDB-3LV2) complex^[7]. Electron density for KAPA in the active site. C) 2Fo-Fc electron density (1σ) for KAPA, PLP, and Lys383. D) Detail of KAPA binding vicinity.

Figure 2.

Structure of complex with Compound **1**. A) Binding site vicinity including electron density (1σ $2F_o-F_c$) for bound Compound **1** (magenta). Density shows binding without disruption of the Schiff base formed between the K383 and the PLP.(cyan) B) Active site detail in the same orientation as shown in Figure 1. Compound **1** is labeled IN1.

Figure 3.

Left: UV-Vis spectroscopy of 0.16 mM PLP bound BioA (Holo BioA) upon mixing with 0.4 mM Compound **2** at different time points. Right: Time-lapsed photos of a PLP-bound BioA crystal soaked in Compound **2** (15% PEG 8000, 100 mM HEPES pH 7.5, 100 mM MgCl₂, and 5 mM Compound **2**) over 2 minutes.

Figure 4.

A)-C) A comparison of complexes of covalent adducts created by reaction of PLP with Compounds 2, 3, and 4. Covalent adducts are labeled PLP-IN2, PLP-IN3, and PLP-IN4, respectively. 1σ $2F_o - F_c$ electron density for K383, the PLP and each ligand is shown (mesh). Electron density clearly shows that the bond to K383 is replaced with a covalent bond to each compound. Tyr25 is shown in two conformations when so observed. Local binding environment of each adduct (2-4) is shown in the same orientation in frames D)-F) to emphasize structural similarities and differences.

Figure 5.

A)-B): Inhibition studies with Compound **2**. Initial rate data of variable amounts of inhibitor and either KAPA (A) or SAM (B). KAPA was varied from 0.94 to 7.5 μM and SAM was varied from 0.3–2.5 mM with the fixed substrates held at 2.34 mM for SAM and 1.9 μM for KAPA. Compound **2** was used at concentrations of 0 μM ($\bullet$), 31.3 μM ($\circ$), 62.5 μM ($\blacktriangledown$), 125 μM (∇). The inhibitor is uncompetitive with respect to KAPA and competitive with respect to SAM with K_i values of $85.4 \pm 3.4 \mu\text{M}$ and $10.4 \pm 0.6 \mu\text{M}$ respectively. Inhibition of ALT by Compound **2**. C)-D): Initial rate data of variable amounts of inhibitor with respect to α -ketoglutarate (C) or alanine (D). The substrate α -ketoglutarate was varied from 6.25 to 50 μM and alanine was varied from 0.625 to 10 mM with the fixed substrates held at 10 mM for alanine or 75 μM for α -ketoglutarate. Compound **2** was used at concentrations of 0 μM ($\bullet$), 29.6 μM ($\circ$), 44.4 μM ($\blacktriangledown$), and 66.6 μM (∇). The inhibitor is uncompetitive with respect to α -ketoglutarate and competitive with respect to alanine with K_i values of $74.0 \pm 8.2 \mu\text{M}$ and $18.9 \pm 1.6 \mu\text{M}$, respectively.

Table 1

Summary of Crystallographic Data

Ligand name	KAPA	Compound 1	Compound 2	Compound 3	Compound 4
PDB-Id	4MQN	4QMO	4MQP	4MQQ	4MQR
Source	APS 17-ID (IMCA)	APS 17-ID (IMCA)	APS 17-ID (IMCA)	APS 17-ID (IMCA)	Rigaku Micromax-007
Detector	DECTRIS PILATUS 6M	DECTRIS PILATUS 6M	DECTRIS PILATUS 6M	DECTRIS PILATUS 6M	Saturn 944+ CCD
Wavelength (Å)	1.000	1.000	1.000	1.000	1.541
Space group	P2 ₁ 2 ₁ 2 ₁				
molecules per ASU	2				
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	62.55, 66.15, 205.29	62.94, 66.08, 201.90	63.02, 65.92, 201.96	62.95, 66.35, 203.97	63.13, 66.48, 203.68
Resolution (Å) (Highest shell)	55.60-1.80 (1.806-1.800)	100.95-1.70 (1.706-1.700)	201.96-1.83 (1.93-1.83)	63.05-1.55 (1.555-1.550)	63.20-2.10 (2.18-2.10)
R _{merge}	0.075(0.432)	0.060 (0.445)	0.063(0.284)	0.088(0.366)	0.055(0.182)
Mean I/σ(I)	16.6(4.0)	17.4 (3.8)	18.0(5.5)	14.0(2.7)	16.6(2.8)
No. observations	515130	403601	467939	757129	145139
Completeness	98.9% (98.3%)	97.6% (96.2%)	98.3%(96.6%)	99.0%(86.0%)	88.4%(73.4%)
Multiplicity	6.5 (6.5)	4.4 (4.7)	6.4 (6.3)	6.2 (4.5)	3.22 (2.17)
No. unique reflections	79062	86627	73075	121718	45088
<i>Refinement</i>					
Resolution (Å)	55.60-1.80	100.95-1.70	100.93-1.83	63.05-1.70	63.20-2.10
Rwork/Rfree (%)	17.2/20.4	19.7/22.6	16.6/20.7	18.2/20.8	20.5/25.5
No. Protein atoms	7184	6750	7106	7597	6885
No. Water	527	232	474	747	375
No. ligand molecule	2	1	2 [*]	2 [*]	2 [*]
No. PLP molecule	2	2	2	2	2
No. other molecule	5	3	4	4	4
Ramachandran plot					
Favored	765 (95.6%)	797 (96.6%)	758(95.0%)	707 (96.7%)	784 (94.0%)
Allowed	29 (3.6%)	22 (2.7%)	27(3.4%)	20(2.7%)	35 (4.2%)
Disallowed	6 (0.7%)	6 (0.7%)	13(1.6%)	4(0.5%)	15 (1.8%)
R.m.s deviations					
Bond lengths (Å)	0.009	0.008	0.009	0.009	0.01
Bond angles (°)	1.243	1.32	1.34	1.27	1.311

* The ligand forms a covalent adduct with the PLP cofactor in the active site.

Table 2

The Binding of small molecules to BioA

Compound	Structure	DSF T_m (°C)
1		78
2		67
3		70
4		77
5		83
6		83
7		73
8		83
9		83
10		Not Available