Crystal structures of *Mycobacterium tuberculosis* S-adenosyl-L-homocysteine hydrolase in ternary complex with substrate and inhibitors

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Abstract

S-adenosylhomocysteine hydrolase (SAHH) is a ubiquitous enzyme that plays a central role in methylation-based processes by maintaining the intracellular balance between S-adenosylhomocysteine (SAH) and S-adenosylmethionine. We report the first prokaryotic crystal structure of SAHH, from *Mycobacterium tuberculosis* (*Mtb*), in complex with adenosine (ADO) and nicotinamide adenine dinucleotide. Structures of complexes with three inhibitors are also reported: 3'-keto aristeromycin (ARI), 2-fluoroadenosine, and 3-deazaadenosine. The ARI complex is the first reported structure of SAHH complexed with this inhibitor, and confirms the oxidation of the 3' hydroxyl to a planar keto group, consistent with its prediction as a mechanism-based inhibitor. We demonstrate the in vivo enzyme inhibition activity of the three inhibitors and also show that 2-fluoroadenosine has bactericidal activity. While most of the residues lining the ADO-binding pocket are identical between *Mtb* and human SAHH, less is known about the binding mode of the homocysteine (HCY) appendage of the full substrate. We report the 2.0 Å resolution structure of the complex of SAHH cocrystallized with SAH. The most striking change in the structure is that binding of HCY forces a rotation of His363 around the backbone to flip out of contact with the 5' hydroxyl of the ADO and opens access to a nearby channel that leads to the surface. This complex suggests that His363 acts as a switch that opens up to permit binding of substrate, then closes down after release of the cleaved HCY. Differences in the entrance to this access channel between human and *Mtb* SAHH are identified.

Keywords: s-adenosyl-L-homocysteine hydrolase; *Mycobacterium tuberculosis*; ternary complex; aristeromycin; 2-fluoroadenosine; 3-deazaadenosine

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Abbreviations: SAHH, S-(5'-Adenosyl)-L-homocysteine hydrolase; SAM, S-(5'-Adenosyl)-L-methionine; SAH, S-(5'-adenosyl)-L-homocysteine; ETA, ethylthioadenosine; ADO, adenosine; HCY, homocysteine; DZA, 3-deazaadenosine; ARI, aristeromycin, also 3'-keto aristeromycin; 2FA, 2-fluoroadenosine; NAD⁺, nicotinamide adenine dinucleotide; MDR/XDR, multidrug-resistant/extensively drug-resistant; MIC, minimum inhibitory concentration.

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S-adenosylhomocysteine hydrolase (SAHH) is an enzyme that catalyzes the reversible hydrolysis of S-adenosylhomocysteine (SAH) into free adenosine (ADO) and L-homocysteine (HCY). SAH is produced from S-adenosylmethionine (SAM) as a by-product of SAM-dependent methyltransferase reactions. Methylation plays a role in a wide range of cellular processes, including DNA replication and repair (Chiang et al. 1996), quorum sensing (Schauder et al. 2001), methionine metabolism, polyamine biosynthesis, and phospholipid biosynthesis (Tehlivets et al. 2004). SAH is a strong feedback inhibitor of many SAM-dependent

methyltransferases, so SAHH plays a crucial role in clearing the cofactor product and maintaining the proper equilibrium. The balance between concentrations of SAM and SAH is referred to as the “methylation ratio” (typically maintained at $\sim 10:1$), and perturbation of this ratio has been demonstrated to arrest growth of various cell lines (Kramer et al. 1990). Because of its central role in metabolism, SAHH has been targeted for drugs against a number of diseases, including hypercholesterolemia (Yamada et al. 2007), malaria (Bujnicki et al. 2003), and cancer (Yaginuma et al. 1981). Additionally, inhibitors of SAHH have also been shown to have antiviral activity against a variety of pox viruses by inhibiting mRNA methylation during the replication cycle (De Clercq et al. 1989).

SAHH (Rv3248c) is an important enzyme in *Mycobacterium tuberculosis* (*Mtb*)—the causative agent of tuberculosis (TB). TB is a worldwide health threat, killing over two million people per year, with an estimated one-third of the human population carrying at least latent infections. New targets for antitubercular drugs are needed because of the lengthy treatment course, and more importantly, because of the alarming rise in drug-resistant (MDR/XDR) strains (Centers for Disease Control and Prevention 2006). SAHH represents a potential drug target in *Mtb* because it has been shown to be essential for growth in vitro (Sasseti et al. 2001). Although it is down-regulated two- to threefold during starvation conditions in vitro (Betts et al. 2002), and is not significantly up- or down-regulated in activated macrophages (Schnappinger et al. 2003), it appears to be up-regulated in infected mouse lung tissue (determined using promoter-trap experiments) (Dubnau et al. 2005), a condition which also shows up-regulation of other infection-related genes, like isocitrate lyase. SAHH is also considered “druggable,” given the large number of nucleoside analogs with activity that have been discovered, such as aristeromycin (ARI) (Wolfe and Borchardt 1991), neplanocin A (Yaginuma et al. 1981; Borchardt et al. 1984), and other ADO analogs (Guranowski et al. 1981). However, these are not considered clinically relevant due to cytotoxicity issues (De Clercq et al. 1989). Care must be taken to avoid inhibition of human SAHH and other ADO-binding proteins in the host, since the *Mtb* SAHH has 61% amino acid sequence identity with the human homolog (Thompson et al. 1994).

The crystal structure for SAHH has been solved for three different organisms to date, all eukaryotic: human, rat (*Rattus rattus*), and malaria (*Plasmodium falciparum*) (Turner et al. 1998; Hu et al. 1999; Komoto et al. 2000; Huang et al. 2002; Takata et al. 2002; Yang et al. 2003; Tanaka et al. 2004; Yamada et al. 2005, 2007). The proposed SAHH catalytic mechanism involves a sequence of steps as follows (Fig. 1; Palmer and Abeles 1979; Takata et al. 2002): (1) oxidation of 3' hydroxyl to keto by NAD^+ , (2) abstraction of proton from C4' to form a

Figure 1. Reaction mechanism catalyzed by SAHH.

carbanion intermediate, (3) cleavage of the thioether by elimination to release HCY, (4) hydration of the C4'=C5' double bond by Michael addition of a water, and finally, (5) reduction of 3' keto to form ADO and regenerate NAD^+ .

The activity of ADO analogs as competitive inhibitors can be rationalized through similar recognition and affinity compared to substrate, but inability to complete the reaction (Wolfe and Borchardt 1991). For example, 2',3'-dihydroxycyclopent-4'-enyl adenine (DHCEA) inhibits SAHH via a type-I mechanism (Wolfe and Borchardt 1991) by oxidation of the 3' hydroxyl to a 3' keto group, which reduces the bound NAD^+ to NADH, thus inactivating the enzyme (Turner et al. 1998). This compound represents a family of carbocyclic pyrimidine nucleosides, like neplanocin A (Mosley et al. 2006), that form similar chemically abortive complexes (Yang et al. 2003). ARI is also expected to inhibit SAHH via a type-I mechanism (Liu et al. 1996). Attempts have also been made to synthesize compounds exhibiting type-II inhibition (activation followed by covalent attachment to active site) (Wolfe and Borchardt 1991). For example, 5'-carboxyldehyde and vinyl-fluoride derivatives of ADO appear to react with a protein nucleophile at the 5' position (Liu et al. 1996). There are also more conventional reversible inhibitors, including acyclic analogs such as D-eritadenine, whose affinity appears to be due to reorganized hydrogen bonding, which locks the enzyme into a closed conformation (Huang et al. 2002). In this paper, we report the crystal structure of *M. tuberculosis* SAHH—the first from prokaryotes—in complex with product, ADO, and three inhibitors: ARI, 2-fluoroadenosine

(2FA), and 3-deazaadenosine (DZA) (also shown in Fig. 2). Enzyme activity and whole-cell assays were carried out to evaluate the efficacy of the potential inhibitors.

Although there has been a significant amount of study on SAHH inhibition in other organisms, the inhibitor designs have until now focused primarily on the ADO binding pocket. All known inhibitors of SAHH are analogs of ADO, complexes of which do not reveal the binding mode of the full substrate: SAH. The binding site of the HCY portion of the substrate has not yet been identified, with the pocket appearing sealed off at the 5' position of ADO. To better understand the possible binding mode of the HCY moiety of SAH, we determined the crystal structure of the SAHH:SAH complex by cocrystallization. The structure reveals a putative solvent access channel that aids in the accommodation of the HCY appendage of SAH and the release of HCY after its elimination. We also identify interesting differences between the human and *Mtb* SAHH in this purported solvent access channel and discuss implications for selective inhibitor design.

Results and Discussion

Overall structure

Mtb SAHH crystallized as a homotetramer in the space group $P2_1$. Each subunit of *Mtb* SAHH (495 residues)

Figure 2. Chemical structures of some SAHH inhibitors.

consists of two α/β domains (Fig. 3A), as observed in previous structures, with domain I being a substrate-binding catalytic domain and domain II being a dinucleotide-binding domain (Rossmann fold). Each subunit is bound to one NAD^+ molecule. Domain I consists of residues 11–247 plus 423–466 (281 total), and domain II consists of residues 248–422 (175 total). In addition, there is a C-terminal extension of 29 residues (467–495) observed in SAHH from other organisms (except Archaea, where it is truncated by eight residues) (Porcelli et al. 2005) that covers the NAD -binding site in an adjacent subunit. This interaction is complemented by the other subunit in a local twofold symmetry, making the tetramer a “dimer of dimers” (Fig. 3B). Upon a dimer formation (between chains A and B, for example; Fig. 3B), $\sim 5700 \text{ \AA}^2$ of surface area is buried within each subunit, whereas a total of only $\sim 3440 \text{ \AA}^2$ of surface area is buried between chain A and the other two subunits (C and D) in the tetramer combined. The N-terminal 10 residues are disordered. This includes half of the 20-residue N-terminal extension found in *Mtb* and other prokaryotic sequences but not seen in eukaryotes; residues 11–19 form an additional β -strand that packs against and extends the edge of the core β -sheet in domain I. This structure superimposes well on previously determined ligand-bound SAHH structures. The backbone $\text{C}\alpha$ RMSD between the *Mtb* SAHH:ADO structure and the ADO-complexed structure from *P. falciparum* (PDB ID: 1V8B) (55% amino acid identity) is $0.84 \text{ \AA}$ (over 461 residues, or 94% of the sequence), as determined by SSM (Krissinel and Henrick 2004). Similarly, the RMSD to the human SAHH (PDB ID: 1A7A) is $0.77 \text{ \AA}$ over 422 residues, and the RMSD to the rat SAHH (PDB ID: 1KY5) is $0.90 \text{ \AA}$ over 415 residues. A superposition of the backbones of these four enzymes is shown in Figure 4. The domains are in a “closed” conformation, with the hinge between domains flexed by ~ 17 degrees (based on comparison with apo-structure from the rat liver enzyme [Turner et al. 2000], since all four structures reported herein were complexed with a product or an inhibitor). There is a nine-residue insertion (residues 109–117) that is unique to Actinobacteria (Stepkowski et al. 2005), but this occurs in a surface loop and has no role on the active site or tetramer formation.

Domain II binds NAD^+ in a similar fashion to that observed in all previous SAHH crystal structures. The dinucleotide-binding domain is considered an atypical Rossmann fold because of variation in the topological connections of the $\beta\alpha\beta\alpha\beta$ core (Turner et al. 1998). Specific interactions with NAD^+ include the side chains of Thr20, Thr21, Asn253, Val286, Thr219, Thr220, Thr221, Thr338, Asn340, Thr304, Glu305, Ile306, Asn310, and Ile343, as well as numerous backbone atoms. All are conserved identically between human and *Mtb* SAHH, except Asn340 contacting the adenine

Figure 3. Overall structure (backbone representation) of *Mtb* SAHH. (A) Subunit bound to NAD⁺ and ADO. The insertion region in the substrate-binding domain is shown in violet. (B) Tetramer showing subunits A, B, C, and D. Each subunit is bound to one NAD⁺ and ADO.

ring, which replaces Cys278 in human SAHH. In addition, several residues from the C-terminal tail of an adjacent subunit make contact with the NAD⁺, covering over the binding site. These include Tyr493 and Lys489 (from subunit B), which hydrogen bond with the diphosphate and ribose moieties, respectively.

The *Mtb* SAHH structure has a 37-residue insertion (residues 167–203) that is observed in other prokaryotic but not mammalian sequences (Stepkowski et al. 2005). The insertion extends helix $\alpha 10$ (human SAHH numbering) (Turner et al. 1998) by two turns, and then forms an antiparallel strand-turn-helix (see Fig. 4) that packs on the surface of domain I, before rejoining helix $\alpha 11$. The insertion is similar to that observed in the *P. falciparum* structure (PDB ID: 1V8B) (Tanaka et al. 2004). However, in 1V8B, helix $\alpha 10$ is extended by one additional turn before the strand begins, so they do not perfectly superimpose (Fig. 4). The insertion structure lies on the surface of the substrate-binding domain of a subunit and does not interact with adjacent subunits (i.e., does not influence packing of the tetramer). The insertion residues are far

away from the active site (at least 12.7 Å away from the ADO in *Mtb* SAHH).

The ADO-binding portion of the active site (Fig. 5) looks similar to that of previous structures from other organisms complexed with ADO analogs. The active site is enclosed in a buried cavity $\sim 14 \times 9$ Å in size, with the nicotinamide ring of NAD⁺ forming part of the boundary (packing against the substrate ribosyl ring, where it oxidizes and reduces C3'). All of the residues thought to be catalytically important are conserved, including Lys248 and His69 (equivalent to Lys185 and His54 in the rat numbering). The adenine ring is surrounded by hydrophobic residues: Leu68, Thr71, Gln73, Leu410, Met421, and Phe425. Ionizable residues Asp156, Asp252, Lys248, and Glu218 interact with the hydroxyls of the ribose. His69 and His363 form hydrogen bonds with the 5' hydroxyl (2.82 and 2.69 Å, respectively). The only residue that is not perfectly conserved is a substitution of Glu58 (rat) by Gln73 in *Mtb* SAHH, both of which form a hydrogen bond with the N6 amino group on the adenine ring.

Complexes of SAHH with NAD⁺ and inhibitors

In addition to the product complex discussed above (with ADO), crystal structures for *Mtb* SAHH were also determined in complex with 3'-keto aristeromycin (ARI), 2-fluoroadenosine (2FA), and 3-deazaadenosine (DZA), at resolutions of 2.01 Å, 2.42 Å, and 2.20 Å, respectively, and exhibited very similar binding-site geometry, with no significant changes in side-chain conformation. Superpositions with the ADO-bound structure

Figure 4. Superposition of *Mtb* SAHH (violet) with human (red, 1A7A), rat (cyan, 1KY5), and *P. falciparum* SAHH (yellow, 1V8B). The bound ADO and NAD⁺ from the *Mtb* structure are shown for reference. The 37-residue insertion in the *Mtb* and *Plasmodium* structures can be seen forming a strand-turn-helix at the surface (upper left, in violet and yellow). $\alpha 10$, αI (insertion), and $\alpha 11$ have been labeled for reference.

Figure 5. *Mtb* SAHH active site, including residues within 4 Å of ADO and NAD⁺. The figure was made using Ligplot (Wallace et al. 1995).

had RMSD values of 0.18, 0.22, and 0.25 Å for complexes with ARI, 2FA, and DZA, respectively.

The complex with ARI (Fig. 6A) is the first crystal structure of SAHH to be solved with 3'-keto aristeromycin. The ribosyl ring oxygen is replaced with a carbon atom in ARI, which is in van der Waals contact with Leu410 (3.65 Å), as in the ADO complex. Thus, no significant hydrogen bonds observed in the other crystal structures were disrupted by this substitution. His69 and His363 still form hydrogen bonds with the 5' hydroxyl group of ADO. Importantly, C3' is observed to be planar, consistent with O3' being oxidized to the keto form. This confirms the prediction based on the proposed mechanism of action for ARI (type-I inhibitor, activated by NAD⁺) (Wolfe and Borchardt 1991). This abortive complex bears resemblance to the complex of rat SAHH with 3'-keto adenosine (PDB ID: 1KY5) (Takata et al. 2002), in which C3' is also planar. O3' nonetheless continues to hydrogen bond with the nearby Lys248 side chain, which is close enough (3.23 Å) to abstract a proton, as well as Thr219 (2.82 Å). ARI strongly inhibits the *Mtb* SAHH enzyme, with an IC₅₀ of 0.2 μM (see Table 1). This is commensurate with inhibition activity reported for ARI against SAHH from other organisms (1.1 μM vs. *Homo sapiens*,

3.1 μM vs. *P. falciparum*) (Tanaka et al. 2004). The strong inhibitory activity of ARI can be explained based on the mechanism. In ADO, the proton abstraction from C4' is assisted by the presence of an electronegative keto group on one side (at the C3'), and the ribosyl oxygen on the other side, which makes the C4' proton more acidic. In ARI, the ribosyl oxygen is replaced by a less electronegative carbon and hence the intermediate gets stuck in the 3' keto form.

The complex with 2FA (Fig. 6B) is similar to that of ADO, including the pucker of the ribose ring, and the position of the adenine ring (RMSD = 0.22 Å). The fluoro group fits snugly into a hydrophobic pocket, surrounded by Phe425, Leu68, and the backbone carbonyl of His69 (all at 3.13–3.30 Å distance). However, the closest contact is with the hydroxyl group of Thr74, to which the fluorine atom forms a hydrogen bond (2.48 Å). While not as potent as ARI, 2FA exhibits better inhibition (66 μM IC₅₀) compared to ADO, which was only found to

Figure 6. Active site showing complexes with inhibitors and the substrate. NAD is shown in cyan. Electron density of Shake&wARP (Kantardjieff et al. 2002) omit maps are contoured at the 1 σ level. Ligand molecules were omitted from the model before the map calculation. The blob feature in XTALVIEW (McRee 1999) has been applied to limit the electron density display within 2.0 Å of the ligand and the final figure is rendered with Raster3D (Merritt and Murphy 1994). (A) 3'-Keto-aristeromycin (ARI). (B) 2-Fluoroadenosine (2FA). (C) 3-Deazaadenosine (DZA). (D) Ethylthioadenosine (ETA).

Table 1. Enzyme activities of the inhibitors against *Mtb* SAHH

Compound	IC ₅₀	MIC	logP
Adenosine	~500 μ M	Not determined	-1.6
Aristeromycin	0.2 μ M	>100 μ M	-1.0
2-Fluoroadenosine	66 μ M	32 μ M	-1.4
3-Deazaadenosine	20 μ M	>100 μ M	-1.3

IC₅₀s was determined by enzyme assay; MIC represents minimum inhibitory concentration against *M. smegmatis* mc²155 cells in a whole-cell assay. LogP values (estimated partition coefficients) were determined by the XLogP v2.0 method of Wang et al. (2000).

inhibit the enzyme at concentrations above 500 μ M (i.e., via product inhibition). Thus, the fluorine atom, which is the only chemical difference, has a significant effect on binding affinity. A similar IC₅₀ for 2FA has been observed for the human SAHH homolog (63 μ M) (Tanaka et al. 2004). However, in *P. falciparum*, polar substitutions at the 2-position have been found to have increased relative inhibition, which was attributed to the replacement of Thr60 (human) by Cys59, creating additional space in the ADO-binding pocket (Tanaka et al. 2004). Importantly, however, in contrast to ARI, 2FA was found to have bacteriocidal activity against *Mycobacterium smegmatis* in a whole-cell assay (MIC of 32 μ M, whereas ARI and DZA had MICs >100 μ M, Table 1). Fluorine substituted nucleosides are known to be better antibacterials (Pittillo et al. 1964). This may reflect differences in cell-wall permeability or uptake mechanism (e.g., by an active nucleoside transporter, which might not recognize carbocyclic compounds like ARI).

The complex with DZA (Fig. 6C) superposes almost perfectly onto the ADO complex, with an RMSD of 0.25 Å between the ligands. The N6 amino group still hydrogen bonds with the carbonyl oxygen on Gln73 (3.02 Å). The C3 position of the adenine ring (substituted for N3) is 3.63–4.52 Å away from Phe425, Leu68, and Thr74. The IC₅₀ for DZA is 20 μ M (Table 1). DZA did not show whole-cell bacteriocidal activity, which again could be due to inability of DZA to exploit the same uptake mechanism as 2FA.

Homocysteine binding site and putative access channel

An interesting open question about the active site of SAHH is where the HCY portion of the full SAH substrate is bound by the enzyme. In all previous structures of complexes with ADO-based inhibitors, the ligand is tightly enclosed in a buried pocket, with no apparent room for the HCY appendage near the 5'OH. His363 is proximal to the 5'OH of the ribose, the site where the thio-ether linkage to the HCY of the substrate would be expected to be.

To gain a better understanding of the region where the HCY moiety of the substrate can bind, we cocrystallized *Mtb* SAHH with SAH. Density corresponding to the covalently attached HCY was observed in an access channel (Fig. 6D), adjacent to the 5' position on the ADO. The channel is formed by rotation of the peptide plane between C α 363 and C α 364 by nearly 180°, swinging the imidazole ring of His363, which was at 2.69 Å from the 5'OH of ADO (SAHH:ADO:NAD structure), to 8.3 Å away from the S δ of SAH (SAHH:SAH:NAD structure). This rotation opens up an access channel to the buried ADO-binding pocket that can accommodate the HCY moiety of the SAH substrate (cf. Figs. 8, A and B). The channel leads to the surface (~20 Å away), and could permit release of the liberated HCY while the rest of the catalytic cycle continues (i.e., hydration at C5', followed by reduction of keto at C3'). S δ of SAH makes a hydrogen bond with His69 (3.03 Å). The C β and C γ atoms make van der Waals contacts (3.66–4.09 Å) with Gly362, Thr219, Leu407, and the 2'OH of the NAD. Phe364 is also close by (4.65 Å), contributing to the hydrophobicity of the pocket. Density was not well defined enough to accurately determine coordinates for the terminal amino and carboxyl groups (and C α) of HCY. Therefore, the ligand was modeled, refined, and deposited in truncated form as ethylthioadenosine (ETA), which we take to partially represent SAH. The C β atom of HCY occupies the same position as the imidazole ring of His363 in the other complexes (Fig. 7A,B). Therefore, to accommodate the binding of SAH, His363 is forced to flip out of the channel, rotating ~180 degrees around the backbone. In the ADO complexed *P. falciparum* structure (PDB ID: 1V8B), the analogous His345 is also observed to be flipped up into a surface pocket formed jointly by a loop from chain A and a loop from chain B of adjacent subunits. However, the lack of a HCY moiety leaves the access channel empty. These observations confirm the hypothesis that the HCY appendage of the SAH substrate binds in this channel. Furthermore, it suggests that His363 acts as a “gate” that swings open to accept the substrate, but closes down after cleavage and release of the free HCY.

Comparison of the active sites between *Mtb* SAHH and human SAHH

The crystal structure of *Mtb* SAHH was superimposed with the human homolog (PDB ID: 1A7A; complexed with DHCEA) (Turner et al. 1998), and the active sites were examined to look for differences that could be exploited for design of selective inhibitors. All of the residues lining the ADO-binding pocket are identical between *Mtb* and human, limiting the possibilities for inhibitor design. This even includes Thr74, which is

Figure 7. Complex of SAHH with ETA, and with ADO, showing conformational changes of His363 (shown in green), and opening of access channel for binding the HCY moiety. The figures were made using Chimera (Pettersen et al. 2004). (A) Stereoview of the complex with ETA. (B) Stereoview of the complex with ADO alone.

equivalent to Thr60 in human, although it is replaced by Cys59 in *P. falciparum* and has been investigated for design of selective antimalarial drugs (Tanaka et al. 2004). The only exception is Gln73, which replaces Glu59 in the human enzyme, although both form hydrogen bonds with the N6 amino group of the adenine moiety.

However, with His363 flipped up in the SAH-bound SAHH complex, differences in the HCY-binding pocket can be included to design inhibitors selective for *Mtb* SAHH. When this structure is compared with the human SAHH, many residues lining the putative access channel (HCY-binding region) are also found to be conserved, including Cys93, Asn94, Tyr133, Gly157, Asp159, Phe364, and Arg406. The residues corresponding to Phe364 (Phe 302) and Arg 406 (Arg 343) in the human SAHH were previously proposed to be involved in hydrophobic and hydrogen-bonding interactions, respectively, with the side chain of homocysteine (Turner et al. 2000). However, several important differences can be observed. Most notably, the mouth of the channel is significantly more constrained in *Mtb* than in human (cf. Fig. 8, A and

C). First, there are several substitutions by large, bulky hydrophobic residues that build up the wall of the channel, including Trp134 (Leu111 in human), Met162 (Asn137), Arg225 (Asn163), Phe190, and Trp187. The latter two are contributed by the 37-residue insertion (surface helix) that is unique to *Mtb* (relative to the human sequence). This transforms the mouth of the channel from a shallow depression (funnel-like) in human to a deep pocket with narrow opening in the *Mtb* SAHH. In addition, the insertion region projects the side chain

Figure 8. Differences in human and *Mtb* forms of SAHH enzyme near the entrance to the access channel/HCY-binding site. (A) *Mtb* SAHH bound to ETA and NAD showing a constricted solvent access channel lined by residues from the insertion region. Residues 167–203 from strand-turn-helix insertion are shown in cyan, with Glu186 highlighted. The figures were made using SPOCK (Christopher 1998). (B) Surface of *Mtb* SAHH binding site with access channel closed off by His363, and ADO enclosed in a buried pocket. (C) Human SAHH complexed with DHCEA and NAD showing a shallower solvent access channel compared to the *Mtb* SAHH. The surface of the active site has been drawn without His301 to simulate the open form of the channel. His301 would presumably flip out of the channel, allowing the SAH substrate to bind and HCY cleaved from the substrate to be released to solvent.

of Glu186 into the access channel. The distance from the carboxylate group on Glu186 to the 5'OH on ADO is 12.66 Å (Fig. 8B). While this residue was not initially considered relevant (Tanaka et al. 2004), its proximity to the HCY portion of SAH bound in the channel (~10 Å from Cβ; see Fig. 8A) makes it a potentially important residue in the design of competitive inhibitors.

Conclusions

The crystal structure of the SAHH enzyme from *M. tuberculosis*, in complex with ADO, has been solved at 1.6 Å. The structure is largely similar to previously reported SAHH structures from human, rat, and malaria, exhibiting an α/β fold with two domains: an N-terminal substrate-binding domain bound to NAD⁺, and a C-terminal dinucleotide-binding domain. The protein is in a closed conformation, with the two domains rotated to enclose the ADO ligand in a buried pocket. The *Mtb* SAHH has a 37-residue loop that forms a helix-turn-strand on the surface, similar to that found in the malaria SAHH, but not found in the mammalian structures. Complexes with three inhibitors were also reported: ARI, 2FA, and DZA. The 2FA and DZA complexes demonstrate expected interactions between the ligands and side chains lining the pocket, as seen in previous structures. However, the ARI complex is the first reported structure of SAHH with ARI, and shows a 3' keto group with planar carbon in the cyclopentanyl ring, confirming the prediction that it is oxidized by NAD⁺ as a mechanism-based inhibitor. While most of the residues lining the ADO-binding pocket are identical between *Mtb* and human SAHH, allowing few opportunities for design of selective inhibitors, less is known about the binding mode of the HCY appendage of the full substrate. We determined the cocrystal structure of SAHH with SAH. Contiguous density was found adjacent to the 5' position of ADO and modeled as atoms in the body of covalently attached HCY. Binding of SAH forces a rotation of His363 around the backbone to flip out of contact with the 5' position of ADO and opens up access to a nearby channel that leads to the surface. The structure, the first complex with the SAH substrate reported, suggests that His363 acts as a switch that opens up to permit binding of SAH, and closes down after release of the HCY appendage. While most of the residues in direct contact with the HCY are conserved between human and *Mtb* SAHH, the mouth of the access channel nearby is significantly different, with the *Mtb* SAHH having a much more enclosed entrance surrounded by hydrophobic residues, along with Glu186 (not found in human SAHH). If the active site is expanded to include this HCY-binding access channel, these differences could potentially be exploited to design inhibitors that compete with substrate binding and moreover are selective against *Mtb*.

Materials and Methods

A 1.49-kb DNA fragment containing the *SAHH* gene (*Rv3248c*) was amplified by PCR from *Mtb* H37Rv genomic DNA as a template, using the following oligonucleotides as the forward and reverse primers, respectively:

5'-AGATGAAGCCATATGACCGGAAATTTGGTGACC-3'
5'-AGAGTAAGCTTAGTAGCGGTAGAGGTCCGGCTT-3'.

The amplified DNA fragment was digested with NdeI and HindIII and subcloned in the corresponding restriction sites in a pET28b vector to yield an N-terminal 6 X (His) tag. The kanamycin resistant SAHH-pET28b vector was transformed into *Escherichia coli* BL21 (DE3) cells, and grown to the mid-log exponential phase at 37°C in LB media (Difco). Cells were induced at 18°C at an O.D₆₀₀ of 0.8 with 1 mM IPTG and grown for 16 h.

S-Adenosyl-*L*-homocysteine hydrolase purification and preparation of the apo-enzyme

The harvested cell pellet was resuspended in buffer containing 20 mM Tris-HCl pH 8.0, 10 mM imidazole, 0.5 M NaCl, and 10% glycerol with 1 mM phenylmethanesulfonyl fluoride and DNase. The cells were lysed using a French press. SAHH was purified on a Hi-Trap Ni²⁺ chelating metal affinity chromatography column (GE Healthcare). The peak fractions were pooled and dialyzed against 20 mM Tris pH 8.0, 50 mM NaCl, 10% glycerol, 1 mM dithiothreitol, and concentrated to 18 mg/mL in an Amicon Ultra Centrifugal filter device (Millipore). The enzyme was found to be about 95% pure as observed on SDS PAGE.

The purified enzyme was stripped of both forms of the dinucleotide to prepare an apo-enzyme. The apo-enzyme was made as described for the preparation of rat liver apo-SAHH (Gomi et al. 1989). It involved an acidic (pH 3.3) ammonium sulfate precipitation of the enzyme followed by resuspension in a potassium phosphate buffer (pH 7.2) containing 5 mM DTT and 1 mM EDTA. The step was repeated a second time followed by precipitation with a neutral saturated ammonium sulfate solution. The precipitate was redissolved in 20 mM potassium phosphate containing 0.1 mM EDTA. Any undissolved protein was collected by centrifugation and discarded. The apo-enzyme was inactive and could not catalyze the hydrolysis reaction by itself. Activity was reconstituted by incubating it with 2 μ M NAD⁺ for 10 min before carrying out the activity assay. The reconstituted holo-enzyme was used for the enzyme activity assays.

Crystallization

Crystallization screening of *Mtb* SAHH was performed using the sitting-drop and hanging-drop methods with Crystal Screen I and II, Index (Hampton Research) and Wizard I and II (Emerald Biosystems), as well as random screening kits (Lawrence Livermore National Laboratory) using a crystallization robot (Robbins Scientific). Initial crystallization screening was carried out with SAHH alone, and preincubated with ADO or an analog inhibitor for 30 min at a molar ratio of ~1:15. Crystals for ADO-, DZA-, and ARI-bound SAHH were obtained at 18°C when 1:1 protein:reservoir solution drops were equilibrated within a hanging-drop against 500 μ L of mother liquor solution containing 20% w/v polyethylene glycol (PEG) 1000, 200 mM

imidazole (pH 8.0), and 100 mM Ca(OAc)₂. Crystals for 2FA-bound SAHH were obtained in the presence of mother liquor solution containing 0.2 M MgCl₂, 0.1 M Tris pH 8.5, and 25% w/v PEG 3350. Diffraction quality crystals were obtained after 3–4 d. To determine the structure of SAHH in complex with SAH, 10 mM SAH was cocrystallized with SAHH in the same condition as for the complex with 2FA.

Data collection, structure determination, and refinement

Crystals from a droplet were transferred directly to a cryoprotectant, N-paratone (Hampton Research). The crystals were mounted on nylon loops and flash frozen in a liquid nitrogen stream at 100 K before data collection. The high resolution data of the ternary complexes of SAHH:ADO:NAD, SAHH:DZA:NAD, SAHH:2FA:NAD, and SAHH:ARI:NAD were collected at beamline 19-ID on an Area Detector Systems Corporation

Q315 area detector at the Advanced Photon Source, Argonne National Laboratory. The data were reduced using DENZO, and intensities were scaled with SCALEPACK (Borek et al. 2003). Integrated and scaled data indicated that *Mtb* SAHH belongs to the *P*2₁ space group. The ternary SAHH:ARI:NAD, SAHH:DZA:NAD, and SAHH:2FA:NAD complexes were crystallized in the same space group with similar cell dimensions. Solvent content calculations indicated the presence of a tetramer in the asymmetric unit (Matthews 1968). Data for SAHH:SAH cocrystals were also collected at APS on beamline 19-ID. The crystallographic data collection statistics are summarized in Table 2.

The crystal structures of *Mtb* SAHH bound with ADO or its analogs, ARI, 2FA, and DZA, have been determined to 1.60, 2.01, 2.42, and 2.20 Å, respectively. Attempts to crystallize apo SAHH did not yield diffraction quality crystals. The structures of the SAHH:ADO:NAD ternary complex of *Mtb* were solved

Table 2. Crystallographic and refinement statistics

	SAHH:ADO:NAD	SAHH:ARI:NAD	SAHH:2FA:NAD	SAHH:DZA:NAD	SAHH:ETA:NAD
Ligand name	Adenosine	Aristeromycin	2-Fluoroadenosine	3-Deazaadenosine	Ethylthioadenosine
PDB ID	3CE6	2ZJ1	2ZJ0	2ZIZ	3DHY
Data collection					
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Wavelength (Å)	0.96	0.96	0.96	0.96	0.98
Temperature (K)	120.00	120.00	120.00	120.00	120.00
<i>a</i> (Å)	97.04	94.85	96.25	95.74	94.20
<i>b</i> (Å)	112.13	111.85	112.08	112.25	111.69
<i>c</i> (Å)	100.23	100.40	100.45	100.85	100.13
α	90.00	90.00	90.00	90.00	90.00
β	92.41	96.49	95.15	95.84	96.01
γ	90.00	90.00	90.00	90.00	90.00
Resolution (Å)	1.60	2.01	2.42	2.20	2.00
Highest resolution bin (Å)	1.50–1.60	2.01–2.11	2.42–2.49	2.20–2.26	2.00–2.05
Observed reflections	325,473	136,379	74,187	93,598	114,623
Unique reflections	308,888	129,513	70,373	89,824	108,886
Percent completeness	94.90	98.90	91.91	97.90	82.50
R (merge)	0.05	0.07	0.08	0.06	0.13
<I/σI>	13.10	10.12	10.52	13.06	8.36
<i>V</i> _m (Matthews coefficient)	2.51	2.44	2.48	2.48	2.48
Percent solvent	50.92	49.49	50.41	50.42	50.41
Refinement					
Free <i>R</i> -value, random, 5%	23.77	26.35	23.25	28.45	33.11
<i>R</i> -value	20.04	21.20	21.21	21.19	26.02
Protein residues	485	485	485	485	485
Water molecules	1066	450	721	676	370
RMSD bond length (Å) ^a	0.01	0.02	0.01	0.03	0.01
RMSD bond angle (Å) ^a	1.38	1.88	0.83	2.42	1.63
RMSD between subunits (Å)	0.10	0.23	0.31	0.27	0.22
Overall coordinate error (Å) ^b	0.10	0.19	0.35	0.27	0.32
RSCC (Refmac5) ^c	0.95	0.95	0.95	0.95	0.89
Residues φ–ψ angles					
Most favored (%)	91.30	90.70	89.00	89.50	89.40
Allowed (%)	8.50	8.90	10.00	10.10	10.00
Generously allowed (%)	0.20	0.20	0.30	0.40	0.40
Disallowed (%)	0.00	0.00	0.00	0.00	0.10

^aDeviations from restraint targets.

^bEstimated standard uncertainty, diffraction precision index (DPI) based on free *R* (Cruickshank 1999).

^cReal space correlation coefficient, maximum likelihood mFo–DFc map, reported by REFMAC (5.02) (Murshudov et al. 1997).

by molecular replacement method, EPMR, using the human SAHH model (PDB ID: 1LI4) as a search model for the extending data from 25 to 3.5 Å (Yang et al. 2003). Complexes with DZA, 2FA, and ARI were solved by molecular replacement from the ADO complex. Crystallographic refinement was performed using REFMAC (5.02) (Collaborative Computational Project, Number 4 1994). NCS restraints were applied for each domain of the model. After model building and fitting, bias-minimized electron density maps were obtained using Shake&-wARP protocol (Kantardjiev et al. 2002). Model building was done using the programs XTALVIEW (McRee 1999) and Coot (Emsley and Cowtan 2004). After repeated cycles of refinement and manual model building, water molecules were added to the structure using Xfit (McRee 1999). The refinement continued with structure factors measuring 1.0 σ or better within the resolution range 30–2.48 Å to reduce the effects of poorly measured reflections. At the final stage of the refinement, the NCS restraints were removed for the entire model. The refinement statistics and the model stereochemistry are summarized in Table 2.

The SAHH:SAH:NAD complex structure was determined to a resolution of 2.0 Å. Our best data showed overall completeness of 82.5% in the resolution range of 30–2.0 Å. The low completeness may be attributed to the high crystal mosaicity (3.42). The data was processed using d*TREK (Pflugrath 1999). After running Shake&wARP, clear density in the unbiased map was observed contiguous with the 5' carbon of the ADO, showing covalent attachment of additional atoms. Although positive density was apparent for most of the expected atoms of the HCY appendage, the density was not defined well enough to model the terminal atoms (amino and carboxylate groups). Therefore, coordinates were only built and refined for C β , C γ , and S δ . The model was refined with Refmac5, and the statistics are also presented in Table 2.

Enzyme inhibition assay

HCY production was monitored using a spectrophotometric assay. Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), or DTNB), was used to convert HCY into an HCY–TNB complex (Ellman et al. 1961; Lozada-Ramirez et al. 2006) with an absorption maximum at 412 nm. The reaction mixture contained 0.5 μ M apo-SAHH, 250 μ M NAD⁺, 100 μ M DTNB, and 50 mM potassium phosphate (pH 7.2) for a total assay volume of 200 μ L. Readings were observed on a POLARstar OPTIMA plate reader from BMG LABTECH, using an absorbance filter of 410 nm.

The apo-enzyme was incubated with cofactor NAD⁺ and the inhibitors (where applicable) for 10 min. The reaction was initiated by the addition of 100 μ M SAH. The reaction progress was measured for ~2 min. The linear region of the curve was used for measuring the initial velocity parameters. Inhibition constants were determined by assaying 10 nM to 1 mM concentrations of the inhibitors in the presence of a constant SAH concentration.

Whole-cell assay

SAHH shares 90% sequence homology between *Mtb* and *Mycobacterium smegmatis*, and *M. smegmatis* grows faster than *Mtb*. Therefore, *M. smegmatis* cells were used to study the MIC values for the inhibitors using whole-cell assay. All inhibitors were dissolved in water to a concentration of 256 mg/L. First,

100 μ L of Middlebrook 7H9 media (Difco) was dispensed into all 96 wells of a microtiter plate. Then 100 μ L of the 256 mg/L stock inhibitor solution was dispensed into column #2. Twofold serial dilutions were carried out for each column up to column #10, giving inhibitor concentrations from 256 mg/L to 0.5 mg/L. Column #11, containing only media served as a negative control. *M. smegmatis* mc²155 (Snapper et al. 1990) cells, diluted in 7H9 media, were added to all the wells to a final O.D₆₀₀ of 0.001. Cells were grown at 37°C for 72 h. After this period, the wells were measured visually for cell growth. MIC values were determined where the wells had no visible bacterial growth.

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