Crystal Structures of the *Helicobacter pylori* MTAN Enzyme Reveal Specific Interactions between S-Adenosylhomocysteine and the S’-Alkylthio Binding Subsite

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**ABSTRACT:** The bacterial S’-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) enzyme is a multifunctional enzyme that catalyzes the hydrolysis of the N-ribosidic bond of at least four different adenosine-based metabolites: S-adenosylhomocysteine (SAH), S’-methylthioadenosine (MTA), S’-deoxyadenosine (S’-DOA), and 6-amino-6-deoxyfutalosine. These activities place the enzyme at the hub of seven fundamental bacterial metabolic pathways: S-adenosylmethionine (SAM) utilization, polyamine biosynthesis, the purine salvage pathway, the methionine salvage pathway, the SAM radical pathways, autoinducer-2 biosynthesis, and menaquinone biosynthesis. The last pathway makes MTAN essential for *Helicobacter pylori* viability. Although structures of various bacterial and plant MTANs have been described, the interactions between the homocysteine moiety of SAH and the S’-alkythiol binding site of MTAN have never been resolved. We have determined crystal structures of an inactive mutant form of *H. pylori* MTAN bound to MTA and SAH to 1.63 and 1.20 Å, respectively. The active form of MTAN was also crystallized in the presence of SAH, allowing the determination of the structure of a ternary enzyme–product complex resolved at 1.50 Å. These structures identify interactions between the homocysteine moiety and the S’-alkythiol binding site of the enzyme. This information can be leveraged for the development of species-specific MTAN inhibitors that prevent the growth of *H. pylori*.

S’-Methylthioadenosine/S-adenosylhomocysteine nucleosidase plays a key role in multiple pathways in bacterial cells. It catalyzes the hydrolysis of the N-ribosidic bond of four different adenosine-based metabolites, releasing adenine and the ribose-containing products (Figure 1). It has been known that it utilizes SAH, MTA, and S’-DOA from the various SAM utilization pathways.1−3 SAH is a product of every SAM-dependent methyl transfer reaction. These biochemical transformations encompass methylation of the three common biological polymers (proteins, nucleic acids, and complex carbohydrates) as well as various lipids.4 Methylation of these polymers affects fundamental properties in all living organisms such as gene expression, cell signaling, and cellular metabolism. In all of these cases, SAM-dependent methylation is susceptible to product inhibition if SAH accumulates to high levels.5 Continuous breakdown of MTA is also required for the proper functioning of spermidine and spermine synthases that are otherwise inhibited by the accumulation of MTA.6 MTAN activity is also linked to important downstream metabolic pathways such as adenine and methionine salvage and autoinducer I (AI1) and II (AI2) production, which are important quorum-sensing signaling molecules.7 AI2 regulates the expression of genes that are required for interspecies communication, biofilm formation, and bacterial virulence and are therefore important facilitators of bacterial pathogenesis.8,9 The AI2 concentration in the cell depends on the transformation of S-ribosylhomocysteine (SRH) into homocysteine and 4,5-dihydroxy-2,3-pentanedione, a precursor of AI2.10 AI1 compounds are N-acetylhomoserine lactones (AHLs) that allow interspecies communication in primarily Gram-negative bacteria. AHL synthase catalyzes the transformation of SAM into AHL and MTA, so the activity of AHL synthase is sensitive to the accumulation of MTA.11 Choi-Rhee and Cronan have reported that MTAN is required for the catalytic hydrolysis of S’-DOA and that accumulation of S’-DOA inhibits SAM radical enzymes such as biotin synthase.1 Recently, Li et al. determined that the *Campylobacter jejuni* and *Helicobacter pylori* MTAN (HpMTAN) enzymes play an essential role in an alternative menaquinone biosynthetic pathway.12 They reported that, unlike *Escherichia coli* and *Thermus thermophilus*, *C. jejuni* and *H. pylori* biosynthesize menaquinone though an intermediate step that requires MTAN to catalyze the hydrolysis of 6-amino-6-deoxyfutalosine. This role, combined with previous data, suggests that MTAN represents a target for compounds capable of affecting bacterial metabolism and bacterial communication.7,11,13,14 Indeed, very recently, Wang et al. have shown that powerful inhibitors of MTAN inhibit the growth of *H. pylori*.15

Previously determined bacterial and plant MTAN structures show that MTAN is an obligatory homodimer, and the two molecules form a set of two shared active sites where residues...
of one molecule form the adenyl and ribosyl binding pockets and residues of the other molecule form the majority of the 5′-alkylthio binding pocket (Figure 2A). The catalytic mechanism has been thoroughly characterized as well as the interactions required to initiate that reaction (Figure 2B,C). Binding of the substrate to the open form of the enzyme stimulates a conformational change characterized predominately by the kinking of helix α6. This closes the active site and positions residue D198 (HpMTAN numbering) to interact with N7 and the N6 exocyclic amine of the adenine moiety. It has been suggested that D198 acts as a general acid by donating a proton to N7 of the substrate, resulting in the stretching of the N-ribosyl bond and subsequent formation of an oxocarbenium intermediate that undergoes nucleophilic attack by a water molecule. Release of the adenine and SRH products appears to be facile following hydrolysis and relaxation of helix α6.

Although much is known about the structure of MTAN and its interactions with adenosine-containing substrates, specifically inhibiting the bacterial MTANs without also inhibiting homologous human enzymes is a challenge. The human genome encodes both a purine nucleoside phosphorylase and the 5′-MTA phosphorylase (MTAP) that are inhibited by the current generation of MTAN inhibitors. The MTAN inhibitors with the tightest binding are either transition-state or nucleoside analogues that interact with residues forming the ribosyl and adenyl binding pockets within the active site. These regions are largely conserved between the bacterial MTANs and MTAPs. As a result, some of these compounds possess lower Kᵢ values for human MTAP than for MTAN. Comparison of the MTAN and MTAP active site structures shows that the latter possesses a truncated 5′-alkylthio binding pocket that permits use of MTA as a substrate but not SAH. Because the larger 5′-alkylthio binding subsite of MTAN is an important feature that differentiates it from human MTAP, characterization of the specific interactions between the 5′-alkylthio binding site of MTAN and the homocysteine moiety of SAH may offer insight into the design of MTAN inhibitors with improved selectivity for the bacterial enzymes as a basis for new drugs for the treatment of H. pylori infections.
EXPERIMENTAL PROCEDURES

Mutagenesis. Site-directed mutagenesis was performed to make the D198N mutant using a pET-32-based plasmid containing the wild-type pfs gene as a template. The template plasmid encodes a polyhistidine-tagged thioredoxin−HpMTAN fusion protein containing a Prescision protease cut site immediately N-terminal to the first residue of wild-type HpMTAN (wt-HpMTAN). Proteolysis with Prescision protease produces a polyhistidine-tagged thioredoxin and untagged HpMTAN. Sequencing of the plasmid resulting from the mutagenesis experiment confirmed the presence of the D198N mutation.

MTAN Expression and Purification. Wild-type HpMTAN and its mutants were expressed and purified as previously described.18 The plasmid containing the mutated gene was used to transform BL21(DE3) Rosetta cells (EMD Biosciences). Cultures of LB medium containing 0.1 mM chloramphenicol and 0.3 mM ampicillin were incubated at 37 °C. Cells were induced after Abs<sub>600</sub> had reached 0.6−0.8 by the addition of 0.1 mM IPTG and incubated for 18−20 h at 16 °C. Cells were harvested by centrifugation and resuspended in buffer A (20 mM HEPES (pH 7.5), 0.5 M NaCl, 5 mM β-mercaptoethanol, and 25 mM imidazole). The resuspended cells were lysed by sonication and then centrifuged at 15000g. The supernatant was applied to a 5 mL HisTrap column equilibrated with buffer A (GE Healthcare). Elution of recombinant proteins was performed using a linear gradient of imidazole from 25 to 250 mM over 20 column volumes. The fractions containing the purified proteins were treated with Prescision Protease and

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Figure 2. Shared active site in the HpMTAN homodimer and the enzyme mechanism. (A) The closed form of the HpMTAN-D198N active site is shown with SAH (spheres colored by CPK) bound. The cartoons (orange and green) represent the HpMTAN homodimer. The position of helix α6 is indicated for each monomer in light blue. The boxed regions indicate the location of the 5′-alkythio binding subsite that interacts with the homocysteine moiety of SAH. (B) Reactions proposed for the two most commonly studied MTAN substrates. The ligands for each of the determined structures are highlighted in boxes. The substrates are colored black; the nucleophile is colored orange, and the MTAN-derived proton is colored blue. (C) Proposed reaction for the human MTAP enzyme. The coloring of the components is the same as in panel B.

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18. The plasmid containing the mutated gene was used to transform BL21(DE3) Rosetta cells (EMD Biosciences). Cultures of LB medium containing 0.1 mM chloramphenicol and 0.3 mM ampicillin were incubated at 37 °C. Cells were induced after Abs<sub>600</sub> had reached 0.6−0.8 by the addition of 0.1 mM IPTG and incubated for 18−20 h at 16 °C. Cells were harvested by centrifugation and resuspended in buffer A (20 mM HEPES (pH 7.5), 0.5 M NaCl, 5 mM β-mercaptoethanol, and 25 mM imidazole). The resuspended cells were lysed by sonication and then centrifuged at 15000g. The supernatant was applied to a 5 mL HisTrap column equilibrated with buffer A (GE Healthcare). Elution of recombinant proteins was performed using a linear gradient of imidazole from 25 to 250 mM over 20 column volumes. The fractions containing the purified proteins were treated with Prescision Protease and...
dialyzed overnight against buffer A. This protein sample was again applied to a HiTrap column (GE Healthcare) to selectively bind the cleaved histidine tag and the protease. The fractions containing HpMTAN were then subjected to size exclusion chromatography on a Hi-Load Superdex 200 column as a polishing step (GE Healthcare). All protein samples were analyzed for purity using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The absorbance at 280 nm was used to determine the protein concentration using an extinction coefficient of 3105 M\(^{-1}\) cm\(^{-1}\).

**Crytalization.** For the crystallization studies, purified protein samples were dialyzed against crystalization buffer [20 mM HEPES (pH 7.5), 0.2 mM TCEP, and 1 mM EDTA] and concentrated to 16 mg/mL by ultrafiltration (Millipore). Crystals of the MTAN-D198N mutant complexed with either MTA or SAH were grown by the hanging-drop vapor diffusion method. Crystallization drops containing 1 μL of well solution, 1 μL of MTAN-D198N (16 mg/mL), and 0.5 μL of SAH or MTA (10 mM) were equilibrated with 100 μL of well solution. The well solution for producing MTAN-D198N–MTA complex crystals contained 0.2 M magnesium chloride, 0.1 M HEPES (pH 7.5), and 25% (w/v) PEG 3350. The well solution for producing the MTAN-D198N–SAH complex crystals contained 0.05 M magnesium chloride hexahydrate, 0.1 M HEPES (pH 7.5), and 0.25% (v/v) PEG-MME 550. Crystals of the wt-HpMTAN–SRH–adenine complex were produced under the same conditions as the MTAN-D198N–SAH complex crystals. X-ray diffraction experiments were performed on the LS-CAT ID-D beamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). Data were integrated and scaled using HKL2000. Although these crystals were isomorphous with previously determined HpMTAN structures, molecular replacement was necessary and performed with a monomer of HpMTAN [Protein Data Bank (PDB) entry 3NM6] using EPMR. Structure refinement proceeded using Phenix and Coot.

**Kinetic Characterization of wt-MTAN.** All assays were performed in triplicate in 100 mM HEPES and 50 mM KCl buffer (pH 7.2). For determining kinetic parameters, stocks of 1 mM SAH and 1 mM MTA were prepared in the same buffer. The substrates were serially diluted to produce a final concentration range of 5–150 μM in 100 μL of assay solution. The reduction of absorbance at a wavelength of 274 nm was monitored for 15 min at 37 °C using a Biotek (Winooski, VT) Synergy H4 plate reader. The extinction coefficient of 1.65 M\(^{-1}\) cm\(^{-1}\) was used for assays with both SAH and MTA. Nonlinear regression analysis of kinetic data was performed using Prism 5.

## RESULTS AND DISCUSSION

The Homocysteine Moiety of SAH Forms Specific Interactions with the HpMTAN Active Site. Although much is known about the interactions between MTAN and the adenosine moiety of the variety of functional substrates, the mechanism by which MTAN interacts with components of the S′-alkythio moiety of these substrates is not. The strategy used to characterize these interactions was to create an inactive mutant capable of binding substrate but lacking enzymatic activity. On the basis of the previously reported E. coli MTAN (EcMTAN) mutant–MTA complex, an HpMTAN-D198N mutant was created.\(^{23}\) The crystal structure of the HpMTAN-D198N–SAH complex was determined and refined to 1.2 Å resolution (Table 1), allowing for clear assessment of the interactions between the SAH substrate and the S′-alkythio binding subsite.

The difference maps within the region enveloped by the HpMTAN-D198N active site exhibited electron density that allowed unambiguous fitting of each non-hydrogen atom of

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<th>MTAN-D198N/SAH</th>
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\(^a\)The structures of the enzyme-bound compound are shown for the sake of clarity. Parentheses indicate units. Brackets indicate additional information regarding that value. A.S.U means asymmetric unit; r.m.s. means root-mean-square. R_free is calculated from a set of reflections chosen randomly. These data are equal to 5% of the unique reflections and were not used for model refinement or calculation of the R_work value.
SAH, including the previously unobserved α-amino and carboxyl groups of the homocysteine moiety (Figure 3A). The α-amino group of the homocysteine moiety forms two types of interactions with the S′-alkythio binding subsite. The first is a cation–π interaction with the aromatic side chain of F107, which is positioned 3.3 Å from the α-amino nitrogen of SAH and the six carbons of the phenylalanine aromatic ring (Figure 3B). This type of interaction has been observed in other proteins that specifically bind ligands or substrates that contain ammonium moieties, and disruption of the cation–π interaction is known to affect the binding affinity within those systems.34 Excellent examples of this are represented by the structures of the γ-aminobutyric acid (GABA) receptor and acetylcholine binding protein (AChBP) bound to their respective ligands or ligand analogues.35–37 In the AChBP–carbamylcholine structure, the aromatic residues found within the AChBP ligand binding pocket form multiple cation–π interactions with the quaternary ammonium of carbamylcholine.35 Recently, this type of interaction was observed in an insect GABA receptor, and incorporation of artificial fluorophenylalanine amino acids within the ligand binding site of the acetylcholine receptor eliminates a cation–π interaction and produces a consistent increase in the measured EC_{50} values when compared to that of the wild-type acetylcholine receptor.34

The second interaction between HpMTAN-D198N and the α-amino moiety occurs through a water-mediated hydrogen bond to D209. This residue is located near the N-terminus of helix α6, which is disordered in ligand-free MTAN structures but takes a helical form as a consequence of substrate binding.18,23 One consequence of this structural organization is the positioning of D198 near the adenyl moiety to initiate the transfer of a proton to the substrate, while the other consequence is the formation of the through-water hydrogen bond. The distances from the water molecule to the side chain of D209 and to the α-amino moiety of SAH are equal, with each hydrogen bond measuring 2.7 Å (Figure 3B). Additionally, the 4.4 Å distance between the side chain of D209 and the α-amino moiety of SAH suggests a possible ionic interaction. The α-carboxyl moiety of SAH in the HpMTAN-D198N–SAH complex also forms a specific interaction with the enzyme active site. Specifically, the Nε atom of H109 forms a 2.8 Å bonding interaction with SAH.

To assess the potential importance of these interactions in substrate recognition, we performed a sequence alignment of the 1000 MTAN protein sequences in the UniProt database most closely related to that of H. pylori (Figure 3C). This sequence comparison showed that more than 85% of these MTANs possess a phenylalanine at the position corresponding to F107 in HpMTAN, suggesting that the cation–π interaction is an important feature that supports specific enzyme–substrate interactions. The sequence alignment also suggests that the charge of the residue at position 209 is important, as it is either an aspartate or glutamate in roughly 90% of the 1000 analyzed sequences.
MTAN sequences. If any hydrogen bond acceptor or donor were sufficient at this position, one would expect to observe asparagine and glutamine with roughly equivalent probabilities. H109 is conserved in bacteria of the order Campylobacterales, which represents approximately 10% of the 1000 MTAN sequences inspected in this study. In contrast, 80% of the sequences possess a tyrosine residue at the analogous position, which is common to all of the previously determined bacterial MTAN structures. Superposition of HpMTAN with EcMTAN (Figure 3D) suggests that a tyrosine side chain could maintain a hydrogen bond with the α-carboxyl of SAH. This highlights the importance of MTAN possessing a hydrogen bond donor or acceptor at this position. In addition, the lack of a resolved SAH–MTAN complex prior to this study suggests that a hydrogen bond between the tyrosine of EcMTAN and the SAH α-carboxylate is insufficient to structurally resolve this interaction, whereas the interaction between the α-carboxyl moiety of SAH and H109 of HpMTAN–D198N is sufficiently strong to fix the conformation of SAH. To ensure that these interactions are observed in the wild-type enzyme and to identify any structural changes in the S′-alkylthio binding region following substrate hydrolysis, we determined the crystal structure of wt-HpMTAN complexed with adenine and SRH to 1.50 Å resolution.

Active Site Interactions with the Homocysteine Moiety Are Unchanged during Catalysis. Previous attempts to form a wt-MTAN–product complex resulted in a complex with adenine and Tris in the active site.18 The crystallization experiments described here produced wt-MTAN crystals in the closed form with both adenine and the α-anomer of SRH bound in the active site as evidenced by two discrete regions of density in an Fn − Fo difference map (Figure 4A). The presence of the α-anomer is consistent with the proposed catalytic mechanism in which breaking of the N-ribosidic bond produces an oxocarbenium intermediate that undergoes a nucleophilic attack on the α-face of the ribosyl group. This nucleophilic attack is from an ordered water molecule coordinated by conserved residues E13, E175, and R194, which has been observed in all MTAN crystal structures to date (Figure 3B).16,18 In the wt-HpMTAN–SRH–adenine structure, it is clear that conversion from the β-ribosyl substrate to the α-ribosyl product also alters the ribose conformation from C2′-endo to C3′-endo. This stems from maintaining the location of the nucleophilic water, which is now O1 of the SRH product, through continued coordination by residues E13, E175, and R194. This conformational change in the ribose moiety contrasts with the fixed conformation of the homocysteine moiety and the interactions it forms with the S′-alkylthio

Figure 4. Comparison of SAH and MTA as substrates. (A) Superposition of the HpMTAN–D198N–SAH and wt-HpMTAN–SRH–adenine complex structures. The Fn − Fo difference density from the wt-HpMTAN–SRH–adenine diffraction data is shown contoured at 3σ. Both SRH and adenine were omitted from the calculation of the map. The coloring of the protein and SAH is the same as in the other figures. The bronze carbon atoms represent the SRH and adenine products. (B) Superposition of the HpMTAN–D198N–SAH and –MTA complex structures. The protein atoms from the HpMTAN–D198N–SAH structure are colored as in the other figures, and the carbon atoms of the enzyme of the MTA complex structure are slightly lighter in color. Because SAH and MTA atom positions superimpose perfectly, the atoms of MTA are not shown, but the location of MTA is indicated by an Fn − Fo omit map contoured at 3σ where MTA was omitted from the calculation of the map. The hydrogen-bonded water network in the MTA-bound structure is indicated by the dashed gray lines, and that of the SAH complex structure is indicated by the dashed black lines. (C) HpMTAN and human MTAP S′-alkylthio binding subsites differ in structure. A superposition of the HpMTAN–D198N–SAH complex active site with the human MTAP active site showing bound methylthio-Immucillin-A (PDB entry 1K27). Components of the HpMTAN–D198N–SAH complex are colored as in the other figures. The corresponding S′-alkylthio binding subsite of human MTAP is colored yellow. The yellow carbon atoms and surface indicate the MTAP−methylthio-Immucillin-A (ImmA) complex and the active site cavity, respectively. H137, V135, and L279 form the boundary of the S′-alkylthio binding subsite in MTAP. H109, F107, and SAH of the HpMTAN–D198N–SAH complex are also shown. The homocysteine moiety of SAH protrudes more than 4 Å beyond the confines of the human MTAP active site. (D) Comparison of steady-state kinetics using either MTA or SAH shows a slightly higher a...
binding pocket. This suggests that interactions defined by the S'-alkylthio binding subsite allow for less conformational flexibility than those in the ribose binding subsite and that the interactions between the homocysteine moiety and the S'-alkylthio binding subsite likely remain unchanged throughout the enzymatic reaction.

The HpMTAN S'-Alkylthio Binding Subsite Structure Is Substrate-independent. The first X-ray crystal structures of MTAN highlighted the conformational change in helix α6 resulting from the induced fit between MTAN and its substrates. However, inspection of the S'-alkylthio binding subsites from the published MTAN structures suggests that this region is insensitive to the substrate-bound state of the enzyme. To determine if the variability of the S'-alkylthio moieties of the known MTAN substrates affects differences in the structure of the S'-alkylthio binding subsite, we determined the crystal structure of an HpMTAN-D198N–MTA complex to 1.6 Å resolution and compared this structure to the HpMTAN–D198N–SAH complex structure.

When superimposing the MTA and SAH complex structures (root-mean-square displacement for Ca atoms of 0.067 Å), the MTA substrate superimposes perfectly with the corresponding atoms of SAH. Additionally, the S'-alkylthio binding pockets of both structures maintain the same conformation (Figure 4B). In particular, the loop containing residues F107 and H109 maintains the identical conformation in both structures. Other similarities between the SAH and MTA complexes are the solvent structure and the hydrogen-bonded network within the S'-alkylthio binding pocket. Specifically, the hydrogen-bonded network formed by D209, a water molecule, and the α-amino group of SAH is observed in the MTA structure as a D209–water–water network. These solvent interactions and the lack of structural changes in the S'-alkylthio binding pocket suggest that MTAN follows a lock-and-key model for binding the homocysteine moiety that contrasts with the dynamics observed in the residues that form the adenyl and ribosyl binding sites. On the basis of the findings described here, it is possible to draw parallels between the interactions observed in the HpMTAN-D198N–SAH complex and those interactions likely formed in a complex of the HpMTAN with the recently identified MTAN substrate, 6-amino-6-deoxyfutalosine. Primarily, the benzoate moiety of 6-amino-6-deoxyfutalosine would be positioned like the α-amino group of SAH within the S'-alkylthio binding subsite and interact with F107 through a π–π stacking interaction. Because 6-amino-6-deoxyfutalosine, like SAH, terminates with a carboxylate moiety, it likely forms an interaction with H109 similar to that observed in the HpMTAN-D198N–SAH and wt-HpMTAN–SRH–adenine complexes.

To date, two wt-MTAN complex structures have been determined with a ligand containing an aromatic functional group bound within the S'-alkylthio binding pocket but none possessing a carboxylate moiety. The EsMTAN formed complexes with two different methyl-Immcillin inhibitors: BnT-DADMeImmA (PDB entry 3DF9) and (4-chlorophenyl)-thio-DADMe-ImmA (PDB entry 3O4V). These structures show that the phenyl moieties bind in the same location but possess slightly different orientations, which results from differences in the conformation of the pyrrolidine moieties of these transition-state analogues. In the 3DF9 structure, the phenyl moiety forms a herringbone interaction with the residue analogous to F107 and overlaps with the position of the α-amino moiety of SAH when bound to HpMTAN. However, in 3O4V, the chlorophenyl moiety does not form any π–π stacking interactions between the inhibitor and the S'-alkylthio binding pocket, suggesting that other interactions are predominating and forcing an alternative inhibitor conformation or the presence of the chlorine atom coupled to the phenyl moiety is sterically hindering the π–π stacking interaction.

Table 2. HpMTAN Kinetic Parameters

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<td></td>
<td>K_{cat} (µM)</td>
<td>k_{cat} (s⁻¹)</td>
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<td>wt</td>
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<td>F107A</td>
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<td>H109A</td>
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The data presented here suggest a role for the HpMTAN S'-alkylthio binding subsite in specifically coordinating the homocysteine moiety of SAH and suggest that the structure of the S'-alkylthio binding region is independent of substrate identity. Because these interactions...
play an important role in substrate recognition and the structural evidence suggests that the homocysteine moiety contributes only a minor conformational entropy penalty to binding, it is expected that evidence of the impact on substrate binding could be observed using steady-state kinetics. In particular, the three additional interactions formed between HpMTAN and the SAH substrate should produce a lower $K_M$ for the HpMTAN–SAH reaction than for the HpMTAN–MTA reaction.

To perform these experiments, we monitored the hydrolysis of either MTA or SAH by wt-HpMTAN by measuring the absorbance decrease at 274 nm. Fitting the initial velocity data allowed calculation of the kinetic parameters (Figure 4D). The $K_M$ values for SAH and MTA were 10 ± 1 and 39 ± 5 μM, respectively (Table 2). The $k_{cat}$ values for SAH and MTA were 1.8 ± 0.1 and 3.8 ± 0.2 s$^{-1}$, respectively. These values are in accordance with those determined for wild-type Streptococcus pneumoniae MTAN, where Singh et al. showed that wild-type S. pneumoniae MTAN has a 1.8-fold lower $K_M$ when using SAH versus using MTA. Results for both HpMTAN and S. pneumoniae MTAN contrast with those observed for EcMTAN. Lee et al. showed that wt-EcMTAN exhibits a 1.6-fold lower $K_M$ when using MTA than when using SAH as the substrate, but $k_{cat}$ values are similar for each substrate (2.6 ± 0.1 and 3.0 ± 0.1 s$^{-1}$), resulting in slightly higher enzymatic efficiency when using MTA as a substrate.

The reason for the discrepancy in the kinetic parameters is not clear. The kinetics of the S. pneumoniae MTAN are more like those of HpMTAN than those of EcMTAN. However, both S. pneumoniae MTAN and EcMTAN possess a tyrosine at the corresponding position of HpMTAN H109. Therefore, the difference in kinetics observed in various bacterial MTANs cannot be simply ascribed to an increase in the positive electrostatic potential due to the presence of H109 in the HpMTAN active site. Another factor potentially contributing to the kinetic differences may stem from solvent effects in the different $5′$-alkylthio binding pockets. This was previously suggested by Thomas et al. to explain the large entropic and enthalpic variation observed when testing a panel of inhibitors to various bacterial MTANs. That rationale may apply to data presented here. In other protein–ligand systems, the effects of ligand and active site desolvation have been tested experimentally for both hydrophilic and hydrophobic substrate/ligand binding sites, and the conclusions can be applied to the $5′$-alkylthio binding subsite of MTAN. Considering that the portion of the HpMTAN $5′$-alkylthio binding pocket that interacts with the $\alpha$-carboxyl and $\alpha$-amino moieties of SAH is generally hydrophilic, the primary driving force for binding likely stems from the favorable entropy caused by ligand desolvation and active site desolvation of the hydrophilic portion of the active site. This is displayed clearly in Figure 4B, where two ordered water molecules in the MTAN complex that exhibit a rather extensive hydrogen-bonded network superimpose with portions of the SAH molecule in the SAH complex. Displacement of these ordered waters affords an entropic gain, but enthalpic changes are likely minimal because of the maintenance of the specific ionic and hydrogen bonding interactions following SAH binding.

To quantify the effect of these interactions within the HpMTAN $5′$-alkylthio binding pocket, we performed kinetic studies using two single mutants, F107A and H109A (Table 2). As predicted from the crystal structure, the $K_M$ values of both mutants exhibit a minor increase (1.9-fold for F107A and 1.4-fold for H109A) when using SAH as a substrate, while the $K_M$ remains unchanged when using MTA. This indicates that the cation–π interaction of F107 and the ionic interaction of H109 play a role in recognizing the homocysteine moiety of SAH. Similarly, studies of EcMTAN showed that mutants F105A (F107 in wt-HpMTAN) and Y107F (H109 in wt-HpMTAN) exhibited 4- and 1.3-fold increases, respectively, in the $K_M$ values for SAH, suggesting these interactions in the $5′$-alkylthio binding pocket are common features among different bacterial MTANs. The effects of the F107A and H109A mutations on the $k_{cat}$ values are more difficult to interpret. When compared to that of wt-HpMTAN, the $k_{cat}$ values for F107A and H109A using either SAH or MTA as the substrate decreased by 3–4-fold. We did not anticipate that the catalytic rate when using MTA as a substrate would be affected. A possible rationale for the effect on the turnover rate is that the structure of the loop containing F107 and H109 is altered in the mutants, which could affect the turnover of either SAH or MTA substrates.

## CONCLUSIONS

Characterizing interactions between the enzyme and substrate are at the heart of understanding substrate specificity and enzyme catalysis. For the bacterial MTANs, the interactions within the adenine and ribose subunits are well-documented. This study offers the first description of the specific bonding interactions between the $5′$-alkylthio binding subsite of any MTAN and a homocysteine-containing ligand by determining the X-ray crystal structure of a binary complex of an inactive HpMTAN mutant with SAH and the structure of a ternary, product complex. These structures not only highlight previously unobserved interactions between the enzyme and either substrate or product but also show that this portion of the enzyme active site is invariant with respect to its substrate-bound state and substrate identity. Additionally, the wt-HpMTAN–SRH–adenine ternary complex structure shows that the interactions between the homocysteine moiety and the enzyme active site are consistent with those observed for the HpMTAN-D198N–SAH complex. The 4-fold lower $K_M$ and the 2-fold lower $k_{cat}$ observed when using SAH as a substrate versus MTA reflect the additional interactions between the $5′$-alkylthio binding subsite and the homocysteine moiety of either the substrate or product. Taken together, these results suggest that the $5′$-alkylthio binding subsite is a prime target for the design of new MTAN inhibitors that are highly specific to bacterial MTANs but will selectively kill H. pylori while sparing beneficial commensal bacteria endogenous to the human gastrointestinal tract.

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ABBREVIATIONS

MTAN, S′-methylthioadenosine/S-adenosylhomocysteine nucleosidase; SAH, S-adenosylhomocysteine; MTA, S′-methylthioadenosine; S′-DOA, S′-deoxyadenosine; SAM, S-adenosylmethionine; AI1, autoinducer I; AI2, autoinducer II; SRH, S-ribosylhomocysteine; AHL, N-acetylhomoserine lactone; MTAP, S′-MTA phosphorylase; HpMTAN, H. pylori MTAN; EcMTAN, E. coli MTAN; GABA, γ-aminobutyric acid; AChBP, acetylcholine binding protein.

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