Neutron structures of the Helicobacter pylori 5′-methylthioadenosine nucleosidase highlight proton sharing and protonation states

Michael T. Banco, Vidhi Mishra, Andreas Ostermann, Tobias E. Schrader, Gary B. Evans, Andrey Kovalevsky, and Donald R. Ronning

Department of Chemistry and Biochemistry, University of Toledo, Toledo, OH 43606; Heinz Maier-Leibnitz Zentrum, Technische Universität München, 85748 Garching, Germany; Jülich Centre for Neutron Science at Heinz Maier-Leibnitz Zentrum, Forschungszentrum Jülich GmbH, 85747 Garching, Germany; Terrier Research Institute, Victoria University of Wellington, Wellington 5010, New Zealand; and Biology and Soft Matter Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

Edited by Dagmar Ringe, Brandeis University, Waltham, MA, and accepted by Editorial Board Member David W. Russell October 21, 2016 (received for review June 16, 2016)

MTAN (5′-methylthioadenosine nucleosidase) catalyzes the hydrolysis of the N-ribosidic bond of a variety of adenosine-containing metabolites. The Helicobacter pylori MTAN (HpMTAN) hydrolyzes 6-amino-6-deoxyfutalosine in the second step of the alternative menaquinone biosynthetic pathway. Substrate binding of the adenine moiety is mediated almost exclusively by hydrogen bonds, and the proposed catalytic mechanism requires multiple proton-transfer events. Of particular interest is the protonation state of residue D198, which possesses a pKₐ above 8 and functions as a general acid to initiate the enzymatic reaction. In this study we present three corefined neutron/X-ray crystal structures of wild-type HpMTAN cocrystallized with S-adenosylhomocysteine (SAH), Formycin A (FMA), and (3R,4S)-4-[(4-Chlorophenyl)methyl]-3-hydroxypropylidine (p-CPh-Thio-DADMe-ImMa) as well as one neutron/X-ray crystal structure of an inactive variant (HpMTAN-D198N) cocrystallized with SAH. These results support a mechanism of D198 pKₐ elevation through the unexpected sharing of a proton with atom N7 of the adenine moiety moieties unconventional hydrogen-bond geometry. Additionally, the neutron structures also highlight active site features that promote the stabilization of the transition state and slight variations in these interactions that result in 100-fold difference in binding affinities between the DADMe-ImMa and ImMa analogous.

neutron diffraction | enzyme mechanism | proton transfer | nucleosidase | Helicobacter

The Gram-negative bacterium Helicobacter pylori is associated with gastric ulcers as well as chronic gastritis. Menaquinone (vitamin K₂) is an essential metabolite that aids in electron transfer in all organisms. In contrast to most bacteria that use the classical menaquinone biosynthetic pathway, H. pylori and Campylobacter jejuni use what is now termed the “alternative” menaquinone biosynthetic pathway to produce menaquinone from chorismate (1). Therefore enzymes that function within this pathway are attractive candidates for developing H. pylori-specific treatments. One such target in this pathway is a homodimeric enzyme, H. pylori 5′-methylthioadenosine nucleosidase (HpMTAN), that hydrolyzes the N-ribosidic bond of 6-amino-6-deoxyfutalosine (Fig. 1A) (2–4). Additionally, HpMTAN hydrolyzes the N-ribosidic bond of other adenosine-containing metabolites such as S-adenosylhomocysteine (SAH) (Table S1) and 5′-deoxyadenosine (5–7) and therefore functions as a central metabolic hub.

The proposed catalytic reaction of HpMTAN progresses through an S₀₁ mechanism and has been well studied for various MTAN homologs (Fig. 1B) (8–13). Catalysis is initiated by protonation of N7 of the adenine moiety by an aspartic acid residue, D198. Maintaining the protonated form of D198 requires elevation of the side chain pKₐ to a level much higher than the theoretical pKₐ of an aspartic acid side chain. Indeed, assessment of side-chain ionization of the analogous residue in the Escherichia coli homolog determined a pKₐ of 8.2, which can be attributed to the burial of the D198 side chain upon substrate binding (9, 14). Additionally, D198 has been demonstrated to be essential for the enzymatic activity through the use of an asparagine variant (D198N) of HpMTAN that binds substrate but does not promote hydrolysis (5, 12, 15). Following protonation of the substrate by D198, the adenine leaving group becomes electron withdrawing, leading to elongation of the N-ribosidic bond. This elongation promotes bond breakage, producing an oxocarbenium ion intermediate. A bound water molecule in the active site functions as a nucleophile to attack the oxocarbenium ion intermediate. In previous studies of E. coli MTAN (EcMTAN), conserved residues E12 and E175 were shown to be essential for the catalytic reaction based on inactive variants (6). Furthermore, it was suggested that E12 functions as a general base by activating the nucleophilic water molecule, because a second ionizable group with a pKₐ value of 5.6 was identified (9).

In the various MTAN homologs, it has been shown that the formation of the oxocarbenium ion intermediate can progress through either an early or late dissociative transition state. The structure of the transition state is defined by the distance from the N9 position of the adenine leaving group to the anomeric carbon on the ribosyl moiety of a substrate. Characterization of the HpMTAN transition state has been demonstrated previously to be an early dissociative transition state by measuring kinetic isotope effects using both Immucillin-A (1mM) and DADMe-Immucillin-A.

Significance

Gastrointestinal infection by the bacterium Helicobacter pylori is strongly associated with the development of gastric cancer. H. pylori 5′-methylthioadenosine nucleosidase (HpMTAN) is an interesting drug target because of its vital role in the production of menaquinone. HpMTAN offers a unique target for treating H. pylori infections without affecting the survival of the human microbiome. Neutron crystallography was performed to determine hydrogen atom positions that provide insight into the catalytic mechanism and transition state stabilization.

Author contributions: D.R.R. designed research; M.T.B., V.M., A.O., T.E.S., and A.K. performed research; G.B.E. contributed new reagents/analytic tools; M.T.B. and D.R.R. analyzed data; and M.T.B. and D.R.R. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. D.R. is a Guest Editor invited by the Editorial Board.

Data deposition: Crystallography, atomic coordinates, and structure factors reported in this paper have been deposited in the Protein Data Bank (PDB ID codes 5CCE, 5KB3, 5K1Z, 5K1Z, 5CCE, and 5K0X).

To whom correspondence should be addressed. Email: donald.ronning@utoledo.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1609718113/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1609718113

PNAS Early Edition | 1 of 6
The observed positions of the deuterium atom in the adenine binding pocket of HpMTAN for the product complex presented. In both panels, the $F_{o}-F_{c}$ for the nuclear density is contoured to 1σ and is represented in light blue. The $F_{o}-F_{c}$ nuclear and $F_{c}$ X-ray density are represented in dark blue and light green, respectively. (A) The $2F_{o}-F_{c}$ density illustrating the hydrogen-bond network that aids in positioning D198. (B) Difference omit $F_{o}-F_{c}$ nuclear density of the shared D$^+$ ion and of the N9 nitrogen and deuterium atoms contoured to 3σ. The $F_{o}-F_{c}$ X-ray map of the adenine molecule and D198, contoured to 3σ, demonstrates the lack of density for the shared D$^+$ ion. Hydrogen-bonding interactions and the respective distances are indicated.
Joint XN Refinement. The jointly refined XN structures were all refined using nCNS (29). After initial rigid-body refinement, several cycles of positional, atomic displacement parameter, and occupancy refinement followed. Within each refinement cycle the structure was manually checked using Coot (30). The $2F_o-F_c$ and $F_o-F_c$ neutron scattering length density maps then were examined to determine the correct orientation and protonation states of residues with exchangeable protons. All water molecules were refined as D$_2$O. Initially, water oxygen atoms were positioned using X-ray difference maps and then were shifted slightly in accordance with the neutron scattering length density maps. The levels of hydrogen/deuterium (H/D) exchange at OH, NH, and SH sites were refined using D occupancy as the metric. All structures have been deposited in the Protein Data Bank (PDB ID codes SCCD, 5K1Z, 5JPC, 5CCE, and 5KB3).

Results and Discussion

Defining Proton Positions During the Catalytic Reaction of HpMTAN.

It is well accepted that the initiation of the catalytic reaction of HpMTAN is afforded by the donation of a proton from D198 to the N7 position of the substrate. The positioning of D198 described in the transition-state analogs and product complexes is afforded by three hydrogen-bond interactions from the hydroxyl side chain of S197, the backbone amide of A200, and N6 of the adenine moiety (Fig. 2A). The deprotonated adenine N7 nitrogen atom has been calculated to possess negative electrostatic potential before protonation, promoting interaction with and proton transfer from D198 (19). Inspection of the omit difference $F_o-F_c$ nuclear map of the product complex reveals strong difference density for a shared deuterium ion between the carboxylic acid moiety of D198 and N7 of adenine (Fig. 2B). The presence of the shared D$^+$ ion was unexpected because it has not been previously proposed. The D$^+$ ion develops a trifurcated hydrogen bond that is positioned at a distance of 1.8 Å and 2.3 Å from O61 and O62 of D198, respectively, and 1.7 Å from N7 of the adenine. The angle between N7-D$^+--O61$ is 98° and between N7-D$^+--O62$ is 116°, deviating significantly from the more common 180° in countless biological interactions or the 155° angle observed in the MTAN transition-state analog neutron structures. Interestingly, the bonding distances of N-D$^+$ and D$^+--O61$ are nearly equivalent, suggesting that the pK$_a$ values of D198 and N7 are closely matched and implying a possible low-barrier hydrogen bond (31). Visualization of the shared D$^+$ ion further confirms the involvement of D198 in the initiation of the catalytic reaction by protonation of the adenine N7. In addition, this hydrogen-bond network elevates the pK$_a$ of the D198 side chain and ensures that a proton is available in the active site for subsequent enzyme-catalyzed reactions.

Additionally, omit difference $F_o-F_c$ nuclear density was observed for a deuterium atom on the N9 position of the adenine molecule, further highlighting the importance of the shared D$^+$ ion in the catalytic mechanism (Fig. 2B). In an accepted mechanism for MTAN, after the initiation of the catalytic reaction, the presence of the resulting carboxylate moiety of D198 develops an N7-H--O61 hydrogen-bond interaction with the protonated N7 of the substrate. The transient positive charge of the adenine moiety consequently leads to breakage of the N-ribofuranosidic bond, resulting in an adenine molecule with a protonated N7 and an unprotonated N9 (12, 32). This conformation directly conflicts with the protonation states observed in the product complex and suggests that, after disruption of the N-ribofuranosidic bond, the N9 accepts a proton, and N7 begins sharing its proton with the D198 side chain instead of retaining the proton. An intriguing question addressed by the protonation states of the adenine product is the identity of the specific chemical group that donates a proton to N9. It has been proposed that the conserved residue E13 acts as a general base to activate the nucleophilic water molecule that attacks the intermediate during the enzymatic reaction (9, 12, 33). Examination of the structure of the ternary product complex shows that the protonated N9 is 6.7 Å from E13 and lacks neighboring proton acceptors that could allow a proton shuttle-like mechanism. Additionally, all the neutron structures presented here show E13 to be in the deprotonated, carboxylate form. Therefore, it is unlikely the observed pK$_a$ of 5.6 is the result of E13 functioning as a general base in activating the nucleophile but instead ensures a fully deprotonated carboxylate to orient the nucleophile water molecule properly for attack on the oxocarbenium ion intermediate. The nucleophilic water molecule then directly donates a proton to N9 subsequent to nucleophilic attack on the oxocarbenium ion intermediate. These observations of the deuterium positions of the subsequent adenine product better define the proton transfer events involved in catalysis (Fig. 3) (34).

The SRH-binding subsite of the HpMTAN active site contains a variety of polar residues that allow the recognition of the ribose moiety of the substrate, some of which have ambiguous hydrogen-bond donors and acceptors. In the substrate complex, the observed nucleophilic water molecule is positioned by hydrogen-bond interactions with both the guanidinium moiety of R194 and a fully deprotonated carboxylate moiety of E13 with distances of 1.8 Å and 1.6 Å, respectively (Fig. 4). The orientation of the putative nucleophile provided by these residues ensures the positioning of a lone pair of electrons in the nucleophile toward the anomeric carbon of the ribose moiety before the formation of the oxocarbenium ion intermediate. Following nucleophilic attack by the water molecule on the intermediate, a fully deprotonated E13 is observed forming a hydrogen-bond interaction with the O1' hydroxyl of SRH in the product complex. This conserved glutamate residue has been demonstrated to be...
important for the catalytic reaction based on a significant loss of activity in EcMTAN variants E12Q (EcMTAN-E12Q) and E12A (6). Alternative hypotheses for the significant, but not complete, loss of enzymatic activity in the EcMTAN variants. Inspection of each neutron structure highlights E13 in HpMTAN as a hydrogen-bond acceptor to the backbone amides of residue A9, M10, and V78 in addition to the nucleophilic water molecule. The analogous Q12 residue in EcMTAN-E12Q could alter the hydrogen-bond network for the nucleophilic water molecule and reorient it within the active site. Because of this improper orientation of the nucleophilic water molecule, the lone pair of electrons on the oxygen atom of the nucleophile needed to form the new bond with the C1' of ribose is oriented toward the Q12 side chain in the E12Q variant and away from the oxocarbenium ion intermediate, consequently decreasing the likelihood of nucleophilic attack. This hypothesis also can explain why the EcMTAN variants retain a low level of enzymatic activity without a general base at this position. Specifically, thermal motion or random reorientation of the nucleophilic water molecule within the active site immediately following oxocarbenium ion formation in the EcMTAN-E12Q variant could allow a minority of the Michaelis complexes to undergo nucleophilic attack and complete catalysis.

Inspection of the neutron structure for HpMTAN-D198N/SAH provides additional information that supports an expanded role of S197 in the catalytic mechanism of HpMTAN. The involvement of S197 in the catalytic reaction has been studied previously for EcMTAN with an MTAN-S197A variant that possessed only 10% of the wild-type activity (6). Furthermore, S197 was suggested to be the proton donor for D198, allowing the hydroxyl of S197 to be regenerated by the bulk solvent through the classical Grotthuss mechanism (13). In the product complex, S197 and D198 develop a strong, linear, 1.9 Å, O-D···O61 hydrogen-bond interaction (Fig. 5A). Although this interaction assists in positioning the D198 side chain to promote proton donation to the substrate, the presented neutron structure as well as published X-ray structures suggest that S197 may play a role in proton transfer to D198 and the substrate. In the HpMTAN-D198N/SAH neutron structure, the deuterium position on the S197 hydroxyl is reoriented, creating a new hydrogen-bond network involving a water molecule in a small hydrophobic pocket abutting the active site (Fig. 5A). The S197 O-D group rotates nearly 180°, positioning the deuterium away from the N198 side chain as the result of the δNδ group developing an N-D···O hydrogen bond with a distance of 2.4 Å. The other δNδ atom of N198 forms an N-D···N hydrogen bond with N7 of the SAH at a distance of 2.1 Å. In the previously published HpMTAN-D198N X-ray crystal structures, this pocket always contains a single ordered water molecule, but no corresponding density is observed in the wild-type structures (5, 12, 15). Although ordering of this water molecule could simply be a consequence of the D198N mutation, it is expected that this pocket contains a disordered water molecule in the wild-type enzyme. The water in the hydrophobic pocket interacts with S197 by accepting a hydrogen bond from the S197 side chain at a distance of 1.7 Å (Fig. 5B). Additionally, the water molecule within this small hydrophobic pocket develops a hydrogen bond with the backbone carbonyl of F208 as well as forming an O-H···π interaction with the side chain of the highly conserved F208 (35, 36). The hydrogen-bonding pattern suggests a possible mechanism for the binding of a hydronium ion within this pocket and a proton shuttle to D198 mediated by S197. Whether this water molecule plays a role in the enzyme mechanism or is simply a function of the inactivating D198N mutation is unclear. It is intriguing, however, to consider that the binding of a hydronium ion to this site in the wild-type enzyme could be stabilized through a cation-π interaction with F208 and promote D198 protonation via a proton shuttle through S197. Such a role for ordered water molecules has been highlighted in other neutron structures (37). This scenario offers a possible second mechanism by which the enzyme causes a protonated D198 residue upon binding substrate.

**Insights into Transition-State Stabilization and Inhibitor Affinity**. In the transition-state analog neutron structures, specifically FMA and p-CIPh-Thio-DADMe-IImA, the observed deuterium...
positions provide information about the specific hydrogen-bond interactions that contribute to the high-affinity binding of these inhibitors and to stabilization of the transition state. Differences in the hydrogen-bond interactions of the two transition-state analogs were observed in the ribose-binding site, whereas the hydrogen-bond interactions in the adenine-binding pocket were conserved in both transition state complexes. In the HpMTAN/FMA and HpMTAN/p-CipH-Thio-DADMe-ImmA neutron structures, 2F\textsubscript{o}−F\textsubscript{c} nuclear density indicates a protonated N7 atom mimicking the protonated substrate immediately after the initiation of the catalytic reaction. The strong hydrogen-bond interaction between the deprotonated carboxylate group of D198 and the N7 proton of the nonhydrolyzable adenine mimic retains a distance of 2.0 Å and an angle of 155° between the three atoms in both complexes. Interestingly, slight variations in the hydrogen-bond distance were observed between the hydroxyl of S197 and the carboxylate of D198 in both transition-state analog structures as compared with the product complex. In the p-CipH-Thio-DADMe-ImmA complex, the observed O−D−O61 hydroxide bond distance increased to 2.4 Å with a 142° angle. The FMA complex demonstrated a more dramatic change in the O−D−O61 hydroxide bond consisting of 2.6 Å with 112° angles between the three atoms. In both the p-CipH-Thio-DADMe-ImmA and the product complex, the hydroxyl of S197 forms a van der Waals interaction with the C8 hydrogen atom of adenine, which in FMA is replaced by a nitrogen atom. Therefore, the loss of the van der Waals interaction between the S197 hydroxyl and the substrate weakens the hydrogen bond interaction between S197 and D198.

The HpMTAN/p-CipH-Thio-DADMe-ImmA complex demonstrates several hydrogen-bond interactions that contribute to the picomolar affinity of this inhibitor even though HpMTAN is suggested to form an early dissociative transition state. It was shown that the K\textsubscript{d} for p-CipH-Thio-DADMe-ImmA was 570 pM for HpMTAN, an affinity roughly 100-fold higher than that of the ImmA analog that was demonstrated to have a K\textsubscript{d} of 40 nM (16). The differences in the affinities of the transition state analogs to structurally similar homologs of MTAN have been studied previously using computational techniques (38, 39). 2F\textsubscript{o}−F\textsubscript{c} nuclear density for the HpMTAN/p-CipH-Thio-DADMe-ImmA complex demonstrates a deuterium atom located on the N1' of the pyrrolidine moiety, confirming the presence of the cationic nitrogen atom in the catalytic site of HpMTAN (Fig. 6A). Therefore, this neutron structure gives a direct view of the hydrogen-bond interactions that orient a lone pair of electrons on the nucleophile water molecule toward the newly formed oxocarbenium ion intermediate. The oxygen atom of the nucleophile is observed to form a strong N−D−O hydrogen-bonding interaction from both the guanidinium moiety of R194 and the cationic nitrogen atom of the N1' from the pyrrolidine moiety with distances of 1.8 Å and 1.6 Å, respectively. Additionally, the deuterium atoms of the nucleophile form moderate-strength O−D−O hydrogen-bonding interactions with the deprotonated carboxylic acid moieties of E13 and E175 at distances of 2.3 Å and 2.5 Å, respectively. Besides the other various interactions between p-CipH-Thio-DADMe-ImmA and HpMTAN, the observed hydrogen bonded network with the nucleophile contributes significantly to the proper orientation of the water molecule for attack on the oxocarbenium ion intermediate. To confirm that the high-affinity binding of p-CipH-Thio-DADMe-ImmA is caused by the presence of a cationic character and not by possible flattening of the pyrrolidine ring, a 1.4 Å resolution X-ray crystal structure was refined showing that the N1' is sp\textsuperscript{3} hybridized (Fig. S1).

Inspection of the HpMTAN/FMA complex provides evidence regarding the difference in affinities observed between DADMe-ImmA and ImmA transition-state analogs (Fig. 6B).

In the HpMTAN/FMA complex an unusual binding orientation of the nucleophilic water molecule was observed that does not allow a lone pair of electrons to be positioned toward the inhibitor. This unusual orientation of the water molecule is afforded by strong and moderate hydrogen-bond interactions with the guanidinium moiety of R194, the carboxylate moiety of E13, and the O3' hydroxyl of the ribose moiety of FMA. Intriguingly, in the HpMTAN/FMA neutron structure the 2F\textsubscript{o}−F\textsubscript{c} nuclear map shows that the deuterium atom of the O3' hydroxyl for the ribose moiety is rotated 76° from the O62 of E175 toward the S'-alkylthio–binding subsite of the catalytic site. Although an increase in the distance between the hydrogen-bond acceptor and donor was not observed, the new proton position disrupts the hydrogen-bond interaction with O62 of E175 that is observed in all other neutron structures. The new orientation of the deuterium atom for the O3' hydroxyl allows a new O−D−O hydrogen-bond interaction to form with the O2' hydroxyl at a distance of 2.1 Å. The O−D−O62 hydrogen-bond interaction between the O3' hydroxyl and the carboxylate side chain of E175 was shown to be essential for the catalytic reaction, because the removal of the O3' hydroxyl from the ribose moiety and the use of E175 EcMTAN variants eliminate catalytic activity (6, 9). Based on these results, it has been proposed that the O3' hydroxyl becomes ionized during the formation of the transition state to assist in stabilizing the oxocarbenium ion intermediate (11, 32), but the neutron structures presented here demonstrate a protonated O3' hydroxyl and a fully deprotonated E175 carboxylate.

ACKNOWLEDGMENTS. We thank Heinz Maier-Leibnitz Zentrum and Oak Ridge National Laboratory for graciously providing the beam time that was essential for this work. This work was supported by the Center for the Advancement of Science in Space via a cooperative agreement with National Aeronautics and Space Administration Grant N-12528-01 (to D.R.R.) and by National Institute of Allergy and Infectious Disease/NIGMS Grant AI05084 (to D.R.R.). This research used the resources of the Advanced Photon Source, a US Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and by Grant 085P1000817 from the Michigan Technology Tri-Corridor. The research was sponsored in part by the Scientific User Facilities Division, Office of Basic Energy Sciences, U.S. Department of Energy. The IMAGINE Project was partially supported by the National Science Foundation (Grant 0922719).


