Structural Rationale for the Affinity of Pico- and Femtomolar Transition State Analogues of Escherichia coli 5′-Methylthioadenosine/S-Adenosylhomocysteine Nucleosidase* ♦

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Immcillin and DADMe-Immcillin inhibitors are tight binding transition state mimics of purine nucleoside phosphorylases (PNP). 5′-Methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN) is proposed to form a similar transition state structure as PNP. The companion paper describes modifications of the Immcillin and DADMe-Immcillin inhibitors to better match transition state features of MTAN and have led to 5′-thio aromatic substitutions that extend the inhibition constants to the femtomolar range (Singh, V., Evans, G. B., Lenz, D. H., Mason, J., Clinch, K., Mee, S., Painter, G. F., Tyler, P. C., Furneaux, R. H., Lee, J. E., Howell, P. L., and Schramm, V. L. (2005) J. Biol. Chem. 280, 18265–18273). 5′-Methylthio-Immcillin A (MT-Imma) and 5′-methylthio-DADMe-Immcillin A (MT-DADMe-Imma) exhibit slow-onset inhibition with $K_i$ of 77 and 2 pm, respectively, and were selected for structural analysis as the parent compounds of each class of transition state analogue. The crystal structures of Escherichia coli MTAN complexed with MT-Imma and MT-DADMe-Imma were determined to 2.2 Å resolution and opposed to form a similar transition state structure as the parent compounds of each class of transition state analogue. The crystal structures of Escherichia coli MTAN complexed with MT-Imma and MT-DADMe-Imma were determined to 2.2 Å resolution and compared with the existing MTAN inhibitor complexes. These MTAN-transition state complexes are among the tightest binding enzyme-ligand complexes ever described and analysis of their mode of binding provides extraordinary insight into the structural basis for their affinity. The MTAN-MT-Imma complex reveals the presence of a new ion pair between the 4′-iminobitol atom and the nucleophilic water (WAT3) that captures key features of the transition state. Similarly, in the MTAN-MT-DADMe-Imma complex a favorable hydrogen bond or ion pair interaction between the cationic 1′-pyrrolidine atom and WAT3 is crucial for tight affinity. Distance analysis of the nucleophile and leaving group show that MT-Imma is a mimic of an early transition state, while MT-DADMe-Imma is a better mimic of the highly dissociated transition state of E. coli MTAN.

The nucleosides, 5′-methylthioadenosine (MTA) and S-adenosylhomocysteine (SAH) are molecules involved in key cellular functions such as biological methylation (1), polyamine biosynthesis (2, 3), methionine recycling (4, 5), and bacterial quorum sensing (6, 7). The breakdown of these nucleosides differs in microbial and mammalian systems. In many pathogenic microbes, such as Bacillus anthracis, Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae, Mycobacterium tuberculosis, and Helicobacter pylori, MTA and SAH are catabolized by the dual substrate specific enzyme, MTA/SAH nucleosidase (MTAN). MTAN irreversibly cleaves the glycosidic bond of MTA or SAH to form adenine and 5-methylthioribose or S-ribosylhomocysteine, respectively (8). However, in mammalian systems the nucleoside is not present and the breakdown of MTA and SAH requires two separate enzymes, MTA phosphorylase (MTAP) (9) and SAH hydrolase (10), respectively. Given the differences in metabolism, MTA/SAH nucleosidase has been identified and validated as a potential target for the design of broad-spectrum antimicrobials (4, 5).

The structures of MTAN complexed with adenine (MTAN-adenine) (11), formycin A (MTAN-FMA) and 5′-methylthiotubercidin (MTAN-MTT) (12) have been solved and a comparison of the active site architecture reveals that MTAN is most similar to MTAP (11–13). MTAP belongs to the NP-1 class of purine nucleoside phosphorylases (PNP) (14), which are proposed to

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The atomic coordinates and structure factors (codes IY6R (MTAN-MT-Imma complex) and IY6Q (MTAN-MT-DADMe-Imma complex).) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

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1 The abbreviations used are: MTA, 5′-methylthioadenosine; SAH, S-adenosylhomocysteine; MTAN, 5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase; MTAP, 5′-methylthioadenosine phosphorylase; MT-Imma, (1S)-1-9-deazaadenin-9-yl-1,4-dideoxy-1,4-imino-5-methylthio-5-ribitol; MT-DADMe-Imma, (3R,4S)-1-9-deazaadenin-9-yl)methyl-3-hydroxy-4-(methylthiomethyl)pyrrolidine; MTT, 5′-methylthiotubercidin; PNP, purine nucleoside phosphorylase; FMA, formycin A; r.m.s.d., root mean square deviation.
undergo a dissociative $S_{N}1$-type reaction with the formation of a ribooxacarbenium ion followed by the nucleophilic attack by the phosphate (11, 15–20). The structural elements common to the catalytic sites of MTAN, MTAP, and PNP suggest a similar catalytic schema (11). Isotope effects and inhibitor studies have shown the transition states for these enzymes to be ribooxacarbenium-like (17, 20).\textsuperscript{2} Given the similarities in transition state structure, inhibitors developed for PNP and MTAP were tested for their ability to inhibit MTAN.

There has been extensive inhibitor development for MTAP and PNP. Such interest stems from the observation that in humans a genetic deficiency in PNP leads to a build up of dGTP and results in the cell death of proliferating T-cells (22). The inhibition of PNP provides a target for the treatment of T-cell-mediated autoimmune disorders and T-cell malignancies (23). Studies of the human, bovine, \textit{M. tuberculosis}, and \textit{Plasmodium falciparum} PNP transition state structures led to the development of the Immucillin and DADMe-Immucillin transition state analogues (24–32). In general, the Immucillins replace the ribosyl O4’ with a positive charge (N4’) to match the electrostatic state of a partially dissociated oxacarbenium ion. Furthermore, the purine N9 is replaced by a carbon to provide a non-hydrolyzable C–C ribosidic bond which increases the $pK_a$ of N7 to $> 10$ and allows for protonation. The DADMe-Immucillin inhibitors were developed with a methylene bridge between the deazaadenine and pyrrolidine group to mimic the highly dissociated or advanced bond order of the leaving group (33–35). In addition, the positive charge is shifted to the anemic position from the ribosyl O4’ to mimic the delocalization of the positive charge between the O4’ and C1’ atoms. The Immucillin and DADMe-Immucillin class of inhibitors exhibit slow-onset, tight binding with $K_i$ in the picomolar range in bovine, human, and \textit{M. tuberculosis} PNP (25, 33–35). More recently, the Immucillin and DADMe-Immucillin classes of transition state analogues were modified to inhibit human MTA phosphorylase and \textit{Escherichia coli} MTA/SAH nucleosidase (Fig. 1) (36–38). 5’-Methylthio-Immucillin A (MT-ImmA) and 5’-methylthio-DADMe-Immucillin A (MT-DADMe-ImmA) exhibit slow onset, tight binding in \textit{E. coli} MTAN with $K_i = 77$ and 2 pt, respectively (36). Aromatic substitutions to the 5’-thio group provided seventeen inhibitors with dissociation constants from $10^{-12}$ to $10^{-14}$ M. 5’-p-Cl-phenylthio-DADMe-ImmA ($K_i = 47$ fM) is one of the most powerful non-covalent inhibitors ever reported.

MT-ImmA and MT-DADMe-ImmA were selected for structural analysis as the parent compounds of the two transition state analogue classes. The structures of MTA/SAH nucleosidase complexed with the inhibitors MT-ImmA (MTAN-MT-ImmA) and MT-DADMe-ImmA (MTAN-MT-DADMe-ImmA) have been determined to 2.2 Å resolution. Comparisons of MTAN-MT-ImmA and MTAN-MT-DADMe-ImmA with the previously determined MTAN-MTT and MTAN-FMA structures has permitted a dissection of the interactions between the active site and the inhibitors that account for the tight affinity of the transition state analogues. The structures reveal that affinity of MT-DADMe-ImmA likely results from more favorable interactions between the cationic charge on the pyrrolidine moiety and the nucleophilic water either through an energetically favorable hydrogen bond or an ion pair. The structures of MTAN complexed with MT-ImmA and MT-DADMe-ImmA are compared with a structure resembling the Michaelis complex and demonstrate increased and more favorable hydrogen bond contacts together with ionic interactions with the carbocation mimics inherent in the Immucillin structures.

**EXPERIMENTAL PROCEDURES**

**Crystallization of MTAN-MT-ImmA and MTAN-MT-DADMe-ImmA**—\textit{E. coli} MTA/SAH nucleosidase was expressed and purified as described previously (39). The transition state analogues MT-ImmA and MT-DADMe-ImmA were synthesized as reported previously (37). The enzyme was concentrated to 15 mg/ml and incubated with 1 μmol MT-ImmA or MT-DADMe-ImmA for 2 h on ice before crystallization. All crystallization experiments were performed at room temperature using the hanging drop vapor diffusion technique. Rod-shaped MTAN-MT-ImmA crystals (0.15 × 0.15 × 0.15 mm).

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\textsuperscript{2} V. Singh, S. Nunez, J. E. Lee, P. L. Howell, and V. L. Schramm, unpublished results.
Table 1

|                  | MTAN-MT-ImmA | MTAN-MT-DADMe-ImmA |
|------------------|--------------|--------------------|
| **Data collection and refinement statistics** |              |                    |
| **Diffraction statistics** |              |                    |
| Space group      | P2₁2₁2₁      | P2₁2₁2₁            |
| Unit cell        | a = 51.6 Å   | 51.7 Å             |
|                  | b = 69.8 Å   | 69.8 Å             |
|                  | c = 128.3 Å  | 128.3 Å            |
|                  | α = β = γ = 90° | 90°                |
| No. of measured reflections | 142,613 | 95,776             |
| No. of unique reflections | 23,961 | 24,103             |
| Resolution range (Å) | 34.8–2.2   | 28.2–2.2           |
| Rmerge (%)      | 6.7 (18.0)²  | 7.0 (29.6)²        |
| Redundancy      | 5.9          | 3.8                |
| Completeness (%)| 99.7 (100.0)³ | 99.0 (99.7)³       |
| Average I/σ(I)  | 8.6 (4.0)⁴   | 12.2 (4.0)⁴       |
| **Refinement statistics** |              |                    |
| No. of protein atoms | 3,475 | 3,301              |
| No. of water molecules | 164 | 219               |
| No. of ligand atoms | 40    | 40                 |
| Resolution range (Å) | 34.8–2.2   | 28.2–2.2           |
| Rcryst (%)       | 19.4³       | 19.8³              |
| Rfree (%)        | 24.4        | 24.6               |
| Overall B-factor (Å²) | 6.7          | 6.7                |
| Monomer A        | 28.1        | 30.2               |
| Monomer B        | 26.6        | 27.7               |
| Ligand           | 19.2        | 22.6               |
| Water            | 31.5        | 33.6               |
| r.m.s.d. Bonds (Å) | 0.010   | 0.010              |
| Angles (degree)  | 1.48        | 1.49               |
| Dihedrals (degree) | 23.5   | 24.0               |
| Impropers (degree) | 0.93    | 0.97               |
| Cross validated Luzzati coordinate error (Å) | 0.28 | 0.32               |

² Rmerge = Σ [I(k) – <I(k)>] / Σ I(k), where I(k) and <I(k)> represent the diffraction intensity values of the individual measurements and the corresponding mean measurements. The summation is over all unique measurements.

³ Values given in the parentheses refer to reflections in the outer resolution shell: 2.28–2.20 Å.

⁴ Rcryst = Σ ||Fobs| – |Fcalc|| / Σ |Fobs|, where Fobs and Fcalc are the observed and calculated structure factors, respectively.

Rfree, the sum is extended over a subset of reflections (5%) excluded from all stages of the refinement.

0.15 × 0.7 mm) were obtained over 3 weeks using 37% (w/v) polyethylene glycol 200, 100 mM sodium acetate, pH 4.7, 100 mM NaCl, and 10 mM CoCl₂·6H₂O. MTAN-MT-ImmA crystals crystallizes over a 2-week period as thin rods (0.1 × 0.1 × 0.5 mm) in 1.4 M sodium chloride and 0.1 M sodium acetate, pH 4.6.

Data Collection and Processing—The MTAN-MT-ImmA complexed nucleosidase crystal was frozen directly in a stream of nitrogen gas at 100 K without the need for cryoprotection. The MTAN-MT-DADMe-ImmA crystals (12), which allowed us to determine the structures using difference Fourier methods. For each structure the initial MTAN-MT dimer (Protein Data Bank code: 1NC1), from which all water and inhibitor molecules were omitted, was subjected to a round of rigid body refinement in CNS (42), using all data between 35.0 and 2.2 Å with no σ-cutoffs, treating each monomer as a rigid body. σ₂-weighted difference and 2Fcalc–Fobs Fourier maps were calculated. Interactive rounds of manually rebuilding using Xfit at XtalView (43) and amplitude-based maximum-likelihood torsion angle simulation annealing (44) and individual-B-factor refinement with an overall anisotropic temperature and bulk solvent correction were performed. The progress of the refinement was monitored by the reduction in Rcryst and Rfree. σ₂-weighted difference Fourier maps revealed ordered water molecules and strong unambiguous positive Fcalc–Fo differences electron density corresponding to the MT-ImmA or MT-DADMe-ImmA inhibitor. The MT-ImmA coordinates were generated by modifying the Protein Data Bank file of MTA downloaded from the HIC-UP server (45). The coordinates for MT-DADMe-ImmA were generated using the web-based program, CORINA (www2.chemie.uni-erlangen.de/software/corina/index.html). The topology and parameter files were subsequently produced using the XPLOR-2D server (46). Water molecules with proper hydrogen-bonding coordination and electron densities larger than 1 σ on 2Fo–Fcalc and 3oFo–Fo σ₂-weighted maps were progressively introduced while monitoring the decrease in Rfree. After the inclusion of water and inhibitor molecules into the models, alternating rounds of crystallographic conjugate gradient minimization refinement and model rebuilding in Xfit were performed.

Chymotrypsin digestion of the enzyme results in the cleavage of 21 residues of the 6 residue N-terminal 9-histidine fusion tag. The remaining 10 residues of the N-terminal fusion tag were located in a region of weak electron density and only residues 2–232 in monomer A and residues 1–232 in monomer B were sufficiently ordered enough to be built into the model. The side chains of Cys(12) and Thr(218) in both MTAN-MT-DADMe-ImmA monomers were found to exist in two orientations and were modeled and refined as such. The MTAN-MT-ImmA and MTAN-MT-DADMe-ImmA structures were refined with Rcryst = 19.4% and Rfree = 24.4% and Rcryst = 19.8% and Rfree = 24.6%, respectively. Analysis of the structures in PROCHECK (47) and CNS (42) reveal good stereochemistry with no non-glycine residues falling into the disallowed regions of the Ramachandran plot. The refinement statistics are reported in Table I.

Superimposition of Structures—The MT/SAH nucleosidase structures were aligned by non-linear least squares fit of selected main chain (N-Cα-C) atoms in the central β-sheet using the program PROFIT (Version 6.0), written by G. David Smith. The residue ranges used in the refinement of the superposition were Lys(5)–Ile(6), Glu(42)–Leu(46), Leu(62)–Ala(71), Val(129)–Arg(134), Pro(136)–Ala(139), and Val(139)–Val(142).

Conformational Energetics for MTA and Inhibitors—Energetics of the sugar-analogue ring puckering and the C-ribosyl torsion angles for MTA, MT-ImmA, and MT-DADMe-ImmA were calculated in vacuum, using the Sybyl force constants in Spartan, Version 5.13. Energies for sugar puckering were minimized at 10-degree intervals of sugar pucker indexed for the dihedral defined by O4=C1–C3–C2–C3’ in MTA(11)–C1’–C2’–C3’ in MTAN-MT-ImmA and C4’–N1’–C2’–C3’ in MTAN-MT-DADMe-ImmA. Note that the C4’ carbonyl carbon of MT-DADMe-ImmA is uncoordinated in Fig. 1. Energetics for glycosyl torsion angles were minimized at 10-degree intervals for the full rotation of the 9-deazadenine ring with respect to the ribosyl group. Energetic minimizations were conducted for neutral MTA and both neutral and cationic forms of MT-ImmA and MT-DADMe-ImmA. All energetic barriers are assumed to be upper limits, since they are calculated in vacuum and the energies are expected to be lower in water where the electrostatic repulsions/attractions are shielded.

RESULTS AND DISCUSSION

Transition State Analogue-Enzyme Complexes

The overall structures of MTAN-MT-ImmA and MTAN-MT-DADMe-ImmA are similar to the ligand-induced closed conformation structures of MTAN complexed with PMA or MTT (12). The overall structures is a homodimer with the catalytic MTAN-ImmA site per subunit (Fig. 2a) (11). Each subunit contains an α/β structure with a central nine-stranded mixed β-sheet and a smaller five-stranded β-sheet. The central β-sheet is surrounded by six α-helices and one small 3₁₀ helix (Fig. 2b). The dimer interface is primarily hydrophobic, consisting of residues from the α2, α5, α6, and 3₁₀ helices and the loops between β2-β3 and β4-α2. The nucleosidase active site has three subsites to accommodate the adenine, ribose, and 5′-alkylthio substituents of the substrate (Fig. 3). Although, MTAN-MT-ImmA and MTAN-MT-DADMe-ImmA were crystallized at pH 4.7 and 4.6, respec-
the sugar and the 5'-methylthio group. The N4’ imino group of MT-ImmA makes two weak hydrogen bonds to the Oy of Ser76 (3.2 Å) and the nucleophilic water, WAT3 (3.1 Å). WAT3 is stabilized by a network of hydrogen bonds to the carboxylate group of Glu12, the guanidinyl group of Arg103, and the iminonitroso group of O2’ and O3’ atoms. The iminoribitol moiety has a C4’-endo sugar pucker conformation (Fig. 4). The 5’-methylthio group is primarily coordinated by inter- and intrasubunit van der Waals interactions. Residues Met6, Ile60, Phe151, Met173, and Phe207 from one subunit and residues Val102, Phe105, Tyr107, and Pro112 from a neighboring subunit (Fig. 3, c and e) coordinate the 5’-methylthio moiety, which binds with an exocyclic C4’–C5’ bond orientation of $\phi_{oc} = 171^\circ$ (S5–C5’–C4’–O4’) and $\psi_{oc} = -71^\circ$ (S5–C5’–C4’–C3’), corresponding to a trans, gauche conformation. This is similar to the 5’-methylthio conformation seen in the MTAN-MTT x-ray structure (12) and in the small molecule MTA crystal structure (50). However, NMR studies of MTA have suggested that in solution a mixture of conformations are present with a diminished contribution of the trans, gauche C4’–C5’ conformation (51).

**Interactions with MT-DADMe-ImmA—Unambiguous difference Fourier electron density was seen for the MT-DADMe-ImmA molecule in the active sites of both monomers (Fig. 2d). The interactions between the deazaadenine base and the protein are identical to those seen in the MTAN-MT-ImmA structure. Like the MT-ImmA inhibitor, the MT-DADMe-ImmA inhibitor is bound to MTAN in a high anti-conformation; however, the presence of the methylene bridge between the pyrrolidine and the deazaadenine base reduces the ribosidic torsion angle (C4’–N1’–C9’–C8) to $-5^\circ$, compared with $-70^\circ$ in the MTAN-MT-ImmA, MTAN-MTT, and MTAN-FMA structures. The binding of the pyrrolidine is different as this moiety lacks a 2’-hydroxyl. As a consequence the only interactions the saccharide mimic makes with the protein is via its 3’-hydroxyl and protonated N1’ nitrogen atom. The 3’-hydroxyl makes two strong hydrogen bonds to the Oe2 of Glu173 (2.8 Å) and the nucleophilic water (WAT3) while WAT3 also interacts with the ribosyl N1’ nitrogen atom (2.7 Å) (Fig. 3f). As found previously for the MT and MT-ImmA complexed structures, WAT3 is stabilized by hydrogen bonds to the carboxylate group of Glu12, the Oe1 of Glu174, and the Nv1 of Arg98. The hydrophobic side chain of Met173 packs against the C2’ and C3’ hydrophobic face of the sugar. The iminoribitol is in a C1’/N1’-exo sugar conformation (Fig. 4) and not surprisingly the 5’-methylthio group of MT-DADMe-ImmA binds with a $\phi_{oc} = -166^\circ$ and $\psi_{oc} = -56^\circ$ conformation, since the 5’-alkylthio moiety interacts with the same protein residues seen in the MTAN-MT-ImmA structure. The $\phi_{oc}$ torsion angle is given with a $-180^\circ$ to $180^\circ$ convention. Although the $\phi_{oc}$ angle has a negative value in MTAN-MT-DADMe-ImmA, there is only an absolute difference of $23^\circ$ from the MTAN-MT-ImmA 5’-methylthio torsion angle. This corresponds to the same unusual trans, gauche conformation seen in the MTAN-MTT and MTAN-MT-ImmA structures.

**Rationale of Tight Binding Affinities**

MT-DADMe-ImmA is one of the tightest MTAN transition state analogues known, with a $K_i$ of 2 pm. This inhibitor binds over 38 times better than MT-ImmA ($K_i = 77$ pm) and 375,000 times better than MTT ($K_i = 0.75 \mu$m) (49). To understand the tight binding affinities of MT-ImmA and MT-DADMe-ImmA, a superimposition of MTAN-MT-ImmA, MTAN-MT-DADMe-ImmA, and MTAN-MTT structures was performed. The superimposition reveals very little difference between all three structures in terms of main chain atoms. The root mean square deviation (r.m.s.d.) between all main chain atoms (N–Cα–C) in the MTAN-MTT, and the MTAN-
MT-ImmA and MTAN-MT-DADMe-ImmA monomers are 0.12 and 0.19 Å, respectively. The r.m.s.d. between the MTAN-MT-ImmA and MTAN-MT-DADMe-ImmA monomers is 0.17 Å. An examination of the purine, ribose, and 5'-alkylthio-binding sites reveals that the side chain positions are also very well conserved (Fig. 3, a–c). However, there are differences in the positions of the nucleophilic water (WAT3) and ring pucker of the inhibitor that likely account for the tight binding of the transition state analogues.

**Rationale of Tight Binding for MT-ImmA**—The hydrogen bond length differences between MT-ImmA and MTT and the protein are within experimental error (0.3 Å) and therefore...

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**Fig. 3. Comparison of MTAN-MT-DADMe-ImmA, MTAN-MT-ImmA, and MTAN-MTT.** Stick representation of the purine (a), ribose (b), and 5'-alkylthio-binding site (c). The MTAN-MTT, MTAN-MT-ImmA, and MTAN-MT-DADMe-ImmA structures are colored in yellow, gray, and cyan, respectively. For ease of comparison residues 8, 9, and 193 were omitted from b. In c, residues 102, 105, 107, and 113 (marked with asterisks) are donated from a neighboring subunit. Active site contacts for MTAN-MTT (d), MTAN-MT-ImmA (e), and MTAN-MT-DADMe-ImmA (f) complexes. Dotted lines represent protein-protein or protein-ligand hydrogen bonds with distances in angstroms (Å). Residues donated from a neighboring subunit are shown in shaded boxes, and numbers in and outside the brackets refer to hydrogen bonding distances in monomer A and B, respectively. Significant changes in the MT-ImmA and MT-DADMe-ImmA structures relative to the MTAN-MTT structure are shaded gray. The structure with MTT was solved previously (12), and the figure was included here to permit a comparison with MT-ImmA and MT-DADMe-ImmA. The MTAN-MTT active site schematic is reprinted with permission from Ref. 12 (© 2003 Journal of Biological Chemistry).
cannot account for the large differences in binding energy (Fig. 3, d–e). However, there is one transition state feature that accounts for the picomolar binding constant of MT-ImmA. The substrate analogue MTT is a neutral molecule and there is no interaction between the nucleophilic water and the O4′ ribosyl atom, as seen by the 3.5- and 3.7-A separation in monomers A and B, respectively (Fig. 3d). In fact, these two groups likely exert a small repulsive force due to the presence of two electronegative oxygen atoms. The 4′-iminoribitol group of MT-ImmA has a pK₅₅ = 6.9 that is cationic in the active sites of N-ribosyltransferases (52). This positively charged moiety mimics the partial positive charge build up at this position, which attracts the nucleophilic water toward the 4′-iminoribitol group (average 3.1-A separation) (Fig. 3e). It is unlikely that this interaction is a hydrogen bond, since Fersht et al. (53) has shown that a hydrogen bond made between charged and uncharged groups account for only ~4 kcal/mol and is worth only a factor of ~1000 in specificity. The introduction of a hydrogen bond does not provide enough binding energy to explain the ~9,700-fold increase in affinity for MT-ImmA compared with MTT. An electrostatic interaction between WAT3 and the 4′-iminoribitol group likely explains the improved affinity. Typically water is not a strong nucleophile and activation is necessary for catalysis to occur. The Oε1 and Oε2 carboxylate atoms of Glu74 are in good position to deprotonate WAT3. The negatively charged water nucleophile could then make a stabilizing ion pair with the 4′-iminoribitol cation. In the MTAN-MTT structure, WAT3 does not make an electrostatic interaction to the ribosyl O4′ atom. The replacement of the N7 purine atom with a carbon prevents a proton donation at N7 and the build up of positive charge at the ribosyl O4′. Although solving for Coulomb’s Law is difficult in proteins given the unknown nature of the dielectric constant, the improved binding energy in MT-ImmA can be approximated by bringing a negatively charged hydroxyl and positively charged 4′-iminoribitol from infinity or an unbound state to 3.1 Å. Such a calculations show an increased binding energy of 26.8 kcal/mol, using point charges and a dielectric constant of 4. Although this calculation is likely overestimated, the binding energy is more than sufficient to account for the ~9,700-fold improvement of MT-ImmA binding over MTT. Interestingly, stabilization of the 4′-iminoribitol charge in MT-ImmA through an ion pair enhancement is also seen in the human MTAP-MT-ImmA complex (37). The distance between the phosphate oxygen and the 4′-iminoribitol shorts from 3.9 to 3.0 Å, thus providing over 10 kcal/mol binding energy to account for the 500,000-fold improvement in affinity.

**Rationale for Tight Binding in MT-DADMe-ImmA—MT-DADMe-ImmA lacks the O2′ atom and the C1′ carbon is replaced by a charged nitrogen. The loss of the 2′-hydroxyl eliminates three hydrogen bonds to Met173, Glu174, and WAT3. The movement of the nitrogen to the anomeric position removes two more hydrogen bonds from the 4′-pyrrolidine position to Ser76 and WAT3. One would hypothesize that MT-DADMe-ImmA would bind poorer to MTAN than MT-ImmA, but in fact binding is ~38 times tighter. A superimposition of the transition state structures reveals similar hydrogen bond lengths and interactions to those seen in MTAN-MTT and MTAN-MT-ImmA. The picomolar inhibition can be accounted for by two features of the transition state analogue. The pK₅₅ of the 1′-pyrrolidine nitrogen is ~9.2 and therefore fully cationic at physiological pH. The presence of the cationic N1′ atom and the deletion of the 2′-hydroxyl allows a better capture of the transition state features by allowing the nucleophilic water to move to within 2.6 and 2.8 Å of the 1′-pyrrolidine nitrogen in monomer A and B, respectively. This essentially positions WAT3 ~1.0 and 0.5 Å closer to the nucleoside than in the MTAN-MTT and MTAN-MT-ImmA structures, respectively (Fig. 3, d–f). Assuming that WAT3 is deprotonated and makes a strong ion pair with the N1′ atom, Coulomb’s Law predicts ~5.1 kcal/mol increased binding energy (point charge interactions separated from 3.1 to 2.6 Å in a dielectric of 4). This binding energy is more than required to explain the ~38-fold improvement in binding constant between MT-DADMe-ImmA and MT-ImmA. Alternatively, the interaction between WAT3 and the 1′-pyrrolidine nitrogen can be an energetically favorable hydrogen bond (~2.6 Å). Energetically favorable hydrogen bonds typically have matched pK₅₅ values between the two heteroatoms sharing the hydrogen. In addition, the distance between the two heteroatoms approaches a value less than the sum of their van der Waals radii (~2.5 Å) (54). These types of hydrogen bonds often account for binding energies of up to 10~20 kcal/mol and have been suggested to play a major role in stabilizing transition states and in the stabilization or destabilization of the ground states of enzyme reactions (54–57). The presence of an energetically favorable hydrogen bond could explain the improvement in the Kᵢ of 77 to 2 pM between MT-ImmA and MT-DADMe-ImmA.

Nucleosides such as MTA or SAH in solution have been shown to exhibit variable sugar ring puckers, with the C3′-endo saccharide conformation being the predominant sugar pucker (51). In all crystal structures of MTAP and MTAN, with the exception of MTAN-MT-DADMe-ImmA, the nucleoside ligands are bound in a C4′-endo ribose ring conformation (12, 13, 15, 37). In MTAN-MT-DADMe-ImmA, the addition of the methyl bridge between the pyrrolidine and deazaadenine groups and the movement of the positive charge to the anomeric position allows MT-DADMe-ImmA to adopt a C1′/N1′-exo conformation (Fig. 4). The difference in sugar pucker observed in the MTAN-MT-DADMe-ImmA structure prompted us to examine the energetic contributions to binding of the ribose moiety. Energetic calculations comparing the sugar puckers for neutral MTA and MT-ImmA as well as cationic MT-DADMe-ImmA (their dominant forms in solution) reveal that the energetic minimum for MTA is 3′-endo, in agreement with NMR data. Neutral and cationic MT-ImmA both have their energetically
minima in the 3'-exo conformations, while the cation of MT-DADMe-ImmA shows an energetic minimum at 0 degrees. Since the actual transition state is a riboxacarbenium ion with an energetic minimum near 0 degrees because of the sp²-hybridized C1 at the transition state, we can postulate that the best mimic of the transition state, both in geometry and charge is the MT-DADMe-ImmA. Based on our calculations, ribosyl geometry alone is estimated to contribute 1 to 2 kcal/mol in binding energy for both the MT-ImmA and MT-DADMe-ImmA cations. A similar energetic analysis of the glycosyl torsion angles of MTA, MT-ImmA and MT-DADMe-ImmA (neutral and cationic inhibitors) reveals rotational barriers are as high as 2.2 kcal/mol for MTA but are less than 1.5 kcal/mol for MT-ImmA and MT-DADMe-ImmA.

Taken together and given that MT-ImmA and MT-DADMe-ImmA bind to the enzyme better than the substrate, MTA, by 5.1–7.3 kcal/mol, the largest force in transition state analogue binding, especially for MT-DADMe-ImmA, is the cationic mimic of the transition state. The importance of the cationic nature of the MT-DADMe-ImmA is clearly demonstrated in the crystal structure where the ion pair between the catalytic water (WAT3) and the cation of MT-DADMe-ImmA is closer than in the MT-ImmA complex and is clearly a dominant force.

Rationale for Femtomolar Affinity Binding of 5'-Substituted DADMe-Immucillins—In the DADMe-Immucillin class of inhibitors, femtomolar inhibition was attained by aromatic substitutions to the 5'-alkylthio moiety of DADMe-ImmA (36). The most powerful inhibitor is 5'-p-chlorophenylthio-DADMe-ImmA with a $K_i^*=47$ fm. Although a crystal structure complex with this inhibitor has not been solved, the rationale for tight binding is hypothesized to be similar to MT-DADMe-ImmA. The majority of the binding energy is due to an enhanced ion

FIG. 5. Reaction coordinate diagram for MTAN. The distances, shown in Å, for substrate complexes are based on the E. coli MTAN-MTT crystal structure (12). The transition state structure was proposed based preliminary MTAN kinetic isotope effect data. The distances for the E-P complex are based on the M. tuberculosis PNP-product complex (34). The MT, MT-ImmA, and MT-DADMe-ImmA bond length and distances are averaged between monomer A and B.
pair or energetically favorable hydrogen bond interaction between the nucleophilic water and the pyrrolidine moiety. The addition of a p-chlorophenyl ring to the 5′-thio group allows a 3.2 Å base stacking or herringbone interactions to residues Phe100 and Phe207 in the 5′-alkylthio-binding site. Aromatic-aromatic interactions can provide 1–2 kcal/mol binding energy (58). Thus Phe100 and Phe207 interactions to the p-chlorophenyl ring can provide up to 4 kcal/mol binding energy to sufficiently account for the 43-fold binding improvement over MT-DADMe-ImmA of 5′-p-chlorophenylthio-DADMe-ImmA.

Evaluation of Transition State Analogues along the Reaction Coordinate

MTA/SAH nucleosidase is an irreversible hydrolase that breaks the ribosidic N9–C1′ bond. Given the structural similarities between MTAN and the NP-1 family of nucleoside phosphorylases, the nucleosidase is proposed to share a common catalytic mechanism (11, 12). The hydrolysis of the N9–C1′ bond of MTA and SAH is proposed to proceed through a dissociative S_n1-type nucleophilic mechanism. Theoretically, there are two energy barriers in the reaction coordinate profile for S_n1-type reactions. The first energy barrier is the rate-limiting step and corresponds to the formation of the oxacarbenium intermediate, while the second energy barrier is lower and corresponds to the nucleophilic attack by water. In human P. falciparum purine nucleoside phosphorylase kinetic isotope effects have shown that these reactions proceed through a stepwise S_n1-type mechanism with a fully dissociated oxacarbenium ion, while in C. fasciculata nucleoside hydrolase, a single transition state is formed before complete dissociation of the ribosyl group (20, 59).

Preliminary kinetic isotope effect data on MTAN confirm a transition state structure with a well removed leaving group and minimal interactions to the nucleophile. As a consequence, the MTAN reaction coordinate is represented by two energy barriers and a small energy well for the oxacarbenium intermediate. In ribooxacarbenium intermediates, the positive charge on the O4′ atom and after passing the first energy barrier, full oxacarbenium character develops until formation of the intermediate. In ribooxacarbenium intermediates, the positive charge distribution between the O4′ and C1′ positions with most of the charge residing on C1′ (60). In addition, the oxacarbenium ion moiety adopts a C3′-exo sugar pucker. After crossing the first energy barrier, the distance between the anomic carbon and the nucleophilic water decreases on the path to products. The enzyme-product (E-P) complex would likely show a ribosidic N9 to C1′ distance that is fully dissociated (~3.8 Å) and the formation of a covalent bond between the water and anomic carbon, as seen in the E-P complex of M. tuberculosis PNP (34).

MTA-MTT bound to MTAN has a 1.5 Å covalent C9–C1′ bond and a distance of 3.1 Å between WAT3 and the anomeric carbon. The higher affinity MT-DADMe-ImmA bound to MTAN has an increased linear distance from the N1′ atom to the C9 (2.8 Å) due to the methylene bridge and a 2.6 Å distance between the nucleophile and the positively charged 1′-pyrrolidine nitrogen. Clearly the short bond to the leaving group and the long distance to the nucleophile places MT-ImmA under the first energy barrier and a mimic of an early transition state (Fig. 5). The increased distance between the anomic position and the leaving group places MT-DADMe-ImmA higher on the energy profile than MT-ImmA and a closer mimic to a highly dissociated transition state.

CONCLUSIONS

The ImmA and DADMe-ImmA series of compounds are the most powerful transition state analogues available for MTA/SAH nucleosidase. MT-DADMe-ImmA binds almost 6 orders of magnitude tighter than the natural substrate, MTA. These inhibitors were designed to mimic the ribooxacarbenium ion character in the transition state structure. The crystal structures of E. coli MTAN complexed with MT-ImmA and MT-DADMe-ImmA have allowed a careful comparison to other MTAN structures and provide a structural basis for the tight binding. The tight binding of MT-ImmA and MT-DADMe-ImmA can be attributed to a key interaction between the positive charge of the analogue and the nucleophilic water. In MTAN-MT-ImmA this interaction is likely an enhanced ion pair, while in MTAN-MT-DADMe-ImmA an ion pair or an energetically favorable hydrogen bond provides the binding energy necessary for picomolar inhibition. An overall analysis of the distances between the C1′–C9 deazaadenine atoms, and the C1′ and nucleophilic water reveals that MT-ImmA and MT-DADMe-ImmA are mimics of an early and highly dissociated transition state of E. coli MTAN, respectively.

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Structural Rationale for the Affinity of Pico- and Femtomolar Transition State Analogues of Escherichia coli 5'-Methylthioadenosine/S-Adenosylhomocysteine Nucleosidase

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