Inhibition and Structure of *Toxoplasma gondii* Purine Nucleoside Phosphorylase

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The intracellular pathogen *Toxoplasma gondii* is a purine auxotroph that relies on purine salvage for proliferation. We have optimized *T. gondii* purine nucleoside phosphorylase (TgPNP) stability and crystallized TgPNP with phosphate and immucillin-H, a transition-state analogue that has high affinity for the enzyme. Immucillin-H bound to TgPNP with a dissociation constant of 370 μM, the highest affinity of 11 immucillins selected to probe the catalytic site. The specificity for transition-state analogues indicated an early dissociative transition state for TgPNP. Compared to *Plasmodium falciparum* PNP, large substituents surrounding the 5'-hydroxyl group of inhibitors demonstrate reduced capacity for TgPNP inhibition. Catalytic discrimination against large 5'-groups is consistent with the inability of TgPNP to catalyze the phosphorylation of 5'-methylthioinosine to hypoxanthine. In contrast to mammalian PNP, the 2'-hydroxyl group is crucial for inhibitor binding in the catalytic site of TgPNP. This first crystal structure of TgPNP describes the basis for discrimination against 5'-methylthioinosine and similarly 5'-hydroxyl-substituted immucillins; structural differences reflect the unique adaptations of purine salvage pathways of *Apicomplexa*.

*Toxoplasma gondii*, the etiologic agent of toxoplasmosis, is an opportunistic pathogen that is widespread among various warm-blooded animals, including domestic felines and humans (1). Approximately 1 billion people worldwide, including 22.5% of the population in the United States, are seropositive for *T. gondii* (2). Toxoplasmosis affects immunocompromised individuals, such as AIDS patients, organ transplant recipients, and the fetuses of newly infected mothers (2, 39–41). Although the incidence of congenital and AIDS-associated toxoplasmosis is low, recent waterborne outbreaks have shown that toxoplasmosis in immunocompetent individuals is more common than initially realized. Unfortunately, current therapeutic options for toxoplasmosis are limited. Antifolate drugs such as pyrimethamine are effective against the tachyzoite stage of *T. gondii* and can also affect the bradyzoite stage that causes chronic infection in the host. Lifelong maintenance with a combination of pyrimethamine-sulfadiazine for toxoplasmic encephalitis often leads to side effects, including severe allergic reactions and hematotoxicity (3). Alternative chemotherapeutic strategies are needed to prevent the onset of these adverse reactions (4, 5).

*Toxoplasma gondii* is a member of the phylum *Apicomplexa*, which also includes *Plasmodium*, the causative agent of malaria. Both parasites replicate rapidly and require large amounts of purines for the synthesis of their nucleic acids and other vital components. These obligate intracellular parasites cannot synthesize purines de novo and depend on purine salvage from the host.

*Toxoplasma gondii* nucleobase and nucleoside transporters have been identified and include TgNB1T, TgAT1, and TgAT2 (6–8). TgAT2 has an affinity for nucleosides, with a high affinity for adenosine (6, 9, 10). The rate of adenosine incorporation is higher than that of other purines, and adenosine kinase (AdK) has a high level of activity relative to other enzymes in the pathway, indicating that adenosine is the major purine utilized by *T. gondii* (9). In contrast, *Plasmodium falciparum* has no AdK activity (11), and no AdK gene has been identified in the *Plasmodium* genome (12). However, in the presence of excess adenosine, *P. falciparum* can use AMP synthesized by human erythrocyte AdK, which is followed by parasite uptake of AMP from the erythrocyte cytosol (11).

*Toxoplasma* can replicate normally in vitro using adenosine kinase or in the absence of adenosine kinase by using pathways that require hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) activity (13). *Toxoplasma gondii* organisms with a ΔAdK background are viable, but genetic ablation of AdK plus PNP inhibition kills the parasite (13). PNP converts inosine to hypoxanthine and guanosine to guanine. TgPNP, which is structurally similar to *P. falciparum* PNP (PfPNP), has a homohexameric structure, in contrast to homotrimeric mammalian PNP (14, 15). The structure and function of PfPNP is unique because of its acceptance of 5'-methylthioinosine (MTI), a specific metabolite of many *Plasmodium* species but one that is not present in the human host or in *T. gondii* (15, 16). The TgPNP amino acid sequence is 41% identical to that of PfPNP, and a previous study showed that MTI is a poor substrate for TgPNP (13).

Immmucillin-H (Imm-H) is a transition-state analogue inhibitor of PNP that causes purine starvation in cultured *P. falciparum* (17, 18) and inhibits both TgPNP and host cell PNP activity (13). To understand the structural basis of substrate and inhibitor recognition, TgPNP was co-crystallized with immucillin-H. The structure of TgPNP in complex with immucillin-H and phosphate

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revealed a reduced capacity to bind 5'-substituted nucleosides. Two representative 4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene) (DADMe)-immucillins (19) were also tested. These analogues have affinity similar to that of immucillin-H for PfPNP and a higher affinity for human PNP (Fig. 1). Surprisingly, these analogues show low affinity for TgPNP. Several PNP family transition-state analogues were used to explore catalytic site interactions of TgPNP (Fig. 1). The results indicate an early dissociative transition state for TgPNP.

FIG 1 Structure of transition-state analogues used in this study to reveal differences in the TgPNP transition state and substrate specificity. The dissociation constants for TgPNP (Tg), PfPNP (Pf), and HsPNP (Hs) were obtained using inosine as the substrate. Dissociation constant is defined as the tightest inhibition constant, either \( K_i \) or \( K_{i*} \). An asterisk in parentheses indicates that the \( K_{i*} \) value is shown.

MATERIALS AND METHODS
Reagents. Xanthine oxidase, inosine, ampicillin, isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG), and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). One Shot Top 10 chemically competent *Escherichia coli* cells, DNase I, Superscript III reverse transcriptase, Platinum Taq high-fidelity master mix, and PtrcHis 2 Topo vectors were pur-
chased from Invitrogen (Carlsbad, CA). BL21-codon plus (DE3)-RIPLE E. coli competent cells were purchased from Stratagene (Santa Clara, CA). RNasey minikits and nickel-nitriolitriacetic acid (Ni-NTA) agarose were purchased from Qiagen (Valencia, CA). Imm-H, 5'-o-Imm-H, 5'-fluoro-Imm-H (5'-F-Imm-H), 5'-COOH-Imm-H, 2'-o-Imm-H, DADMe-Imm-H, DADMe-Imm-G, 5'-methyl-Imm-H (5'-MT-Imm-H), 5'-CONH2-Imm-H, 5'-thio-Imm-H, and 1',9-Me-Imm-H were synthesized as described previously (15, 20, 21). Crystallography reagents and plates were purchased from Hampton Research (Aliso Viejo, CA).

CDNA synthesis and PCR analysis of TgPNP. Toxoplasma gondii RH tachyzoite cDNA was synthesized from total cellular RNA, which was prepared using chloroform-TRIzol (1:5, vol/vol). RNA was quantified using a Nanodrop spectrophotometer and then treated with DNase 1 (RNase-free) at 37°C for 15 min prior to cDNA synthesis. RNA was purified using a Qiagen RNeasy extraction kit according to the manufacturer’s protocol. Aliquots containing 3.5 μg of RNA were stored at −80°C until needed. First-strand cDNA was generated using Invitrogen SuperScript III reverse transcriptase and oligo(dT)20 as described by the manufacturer (22). PCR products from CDNA and genomic DNA (gDNA) were assessed on an agarose gel and analyzed via automated DNA sequencing (Albert Einstein College of Medicine DNA Sequencing Facility, Bronx, NY).

Development of TgPNP constructs. The coding region of TgPNP, without the stop codon, was amplified by PCR with high-fidelity Taq with the sense primer 5’-AGGGCATTGAAAGTCGACGTCGCTC-3’ and anti-sense primer 5’-GTACTGGCGACCGAGATCT-3’. The coding region was then cloned into the pTrcHis2-TOPO vector (Invitrogen) with a C-terminal hexahistidine tag and an ampicillin selection cassette. Each plasmid was transformed into E. coli strain BL21-codon plus (DE3)-RPL (Stratagene). The TgPNP clone used in this study contains a Val233Ile substitution not found in the TgPNP amino acid sequences reported earlier (13). This conservative substitution is remote from the catalytic site and is not expected to cause significant differences in enzymatic activity. It should be noted that the genes for strains GT1 TGGT1_307030 and ME49 TGME49_307030 found at the Toxoplasma Genomics Resource website, www.toxoDB.org, are incorrectly predicted to encode a 330-amino-acid protein, in contrast to the 247-amino-acid protein previously characterized (13) and predicted for the T. gondii VEG strain TGVEG_050700.

Expression and purification of TgPNP for kinetic studies. Fresh enzyme was expressed and purified before each experiment. The recombinant enzyme was expressed by inducing a 100-mL bacterial culture with 1 mM IPTG at 37°C for 18 h. Cells were ruptured by resuspension in Bug-Buster (Novagen, Darmstadt, Germany) according to the manufacturer’s instructions, and the cell debris was removed by centrifugation at 16,000 × g for 20 min at 4°C. Recombinant TgPNP was purified using Ni-NTA affinity chromatography spin column (Qiagen) according to the manufacturer’s instructions. Purified recombinant protein was buffer exchanged against 50 mM Na2HPO4·KH2PO4 (pH 5.0), 1 mM Dithiothreitol (DTT), and 50 mM NaCl. This buffer condition was selected after screening (described below). The enzyme concentration was determined from the extinction coefficient (24,005 M−1 cm−1) at 280 nm.

Buffer optimization for protein stability. Recombinant TgPNP was expressed and purified as described above. Immediately after purification, the recombinant protein was diluted 100-fold into 50 mM test buffer containing 50 mM NaCl and 1 mM DTT (see Table S1 in the supplemental material), and an aliquot from each condition was tested for PNP activity as described below. The remaining protein was incubated for 6 h at 4°C and then retested for PNP activity.

Enzymatic assays and inhibition studies. Recombinant proteins were used for enzymatic assays directly following purification. Kinetic assays were carried out in 50 mM KH2PO4 at pH 7.4, measuring phosphorylase of inosine by PNP in a coupled reaction with 60 μM/ml of xanthine oxidase to convert hypoxanthine to uric acid. Formation of uric acid was measured at a wavelength of 293 nm (ε293 = 12.9 mM−1 cm−1) (17, 18). Assays were performed with excess substrate in the presence of inhibitors. Inhibition assays for determining the equilibrium dissociation constant for the inhibitor (Ki) and for initial- and slow-onset inhibition constants (Ki*) were performed using various concentrations of Imm-H, 5’-o-Imm-H, 5’-F-Imm-H, 5’-COOH-Imm-H, 2’-o-Imm-H, DADMe-Imm-H, DADMe-Imm-G, 5’-MT-Imm-H, 5’-CONH2-Imm-H, 5’-thio-Imm-H, and 1’,9-Me-Imm-H, along with 500 μM inosine. Inhibition constants were determined as described previously (23). Initial-onset inhibition was analyzed using the following equation: \( v_0 = (k_{cat} \times [S]) / (K_i + [I]) + [S] \), where \( v_0 \) represents the steady-state rate, \( k_{cat} \) represents the catalytic rate, [S] represents the substrate concentration, \( K_i \) represents the Michaelis constant for inosine, and [I] represents the inhibitor concentration. Human and P. falciparium PNP were used as controls and were expressed and purified as described elsewhere (15, 16).

Protein crystallization and data collection. Bacterial cultures for expressing TgPNP were grown in Luria Bertani-ampicillin broth at 37°C to an optical density at 595 nm of 0.6, induced with IPTG at a final concentration of 1 mM, and grown at 25°C for an additional 18 h. Cells were harvested by centrifugation (4,000 × g for 30 min) and then ruptured by passage through a French press. The resulting cell debris was pelleted by centrifugation (16,000 × g for 30 min), and the remaining supernatant was purified over a 3-mL Ni-NTA affinity column (Qiagen) with elution by a step gradient of 50, 75, 100, 200, 300, and 500 mM imidazole in 50 mM HEPES (pH 8.0), 300 mM NaCl, and 1 mM DTT. The purified recombinant protein was dialyzed overnight against two different conditions: ammonium acetate buffer (50 mM ammonium acetate [pH 5.0], 50 mM NaCl, and 1 mM DTT) and phosphate buffer (25 mM Na2HPO4·KH2PO4 [pH 5.0], 50 mM NaCl, and 1 mM DTT). The final concentration of TgPNP for crystallization was 10 mg/mL in the presence of 3 mM Imm-H and 10 mM phosphate. The crystallization condition of 25% polyethylene glycol monomethyl ether 2000, 100 mM Tris (pH 8.5), and 0.2 M trimethylamine N-oxide dihydrate was determined using Hampton Research Index HT screening by sitting-drop vapor diffusion. Crystals were transferred into a fresh drop of the crystallization solution containing 25% glycerol and rapidly frozen in liquid nitrogen. X-ray diffraction data for Imm-H bound to TgPNP were collected using Beamline X29A at the Brookhaven National Laboratory. All data were processed using the HKL2000 program suite, and the data-processing statistics are provided in Table 1 (24).

Structure determination and refinement. The crystal structure of TgPNP bound to immucillin-H was determined by molecular replacement in MolRep using the published structure of PnPNP bound to immucillin-H (PDB code 1NW4) as the search model, followed by model building using Phenix (25, 26). The model without immucillin-H was first rebuilt using the crystallographic object-oriented toolkit (COOT) and refined in Refmac5 (27, 28). Immucillin-H was added last using an Fc-F map and refined in Refmac5 (28). The refinement statistics are summarized in Table 1.

PDB accession number. The coordinates and structure factors for Imm-H-bound TgPNP were deposited in the Protein Data Bank (PDB) under accession code 3MB8.

RESULTS

Buffer optimization for protein stability. Recombinant TgPNP enzyme activity was unstable, precluding previous attempts at crystallization. Buffer composition and pH were optimized to stabilize the enzyme activity (see Table S1 in the supplemental material). With inosine as the substrate and Na2HPO4·KH2PO4 buffer at pH 5 and pH 6 or ammonium acetate buffer at pH 7, PNP activity was retained (see Fig. S1).

Kinetic analysis of TgPNP. TgPNP accepts multiple substrates, including guanosine and inosine (13). Inosine is a purine salvage metabolite and was used to establish kinetic parameters. The catalytic efficiency (\( k_{cat}/K_m \)) with inosine was 5.5 × 104 for TgPNP and 1.5 × 105 for PnPNP, similar to the values reported previously for these enzymes (Table 2) (13, 15).
TABLE 1 Data collection and refinement statistics

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<sup>a</sup> Numbers in parentheses are for the highest-resolution shell. One crystal was used for each data set. The PDB code for Imm-H-bound TgPNP is 3MB8.

Transition-state analogue screening. Transition-state analogues that mimic the specific geometry and electrostatic features of a molecule can be used to predict the position of the transition state in the reaction coordinates (29). The dissociation constants of an array of transition-state analogue inhibitors were measured to explore the specificity of TgPNP. Immucillin-H bound TgPNP tightly, with a K<sub>i</sub> of 0.37 nM, similar to the K<sub>i</sub> of 0.86 nM for PNP (Fig. 1; also see Table S2 in the supplemental material) (13). DADMe-Imm-H and DADMe-Imm-G are expanded-spectrum immucillins and are powerful inhibitors of human PNP, with K<sub>i</sub>* values of 0.0085 and 0.007 nM, respectively. These analogues inhibited PNP with an affinity similar to that of Imm-H (Fig. 1; also see Table S2) (15). Surprisingly, these transition-state analogues bound poorly to TgPNP, with K<sub>i</sub> values of 3,600 nM for DADMe-Imm-H and 1,500 nM for DADMe-Imm-G. Both Imm-H (K<sub>i</sub> = 0.37 nM) and Imm-G (K<sub>i</sub>* = 1.9 nM) are powerful inhibitors of TgPNP (13). Imm-H and Imm-G also bound PNP tightly, with K<sub>i</sub>* values of 0.6 and 0.9 nM, respectively (15). DADMe-Imm-H and DADMe-Imm-G mimic a fully dissociated S<sub>i</sub>1 ribocation transition state.

High-affinity transition-state analogues for human PNP (HsPNP) were designed with a methylene bridge between the purine and ribocation group, and these analogues lacked the 2'-hydroxy group (Fig. 1) (30). The linear distance between the deazapurine ring and the C1’ of Imm-H was found to be 1.5 Å, characteristic of an early transition state, whereas the distance to the N1’ of DADMe-Imm-H was determined as 2.5 Å, which was characteristic of a late transition state (30). To evaluate if the presence of the methylene bridge is responsible for the loss of affinity, 1',9-Me-Imm-H was tested with TgPNP. No inhibition was observed at 50 μM with 1',9-Me-Imm-H, although 1',9-Me-Imm-H inhibited HsPNP with a K<sub>i</sub> of 250 nM. These results indicate an early dissociative transition state for TgPNP. In addition, 2'-D-Imm-H displayed a 522-fold decrease in affinity for TgPNP compared to Imm-H, indicating that the 2'-hydroxyl group is necessary for tight binding (Fig. 1; also see Table S2 in the supplemental material). This specificity suggests a physiological role for TgPNP in nucleoside salvage. TgPNP did not show slow-onset inhibition with any inhibitor.

5'-MT-Imm-H was specifically designed to examine the substrate specificity of PNP for MTI. MTI is a poor substrate for TgPNP; thus, 5'-methylthio-purines are unlikely to be formed during T. gondii metabolism (13). Consistent with this, 5'-MT-Imm-H was found to be a poor inhibitor of TgPNP, with a K<sub>i</sub> of 5,600 nM (Fig. 1; also see Table S2 in the supplemental material). The inhibitory activity of 5'-CONH<sub>2</sub>-Imm-H was similar to that of 5'-MT-Imm-H, with a K<sub>i</sub> of 8,900 nM, and no inhibition was observed with 5'-thio-Imm-H at 50 μM. Other 5'-modifications revealed a high selectivity for a hydroxyl group in the 5’ position (Fig. 1; also see Table S2).

Crystal structure of TgPNP-bound Imm-H. The hexameric quaternary structure was similar to that of the Apicomplexa phyllum member P. falciparum PNP and to E. coli PNP (Fig. 2A) but differed from that of mammalian trimeric family 1 PNP (15, 31). TgPNP cosrystalized in the presence of Imm-H showed a monomeric structure consisting of a core containing 11 β sheets (Fig. 2B). TgPNP consists of a trimer of dimers, with six catalytic sites formed by residues at the interface of the monomeric pairing (Fig. 2A). Each subunit primarily houses one inhibitor molecule, with residues His9 and Arg47 from the adjacent subunit binding to the 5'-hydroxyl of Imm-H and the phosphate ion, respectively (Fig. 3).

Clear electron density at 1.8 Å resolution was observed for the transition state analogue and phosphate in the active site of the enzyme. The deazahypoxanthine base was bound in a pocket formed by nonpolar residues Phe162, Pro210, Ile182, and Trp213 and by polar residue Asp207. The N1’ proton of the deazapurine hydrogen bonded with structurally conserved water at 2.8 Å, which was stabilized in a water lattice with another water molecule and the backbone oxygen of Tyr161 (Fig. 4). Asp207 was observed to interact with the N7 proton of Imm-H at 3.0 Å. It formed water-mediated hydrogen bonds with both O6 and O5’ of the
inhibitor and is favored to be the general acid/base for protonation of N7 with formation of the transition state (15, 32, 33).

Tyr218 formed a water-mediated interaction with the 5'-hydroxyl of the iminoribitol group (2.7 Å) and formed hydrogen bonds with the O2 of a glycerol molecule (2.6 Å) from the crystallization buffer in the active site. His9 NE2 from the adjacent subunit was within hydrogen bonding distance to the 5'-hydroxyl (2.7 Å). Glu185 was bound to both the 2'- and 3'-hydroxyls of the iminoribitol group. The phosphate ion was stabilized in an anionic pocket consisting of Arg90, Arg47b, Arg29, and the α-polar residue Thr93 beneath the inhibitor. The OG1 of Thr93 was within hydrogen bonding distance to the N1' of the ribitol group (3.2 Å). Ion pairing to the phosphate ion occurred with residues

FIG 2 (A) X-ray crystal structure of the TgPNP hexamer with immucillin-H and PO₄³⁻ bound in the active site. The hexamer structure was generated by applying crystallographic symmetry to the dimers composed of the light blue and dark blue subunits on the bottom left, yielding the purple, green, yellow, and orange monomers. (B) TgPNP monomer with immucillin-H and PO₄³⁻ bound in the active site (in yellow). The monomer consists of eight α helices in light blue and surrounds 11 β sheets in the red subunit. Loop regions are shaded in purple. The figure was prepared with MacPyMol.

FIG 3 Stereo views of the catalytic site contacts in TgPNP with the transition-state analogue inhibitor immucillin-H and PO₄³⁻. The structure shows light blue side chains of the parental monomer surrounding the bound Imm-H (yellow); the green subunits on the bottom right indicate residues contributed from the adjacent subunit. Residues participating in binding Imm-H and PO₄³⁻ are labeled in the active site of TgPNP. The figure was prepared with MacPyMol.

FIG 4 Schematic diagram of catalytic site contacts for immucillin-H and PO₄³⁻ at the active site of TgPNP. Amino acids are from the parent subunit unless labeled with a b, which marks residues from the adjacent subunit. Dashed lines indicate hydrogen bonding. Distances are shown in angstroms.
whereas surrounded by hydrophilic residues in a solvent-filled cavity, Tg PNP-bound Imm-H, the 5\'-hydroxyl was in a position that is code 1NW4). The two homologous enzymes shared 41% sequence (15).

hydrophobic cavity encompassed by Val66, Val73, and Tyr160 in the crystal structure of 1NW4. When 5\'=\text{-MT-Imm-H} was in the electronic state, a distance of 4.1 Å from C5 and 5.0 Å from O5' of Imm-H in the crystal structure of 1NW4. When 5\'-MT-Imm-H-bound PNP (PDB code 1IQG) was overlaid with Imm-H-bound PNP, Val66 and Val73 were found to be within Van der Waals distances of 2.5 and 1.8 Å from Ile68 and Ile75, creating a steric clash (Fig. 5B). The 5\'-hydroxyl and 5\'-methylthio groups of Imm-H and 5\'-MT-Imm-H were in different orientations. In TgPNP-bound Imm-H, the 5\'-hydroxyl was in a position that is surrounded by hydrophilic residues in a solvent-filled cavity, whereas PNP-bound 5\'-MT-Imm-H (1IQG) was observed to have the 5\'-methylthio group rotated 135° away, toward a closed hydrophobic cavity encompassed by Val66, Val73, and Tyr160 (15).

**DISCUSSION**

**Improvement of TgPNP stability after purification.** The stability of TgPNP was improved by buffer screening. The $k_{cat}/K_m$ was similar to that of earlier reports, but the stabilized protein had a lower $k_{cat}$ (Table 2) (13). Buffer screening enabled the synthesis of viable TgPNP crystals for crystallographic structural determination and inhibition studies.

**Transition-state analogue screening.** Transition-state structures can be predicted based upon affinity to related transition-state analogues. The transition-state characteristics are compared by analyzing dissociation constants ($K_i$) of an array of representative transition-state analogues (29). These inhibitors mimic known early or late dissociative transition states. Transition-state structures of N-ribosyl phosphorylases that metabolize nucleosides can be either early or late dissociative and are distinguished by the distance between the ribosyl anomic carbon and the N9 of the purine base-leaving group (23). The narrow-spectrum PNP transition-state analogue immucillin-H was designed from the transition state of bovine PNP, which revealed an early transition state with ribocation characteristics but a relatively close 1.8-Å distance between the leaving-group nitrogen and the anomic carbon (34). In contrast, HsPNP has a fully dissociated purine leaving group with a fully developed ribocation, with a distance greater than 2.6 Å between the leaving-group nitrogen and the anomic carbon (30). An expanded-spectrum HsPNP transition-state analogue inhibitor, DADMe-Imm-H, was designed to match this characteristic by the addition of a methylene bridge between the purine and the sugar moiety (19) (Fig. 1). TgPNP was strongly inhibited by immucillin-H, with a $K_i$ of 0.37 nM. Surprisingly, TgPNP was weakly inhibited by DADMe-Imm-H and DADMe-Imm-G, with $K_i$ values of 3,600 and 1,500 nM, respectively, indicating an early dissociative transition state for TgPNP. As shown in Fig. 1, no inhibition was observed with 1',9-Me-Imm-H at 50 μM, which also supports the formation of an early transition state. Despite the high degree of residue conservation found in the active sites of HsPNP and TgPNP, inhibitor specificity studies support distinct transition states. Moreover, different affinities of PNP and TgPNP for the analogues tested indicate divergence of the transition states among Apicomplexa PNP.

**TgPNP substrate specificity.** Consistent with the observation that TgPNP exhibits substrate specificity for inosine but not for MTI, TgPNP inhibition assays with immucillin-H and 5\'-MT-Imm-H yielded $K_i$ values of 0.37 and 5,600 nM, respectively. Because the 5\'-CONH$_2$ group is able to hydrogen bond with His9, 5\'-CONH$_2$-Imm-H was tested as an alternative transition-state analogue to the 5\'-methylthio group of 5\'-MT-Imm-H. However, 5\'-CONH$_2$-Imm-H showed weak binding, with a $K_i$ of 8,900 nM, suggesting that this 5\'-group is too bulky. For both 5\'-MT-Imm-H and 5\'-CONH$_2$-Imm-H, the weak binding reflects space constraints in the 5\'-group binding cavity. This restriction at the 5\' position is supported by the results of tests involving several distinct inhibitors with specific 5\' modifications (Fig. 1; also see Table S2 in the supplemental material). TgPNP exhibits a high degree of selectivity for a hydroxyl group in the 5\' position, as evidenced by results showing that all 5\' modifications resulted in a decrease in binding affinity compared with Imm-H (Imm-H < 5\'-d-Imm-H < 5\'-F-Imm-H < 5\'-COOH-Imm-H < 5\'-MT-Imm-H < 5\'-CONH$_2$-Imm-H < 5\'-thio-Imm-H).

**TgPNP crystal structure.** The residues in the active site of

**FIG 5** (A) Cross-eyed stereo-view superposition of TgPNP Imm-H-PO$_4^{3-}$ (purple; PDB code 3MB8) and PNP Imm-H-PO$_4^{3-}$ (light blue; PDB code 1NW4) to show differences in amino acids surrounding the inhibitor. (B) Cross-eyed stereo-view superposition of TgPNP Imm-H-PO$_4^{3-}$ (purple; PDB code 3MB8) and PNP 5\'-MT-Imm-H-PO$_4^{3-}$ (yellow; PDB code 1IQG) to show the relative angular shifting of residues in the active site when 5\'-MT-Imm-H is bound.

Arg90 NH1 and NH2 (3.1 to 2.8 Å), Arg47b NH1 and NH2 (2.9 to 3.0 Å), and Arg29, with distances of 2.7 Å for both NH1 and NH2.

Comparison of the active sites of TgPNP and PNP. In the structures depicted in Fig. 5, residues His9, Ile68, Ile75, Phe162, and Tyr218 are shown to illustrate the similarities found in the active sites of TgPNP to those of PNP bound to Imm-H (PDB code 1NW4). The two homologous enzymes shared 41% sequence identity, and their Cα backbone superimposed well, with root mean square deviations (RMSD) of 0.7 Å out of 1,208 atoms for 1NW4 and 0.7 Å out of 1,220 atoms for 1IQG. Even though the sequence identity was only 41%, with the exception of Ile68, Ile75, Phe162, and Thr93, there was a high degree of identity with respect to the active pocket residues between the two isozymes. The corresponding residues for PNP were Val66, Val73, Tyr160, and Ser91. The first three residues are proposed to play a key role in binding the 5\'-methylthio group in the pocket (15). A water molecule was found to be conserved between TgPNP-bound Imm-H and PNP-bound Imm-H (1NW4). Both Ile68 and Ile75 were found to carry an additional methyl group compared to Val66 and Val73. These bulky groups protrude an additional 1.5 Å into the active site, a distance of 4.1 Å from C5' and 5.0 Å from O5' of Imm-H in the crystal structure of 1NW4. When 5\'-MT-Imm-H-bound PNP (PDB code 1IQG) was overlaid with Imm-H-bound PNP, Val66 and Val73 were found to be within Van der Waals distances of 2.5 and 1.8 Å from Ile68 and Ile75, creating a steric clash (Fig. 5B). The 5\'-hydroxyl and 5\'-methylthio groups of Imm-H and 5\'-MT-Imm-H were in different orientations. In TgPNP-bound Imm-H, the 5\'-hydroxyl was in a position that is surrounded by hydrophilic residues in a solvent-filled cavity, whereas PNP-bound 5\'-MT-Imm-H (1IQG) was observed to have the 5\'-methylthio group rotated 135° away, toward a closed hydrophobic cavity encompassed by Val66, Val73, and Tyr160 (15).
Imm-H bound to =
of immucillin-H in -hydroxyl group
Tyr160 in makes conserved water-mediated contacts that substitute for Residue Tyr218, which is located within the Tg PNP active site, makes conserved water-mediated contacts that substitute for Tyr160 in PNP.

In the structure of PNP bound to 5'-MT-Imm-H, the 5'-methylthio group is turned 135° relative to the 5'-hydroxyl group of immucillin-H in Tg PNP Imm-H, abutting residues in a densely packed hydrophobic area in the catalytic site. The 5'-hydroxyl group of immucillin-H is surrounded by largely hydrophilic residues within a solvent-filled cavity in PNP. These findings agree with reported positions of both immucillin-H and 5'-MT-Imm-H bound to PNP (15).

T. gondii and polyamine salvage. Similar to other members of the phylum Apicomplexa, such as Plasmodium and Cryptosporidium, T. gondii is a purine auxothroph that relies on purine salvage for its metabolic requirements (35, 36). The difference in the specificities of Tg PNP and PNP relates to distinct metabolic functions within the respective parasites. Toxoplasma gondii lives in nucleated mammalian cells. As polyamines are abundant in these cells, it is believed that polyamine salvage replaces de novo polyamine synthesis (13–15). Under conditions of polyamine abundance, no 5'-methylthioadenosine, a by-product of polyamine synthesis, is formed in Toxoplasma. In contrast, P. falciparum must synthesize polyamines because erythrocytes are polyamine deficient. The resulting 5'-methylthioadenosine formed is metabolized by unique dual-specificity N-(2-acetamido)iminodiacetic acid and PNP enzymes in most Plasmodium species, hence the specificity for 5'-methylthioinosine in PNP.

Conclusions. Due to a lack of forward-directed polyamine biosynthetic enzymes, T. gondii is unable to generate 5'-methylthiopurines (37, 38). Examination of the structure of Tg PNP indicates that the residues in the active site cannot accommodate 5'-methylthioinosine, but the solvent-filled pocket surrounding the 5'-OH group of immucillin-H suggests that the active site is able to accept a larger 5' group. Inhibitor screening of 5' analogues revealed a high selectivity for a hydroxyl group in the 5' position. Any modification resulted in a decrease in binding affinity compared with binding to immucillin-H. Moreover, the results presented here show that Tg PNP is strongly inhibited by immucillin-H but weakly inhibited by DADMe-Imm-H and DADMe-Imm-G. In addition, no inhibition was detected with 1',9-Me-Imm-H, indicating an early dissociative transition state for this enzyme. This information reveals a surprising degree of catalytic divergence among Apicomplexa PNP.

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