Anopheles gambiae Purine Nucleoside Phosphorylase: Catalysis, Structure and Inhibition

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ABSTRACT: The purine salvage pathway of Anopheles gambiae, a mosquito that transmits malaria, has been identified in genome searches on the basis of sequence homology with characterized enzymes. Purine nucleoside phosphorylase (PNP) is a target for the development of therapeutic agents in humans and purine auxotrophs, including malarial parasites. The PNP from Anopheles gambiae (AgPNP) was expressed in Escherichia coli and compared to the PNPs from Homo sapiens (HsPNP) and Plasmodium falciparum (PfPNP). AgPNP has $k_{cat}$ values of 54 and 41 s$^{-1}$ for 2′-deoxyinosine and inosine, its preferred substrates, and 1.0 s$^{-1}$ for guanosine. However, the chemical step is fast for AgPNP at 226 s$^{-1}$ for guanosine in pre-steady-state studies. 5′-Deaza-1′-aza-2′-deoxy-1′-(9-methylene)-Immuclin-H (DADMe-ImmH) is a transition-state mimic for a 2′-deoxyinosine ribocation with a fully dissociated N-ribosidic bond and is a slow-onset, tight-binding inhibitor with a dissociation constant of 3.5 pM. This is the tightest-binding inhibitor known for any PNP, with a remarkable $K_{i}/k_{cat}$ of $5.4 \times 10^7$, and is consistent with enzymatic transition state predictions of enhanced transition-state analogue binding in enzymes with enhanced catalytic efficiency. Deoxyguanosine is a weaker substrate than deoxyinosine, and DADMe-Immuclin-G is less tightly bound than DADMe-ImmH, with a dissociation constant of 23 pM for AgPNP as compared to 7 pM for HsPNP. The crystal structure of AgPNP was determined in complex with DADMe-ImmH and phosphate to a resolution of 2.2 Å to reveal the differences in substrate and inhibitor specificity. The distance from the N1′ cation to the phosphate O4 anion is shorter in the AgPNP-DADMe-ImmH-PO$_4^-$ complex than in HsPNP-DADMe-ImmH-PO$_4^-$, offering one explanation for the stronger inhibitory effect of DADMe-ImmH for AgPNP.

Malaria is one of the oldest known human diseases, first described in the fifth century B.C. by Hippocrates (1). It was then, as it is now, a seasonal disease of the poor, being one of the principal causes of mortality in Africa, southeast Asia, and Latin America (2). Current World Health Organization figures estimate that malaria infection causes 300 million cases of acute illnesses and at least one million deaths annually, primarily of children under 5 years of age (2). In addition to the human toll, the economic costs have been estimated as 1% of world gross domestic product (GDP) per year (3). Attempts to break the cycle of malaria transmission include mosquito control, bed nets, antibiotics, and education, but the lack of consistent utilization of these methods has resulted in continued epidemics of malaria (4). Anti-malarial drugs such as chloroquine were initially successful, but drug resistance has developed and contributes to the current resurgence of the disease (5).

Malaria is caused by several species of Plasmodium that are transmitted by Anopheles mosquitoes when they take a human blood meal. Plasmodium falciparum is the most prevalent and most malignant of the malaria-causing pathogens. P. falciparum is a purine auxotroph, salvaging purines from its host for synthesis of RNA and DNA (6–8).

Inhibition of purine salvage with transition-state analogue inhibitors is lethal for cultured malaria (9). Metabolic pathways for purine salvage are substantially different in P. falciparum and humans. The adenosine deaminase and purine nucleoside phosphorylase (PNP) from the parasite accepts 5′-methylthionucleosides as favorable substrates, while the respective human enzymes do not (10). On the basis of the differences between PfPNP and HsPNP we investigated the PNP from a mosquito vector involved in malaria transmission.

Purine metabolism in mosquitoes is now complemented by the Anopheles gambiae (sensu stricto strains) PEST genome sequence (11), thus allowing comparison of parasite,
FIGURE 1: Proposed pathway for purine salvage in A. gambiae. On the basis of genomic sequence homology, the recycling of adenosine and MT-adenosine to AMP are likely to be similar for humans and the malarial mosquito vector.

vector, and human PNPs. Anophele genome analysis, in comparison to the previously completed insect genomes (12) and the genes from previously characterized purine salvage pathways, revealed mosquito orthologues of adenosine deaminase (ADA), adenosine kinase (AK), methylthioadenosine phosphorylase (MTAP), adenine phosphoribosyltransferase (APRT), and purine nucleoside phosphorylase (PNP) (Figure 1). The anopheline PNP (AgPNP) was kinetically characterized and found to be distinct from other PNPs. AgPNP was found to be trimetric by crystallographic analysis, similar to human and bovine PNPs. Transition-state analogue inhibitors of human and malarial PNPs were also found to be powerful inhibitors of AgPNP.

MATERIALS AND METHODS

Reagents and General Methods. HsPNP and PfPNP were prepared as previously reported (13). Adenosine, inosine, guanosine, 2′-deoxyadenosine, 2′-deoxyguanosine, potassium phosphate, xanthine oxidase, lysozyme, and EDTA were purchased from Sigma—Aldrich Chemical Co. Escherichia coli competent cells (DH5α and BL21-AI), Trizol, DNase I, Superscript III reverse transcriptase, Platinum PCR super master mix High Fidelity, LR-Clonase II, 1 cm2 plates, and pDEST-14 vectors were purchased from Invitrogen (Carlsbad, CA). Miniprep kits and Ni-NTA agarose beads were obtained from Qiagen (Valencia, CA). DNase I and protease inhibitor tablets were purchased from Roche Applied Science (Indianapolis, IN). Restriction enzymes were purchased from New England Biolabs (Ipswich).

All other reagents were of the highest quality commercially available. Protein concentrations were determined by UV absorbance, by use of the extinction coefficients 28 830, 17 930, and 18 400 M⁻¹ cm⁻¹ at 280 nm for HsPNP, PfPNP, and AgPNP, respectively (14). AgPNP has no Trp residues and its concentration was also estimated by the bicinchoninic acid (BCA) assay and Bradford assays. Inhibitor concentrations were determined spectrophotometrically by use of the published millimolar extinction coefficients of 8.5 at 275 nm, 9.54 at 261 nm, and 8.92 at 269 nm at pH 7 for 9-deazaguanosine (ImmA-based inhibitors), 9-deazainosine (ImmH-based inhibitors), and 9-deazaguanosine (ImmG-based inhibitors), respectively (15, 16). Substrate concentrations were determined spectrophotometrically by use of the extinction coefficients 12 200 M⁻¹ cm⁻¹ at 248.6 nm, 15 100 M⁻¹ cm⁻¹ at 260 nm, and 13 700 M⁻¹ cm⁻¹ at 252.5 nm for substrates containing hypoxanthine, adenine, and guanine, respectively. Escherichia coli cells DH5α and BL21-AI cells were grown in Luria broth (LB) with 100 µg/mL ampicillin (AMP), at 37 °C.

In Silico Purine Salvage Pathway Identification. The protein sequences Mus musculus ADA, Homo sapiens AK, Saccharomyces cerevisiae APRT, H. sapiens MTAP, and H. sapiens PNP (HsPNP) were used as query sequences in a tBLASTn search of the Anopheles gambiae genome through the NCBI web site (default settings). This database search identified hypothetical orthologues of each of these proteins with 20%, 51%, 39%, 73%, and 54% identity, respectively. Sequences were analyzed for the presence of introns, and translations of the exon regions were used for further comparisons.

RNA Isolation, cDNA Synthesis, and PCR Analysis. Genomic DNA from A. gambiae G3 was obtained from MR4. Total RNA was prepared directly from 7–10 frozen adults of A. gambiae G3 generation F5 (MRA-123B, MR4) in 2 mL of Trizol, and RNA was extracted according to the manufacturer’s instructions. Subsequent cDNA synthesis was performed with 1 µg of total RNA. All RNAs were treated with DNase I (RNase-free) before cDNA synthesis, according to the manufacturer’s instructions. First strand cDNA was then synthesized by use of Superscript III reverse transcriptase and a gene-specific primer mix with 2 pmol of each primer. Gene-specific oligonucleotide primers for A. gambiae PNP gene and S7 (ribosomal protein gene), used as a control, are described in the Supporting Information. PCR reactions were performed with Platinum PCR super master mix High Fidelity as described by the manufacturer. PCR products from cDNA and gDNA of each gene were sequenced after gel purification.

Anopheles gambiae Purine Nucleoside Phosphorylase Gene Construction. A chemically synthesized gene encoding AgPNP was generated, cloned into a pDONR221 vector, and sequenced by DNA 2.0 (Menlo Park, CA). The gene was synthesized by use of optimized codons for E. coli protein expression and the addition of a 20-residue N-terminal thrombin-cleavable (histidine)x tag, (MetGlySerSerHisHisHisHisSerSerHisGluLeuValProArgGlySerHis).

Protein Expression and Purification. The designed gene was transferred to the pDEST-14 vector with LR Clonase II. The pDEST-14-AgPNP construct was transformed into DH5α competent cells, plated onto LB-Amp, and subse-
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quenty grown overnight in 3 mL of LB-Amp at 37 °C. The overnight growth was pelleted and the plasmid was isolated by use of a miniprep kit. Presence of the desired gene was confirmed by separate digestion with AvaI and BamHI. The vector was then retransformed into BL-21-AI cells. The cells were grown in LB-Amp at 37 °C to an OD_{600} = 0.6, induced with L-arabinose (0.2% final concentration), and then returned to 37 °C to continue growing overnight. Cells were harvested by centrifugation at 4 °C and frozen until purification.

The bacterial pellet was suspended in binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 500 mM NaCl, and 5 mM DTT) with a spatula tip of both DNase I and lysozyme, and protease inhibitor (one tablet per 2 g of cells). The cells were ruptured by passage through a French press, and the cell debris was pelleted by centrifugation at 26000g for 30 min at room temperature. The remaining supernatant was incubated with 2.5 mL of Ni-NTA Agarose beads per 1 g of cell pellet for 30 min with rocking at room temperature. After incubation, the column was poured and washed with two column volumes of binding buffer. Two-column-volume stepwise fractions were taken at 100 mM imidazole and 1 M imidazole in binding buffer with AgPNP eluting in the 1 M fraction. Each fraction was eluted into a tube containing glycrol and EDTA to give final concentrations of 10% (v/v) and 1 µM, respectively. The 1 M fraction was dialyzed against 50 mM Tris-HCl pH 7.9, 10% (v/v) glycrol, 5 mM DTT, and 1 µM EDTA at room temperature. The enzyme was precipitated with 4 M (NH₄)₂SO₄, and stored at 4 °C.

The protein was equally active with or without the (histidine)₆ tag, which could be removed by combining 1 unit of thrombin (GE Healthcare, Piscataway, NJ) for every 1 mg of protein in 1× PBS at 22 °C and incubating the mixture for 1 h, followed by purification over an 8 mL Source 15Q column with elution by a 0–1 M NaCl gradient in the dialysis buffer.

PNP Steady-State Assay. The phosphorolytic activity of AgPNP was determined in assay mixtures containing variable substrate concentrations (1 µM–1 mM) and 50 mM potassium phosphate, pH 7.4, at 25 °C. Inosine and adenosine activities were each measured in the xanthine oxidase-coupled spectrophotometric assays, where the conversion of hypoxanthine to uric acid was monitored at 293 nm (ε₂₉₃ = 12.9 mM⁻¹ cm⁻¹) with 60 milliunits of xanthine oxidase or adenine conversion to 2,8-dihydroxyadenine at 301 nm (ε₂₉₃ = 15.2 mM⁻¹ cm⁻¹) in a coupled assay with 600 milliunits of xanthine oxidase. Guanosine phosphorylase activity was measured spectrophotometrically by following the decrease in absorbance at 257 nm by converting guanosine to guanine (ε₂₅₇ = −5.07 mM⁻¹ cm⁻¹). All reactions were initiated by the addition of enzyme, typically at 2 mM final concentration.

PNP Pre-Steady-State Assay. Enzyme was pretreated to remove hypoxanthine that copurifies with the enzyme. Two cycles of incubating for 1 min with charcoal (10% w/v) in 100 mM potassium phosphate, pH 7.4, at 25 °C were followed by centrifugation to remove tightly bound hypoxanthine. The temperature dependence of single-turnover catalysis of AgPNP with guanosine as a substrate was determined in stopped-flow fluorescence experiment. The fluorescence increase caused by enzyme-bound guanine (AgPNP guanine-ribose-1-PO₄) was monitored at 340 nm by excitation at 295 nm, after ~6 µM of AgPNP in 100 mM KH₂PO₄, pH 7.4, was mixed with saturated guanosine (~3 mM) in the same buffer. The temperature was varied from 5 to 30 °C and at least six runs were averaged at each temperature. The pre-steady-state phosphorolytic activity of AgPNP was determined in assay mixtures containing variable substrate concentrations (0.1–0.4 mM) and 50 mM potassium phosphate, pH 7.4, at 25 °C. The enzyme was then diluted to 4 µM and a final potassium phosphate concentration of 50 mM. Bound guanine (but not free guanine or free or bound guanosine) gives a unique fluorescence signal that can be monitored by emission at 340 nm after excitation at 290 nm; it corresponds to the formation of AgPNP-guanine- (ribose-1-PO₄) after mixing of AgPNP-PO₄ and potassium phosphate (50 mM, pH 7.4) buffered guanosine. All values represent an average of four repeated experiments.

Slow-Onset Enzyme Inhibition Assay. Slow onset of inhibition was measured following the addition of enzyme (0.1 nM) to complete assay mixtures containing 5 mM inosine, 60 milliunits of xanthine oxidase, 50 mM potassium phosphate, pH 7.4, and inhibitor concentrations ranging from 10 µM to 2 nM, at 25 °C (17). The change in absorbance at 293 nm was monitored for 1–2 h to determine both the initial reaction rate and to determine whether a second reaction rate occurred as a result of slow-onset inhibition. The Kᵢ values were determined by fitting the initial rate and inhibitor concentrations to the following expression of competitive inhibition (18, 19):

\[
\left( \frac{V'}{V_0} \right) = (K_m + [S]) / \{K_m + [S] + (K_m[I]/K_i) \}
\]

where \(V_0\) is the initial rate in the presence of inhibitor and \(V_0\) is the initial rate in the absence of inhibitor, \(I\) is the inhibitor concentration, and \([S]\) is the substrate concentration. This expression is valid only under the condition where the inhibitor concentration is 10 times greater than the enzyme concentration. In conditions where inhibitor concentration does not exceed 10 times the enzyme concentration, the effective inhibitor concentration was obtained by

\[
I' = I - (1 - V'/V_0)E_i
\]

where \(I'\) is the effective inhibitor concentration, \(V'/V_0\) and \(V_0\) are the initial rates in the presence and absence of inhibitor, and \(E_i\) is the total enzyme concentration.

In cases where slow onset inhibition was observed, where the inhibitor reached a tighter binding thermodynamic equilibrium with the enzyme, the equilibrium dissociation constant (\(K_i^*\)) was obtained by fitting the final rates to the following equation for competitive inhibition:

\[
\left( \frac{V'/V_0} \right) = (K_m + [S]) / \{K_m + [S] + (K_m[I]/K_i^*) \}
\]

with \([I]\) concentrations being corrected as above (16). The \(K_m\) values for the enzymes acting on inosine are 254 M for the mosquito enzyme, 5 M for the malarial enzyme, and 40 µM for the human enzyme.

Crystallization of AgPNP-DADMe-ImmH Complex. In order to obtain high-quality crystals of AgPNP, the above purification procedure was followed except that the inhibitor was added to the cells prior to lysis and purification. The affinity-purified, His-tagged AgPNP-DADMe-ImmH complex was concentrated to 50 mg/mL and was stable at room temperature for weeks, whereas the protein purified in the
absence of inhibitor was less stable. The purified AgPNP-DADMe-ImmH complex was diluted to 30 mg/mL, and 1 mM KH2PO4 was added before crystallization trials. The AgPNP-DADMe-ImmH-P04 complex was crystallized via sitting drops that were equilibrated by vapor diffusion at 18 °C against an 80 μL reservoir containing 0.1 M sodium cacodylate trihydrate, pH 6.5, and 1.4 M sodium acetate trihydrate, where 1 μL of the protein solution was mixed with 1 μL of the reservoir solution.

Data Collection. Crystals were soaked in mother liquor supplemented with 20% glycerol and flash-cooled to −178 °C prior to data collection. Diffraction from the AgPNP-DADMe-ImmH-P04 crystals is consistent with the space group P41212 (a = b = 106.7 Å and c = 240.4 Å) with three molecules in the asymmetric unit arranged in the range of 1 detector. Each frame was exposed for 2 s with an oscillation at the National Synchrotron Light Source, Brookhaven Da, corresponding to a solvent content of 56%. Diffraction group AgPNP with 1 trihydrate, where 1 cacodylate trihydrate, pH 6.5, and 1.4 M sodium acetate supplemented with 20% glycerol and flash-cooled to 293 K for molecular modeling. Difference Fourier maps calculated for Fobs − Fcalc coefficient produced clear density at 5σ that readily allowed the inhibitor to be added to the model. Density for the 86 N-terminal residues was disordered and could not be built into the final model of the AgPNP-DADMe-ImmH-P04 complex. Water molecules were included in the structure following the refinement of protein and ligands. The final structure had R-factor and Rfree of 17.5% and 21.7%, respectively. All structural figures were made with PYMOL (27).

RESULTS AND DISCUSSION

Anopheles gambiae Purine Salvage Pathway. Reports of mosquito growth on a semisynthetic medium suggested dietary requirements for AMP (or IMP), dTMP, and UMP (or CMP) or the corresponding nucleosides (28). Requirement for these purine metabolites suggested that purine and pyrimidine salvage pathways may play an important role in Anopheles nucleotide metabolism. Anopheline orthologues of previously characterized ADA, AK, MTAP, APRT, and PNP proteins were identified from the genome. The presence of genes corresponding to all five of these enzymes suggests that the purine salvage metabolic pathway resembles that found in humans (Figure 1). Alignments of these translated sequences show conservation of catalytic residues and allow assignment of consensus sequences and comparison between species (Figure 2). The AgPNP sequence is approximately 50% identical to human, bovine, and Drosophila melanogaster PNP sequences (54%, 55%, and 50% respectively).

General Characteristics of AgPNP. The presence of the PNP gene and its transcripts in A. gambiae were confirmed by PCR of A. gambiae genomic DNA and cDNA with a single set of primers (Supporting Information). RT-PCR analysis of the mosquito total RNA reveals that at least two splice variants of AgPNP are transcribed, a shorter variant (without the 68 N-terminal residues) and a longer variant (with the 68 N-terminal residues). The sequence corresponding to the longer variant of AgPNP protein was used to design a synthetic gene with an N-terminal histidine tag optimized for E. coli codon usage. This protein was highly overexpressed, yielding almost 80 mg of AgPNP/L of cultured cells after purification to >95% purity. Purification at room temperature and storage as an ammonium sulfate precipitate provided stable enzyme.

AgPNP Kinetic Parameters. The catalytic efficiencies (kcat/Km) of AgPNP were 7.0 × 104 M−1 s−1 for Ino and 2.8 × 105 M−1 s−1 for dIno (Table 2). Steady-state turnover numbers for inosine (Ino) and 2′-deoxyinosine (dIno) were 41- and 10-fold greater than for guanosine (Guo) and 2′-deoxyguanosine (dGuo), with the deoxy substrates having slightly greater kcat values than their ribo analogues (Table 2). No activity was detected for adenosine or 2′-deoxyadenosine, similar to the specificity for HsPNP. AgPNP’s strong preference for Ino and dIno over other purines is unique among both trimeric and hexameric PNPs (Table 3) (17, 29–32). The AgPNP kcat for inosine of 41 s−1 is similar to other trimeric PNPs, including those from H. sapiens (33) and Bos taurus (30), but greater than that for P. falciparum (17), which are reported to be 56, 16, and 0.34 s−1, respectively (Table 4). Transition-state theory proposes that the greater the enzymatic rate enhancement for a given reaction, the greater the binding potential for a transition-state analogue (34). The kcat value provides only an apparent
value for enzymatic rate acceleration, since other PNPs have $k_{\text{cat}}$ values limited by the rate of purine product release. The rates for chemical steps on mammalian PNPs have been reported to be 2-10-fold greater than $k_{\text{cat}}$ (35, 36). For AgPNP, the phosphorolysis of guanosine in a single-turnover on-enzyme reaction at 25°C is 226-fold faster than $k_{\text{cat}}$ in pre-steady-state studies (Table 2). Thus, the catalytic potential for AgPNP is greater than in mammalian enzymes, and transition-state analogue inhibitors might be expected to bind more tightly to AgPNP than to other PNPs due to the greater enzymatic rate enhancement.

**Inhibition of AgPNP.** The inhibition constants of AgPNP were determined for the Immucillins, known to be transition-state analogue inhibitors of HsPNP and PfPNP (Figure 3, Table 5). Unlike other PNPs, only Immucillin-H (1), DADMe-Immucillin-H (DADMe-ImmH; 5), DADMe-Immucillin-G (6), MT-DADMe-Immucillin-H (9), and DADEt-Immucillin-H (10) exhibited slow-onset inhibition. DADMe-ImmH is the most potent inhibitor of HsPNP and was also the best inhibitor of AgPNP, having a $K_i$ of 310 pM and a $k_{\text{cat}} / K_m$ of 109 M$^{-1}$ s$^{-1}$.

**Table 2: Kinetic Constants for AgPNP with Various Substrates**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{chem}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}} / K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>inosine</td>
<td>41 ± 2</td>
<td>585 ± 68</td>
<td>7.0 × 10^4</td>
<td></td>
</tr>
<tr>
<td>guanosine</td>
<td>226 ± 30</td>
<td>1.0 ± 0.2</td>
<td>187 ± 58</td>
<td>5.0 × 10^3</td>
</tr>
<tr>
<td>adenosine</td>
<td>&lt; 0.5</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>2-deoxyinosine</td>
<td>54 ± 2</td>
<td>190 ± 8</td>
<td>2.8 × 10^3</td>
<td></td>
</tr>
<tr>
<td>2-deoxyguanosine</td>
<td>54 ± 0.1</td>
<td>50 ± 11</td>
<td>1.1 × 10^3</td>
<td></td>
</tr>
<tr>
<td>2-deoxyadenosine</td>
<td>&lt; 0.5</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

$^a$nd indicates that the value was not determined. $^b$Reaction rate at 25°C. Pre-steady-state turnover was also measured at other temperatures and gave a turnover of 438 ± 80 at 30°C.

**Table 3: Comparison of Substrate Utilization of Various PNPs**

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Multimeric State</th>
<th>Guo</th>
<th>Ino</th>
<th>Ado</th>
<th>dGuo</th>
<th>dIno</th>
<th>dAdo</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. gambiae</td>
<td>trimer</td>
<td>2</td>
<td>100</td>
<td>0</td>
<td>13</td>
<td>123</td>
<td>0</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>(31, 32)</td>
<td>52</td>
<td>100</td>
<td>0</td>
<td>42</td>
<td>53</td>
<td>nd</td>
</tr>
<tr>
<td>B. taurus</td>
<td>(30)</td>
<td>50</td>
<td>100</td>
<td>0</td>
<td>38</td>
<td>71</td>
<td>nd</td>
</tr>
<tr>
<td>T. vaginalis</td>
<td>(29)</td>
<td>23</td>
<td>100</td>
<td>35</td>
<td>nd</td>
<td>nd</td>
<td>17</td>
</tr>
<tr>
<td>E. coli (32)</td>
<td>hexamer</td>
<td>104</td>
<td>100</td>
<td>133</td>
<td>161</td>
<td>217</td>
<td>133</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>(17)</td>
<td>41</td>
<td>100</td>
<td>0</td>
<td>59</td>
<td>188</td>
<td>nd</td>
</tr>
</tbody>
</table>

$^a$Values are given as percent of $V_{\text{max}}$ relative to inosine. $^b$nd indicates that the value has not been reported in the literature.

**Table 4: Kinetic Constants for Inosine with Various PNPs**

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}} / K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. gambiae</td>
<td>41</td>
<td>585</td>
<td>7.0 × 10^4</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>56</td>
<td>40</td>
<td>1.4 × 10^4</td>
</tr>
<tr>
<td>B. taurus</td>
<td>16</td>
<td>36</td>
<td>4.4 × 10^4</td>
</tr>
<tr>
<td>P. falciparum</td>
<td>0.34</td>
<td>5</td>
<td>6.8 × 10^4</td>
</tr>
</tbody>
</table>

$^a$Values are given as percent of $V_{\text{max}}$ relative to inosine. $^b$nd indicates that the value has not been reported in the literature.
Kᵢ* of 3.5 pM. This is the tightest binding observed for any PNP inhibitor, with 2.4- and 140-fold increased affinity compared to HsPNP and PfPNP, respectively. Compounds 19, 17, and 18 are powerful inhibitors of HsPNP (37) but bind 19-, 4.5-, and 51-fold less tightly to AgPNP than to the human enzyme. Only four additional inhibitors were found to bind more tightly to HsPNP than the mosquito enzyme: Immucillin-H (1), Immucillin-G (2), DADMe-Immucillin-G (6), and 5′-nor-DADMe-Immucillin-H (12), with 7.8-, 6.4-, 3.3-, and 5.1-fold differences, respectively. MT-Immucillin-H (4), which has a Kᵢ of 225 nM for AgPNP, is the only inhibitor that binds more tightly to PfPNP than AgPNP, a trend that is also true for the comparison of PfPNP and HsPNP. AgPNP bound to the remaining inhibitors with higher affinity than to HsPNP or PfPNP, which is consistent with their catalytic properties.

The tight binding of AgPNP to inhibitors without slow-onset kinetics indicates a rapid protein rearrangement to effect tight binding, whereas other PNPs require more time for this step and therefore exhibit slow-onset tight binding. Higher affinity for DADMe-ImmH (5) relative to Immucillin-H (1) suggests that AgPNP has a late, highly dissociative transition state (38).

Crystal Structure of AgPNP-DADMe-ImmH·PO₄ The structure of AgPNP-DADMe-ImmH·PO₄ is a trimer with three monomers in the asymmetric unit. The catalytic sites are formed by residues coming from a pair of monomers near the subunit interfaces. Each monomer of AgPNP has a β-sheet core flanked by seven α-helices. The monomers in the trimer of AgPNP superimpose with an rms deviation of 0.13–0.15 Å comparing Cα positions of 280 amino acid residues. The structure of AgPNP is most similar to bovine
Table 5: Inhibition Comparison of AgPNP versus HsPNP and PfPNP

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>AgPNP</th>
<th>HsPNP</th>
<th>PfPNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissociation constant (nM)</td>
<td>vs HsPNP</td>
<td>vs PfPNP</td>
<td></td>
</tr>
<tr>
<td>Immucillin-H (1)</td>
<td>0.47 ± 0.09</td>
<td>0.06 ± 0.02</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>Immucillin-G (2)</td>
<td>0.27 ± 0.07</td>
<td>0.042 ± 0.006</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>9-deaza-9-(pyrrolidin-2-yl)hypoxanthine (3)</td>
<td>110 ± 10</td>
<td>840 ± 110</td>
<td>273 ± 94</td>
</tr>
<tr>
<td>MT-Immucillin-H (4)</td>
<td>230 ± 60</td>
<td>300 ± 80</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>DADMe-Immucillin-H (5)</td>
<td>0.0035 ± 0.0006</td>
<td>0.0085 ± 0.0002</td>
<td>0.5 ± 0.04</td>
</tr>
<tr>
<td>DADMe-Immucillin-G (6)</td>
<td>0.023 ± 0.009</td>
<td>0.007 ± 0.0001</td>
<td>0.89 ± 0.06</td>
</tr>
<tr>
<td>DADMe-Immucillin-A (7)</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>9-deaza-9-(pyrrolidin-1-ylmethyl)hypoxanthine (8)</td>
<td>4 ± 1.7</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>MT-DADMe-Immucillin-H (9)</td>
<td>0.044 ± 0.004</td>
<td>0.070 ± 0.0009</td>
<td>0.89 ± 0.12</td>
</tr>
<tr>
<td>DADEt-Immucillin-H (10)</td>
<td>0.42 ± 0.23</td>
<td>0.46 ± 0.05</td>
<td>610 ± 150</td>
</tr>
<tr>
<td>3′-epi-DADMe-Immucillin-H (11)</td>
<td>3.1 ± 0.4</td>
<td>4.0 ± 0.4</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>5′-nor-DADMe-Immucillin-H (12)</td>
<td>4.0 ± 0.7</td>
<td>0.79 ± 0.34</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>5′-(R)-methyl-DADMe-Immucillin-H (13)</td>
<td>10 ± 7</td>
<td>34 ± 7</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>5′-(S)-methyl-DADMe-Immucillin-H (14)</td>
<td>2.2 ± 0.7</td>
<td>4.0 ± 0.6</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>5′-deoxy-5′-nor-DADMe-Immucillin-H (15)</td>
<td>0.37 ± 0.13</td>
<td>0.38 ± 0.006</td>
<td>1.16 ± 0.05</td>
</tr>
<tr>
<td>3′-epi-5′-deoxy-5′-nor-DADMe-Immucillin-H (16)</td>
<td>1.1 ± 0.4</td>
<td>1.8 ± 0.2</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td>9-deaza-9-[(1,3-dihydroxypropan-2-ylamino)-methyl]hypoxanthine (17)</td>
<td>4.5 ± 2.0</td>
<td>0.62 ± 0.17</td>
<td>160 ± 80</td>
</tr>
<tr>
<td>9-deaza-9-[(1,3,4-trihydroxybutan-2-ylamino)methyl]hypoxanthine (18)</td>
<td>0.94 ± 0.16</td>
<td>0.21 ± 0.08</td>
<td>300 ± 100</td>
</tr>
<tr>
<td>9-deaza-9-[(2R,3S)-1,3,4-trihydroxybutan-2-ylamino]methyl]hypoxanthine (19)</td>
<td>0.17 ± 0.02</td>
<td>0.0086 ± 0.0006</td>
<td>55 ± 12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Dissociation constant is defined as the tightest inhibition constant, either K<sub>d</sub> or K<sub>i</sub>*. <sup>b</sup> Affinity comparisons indicate that the inhibitor binds more tightly to the mosquito enzyme except as noted. <sup>c</sup>This inhibitor binds more tightly to the human enzyme. <sup>d</sup>This inhibitor binds more tightly to the plasmodium enzyme.

PNP (PDB code 1b80), human PNP (PDB code 1RSZ), human MTAP (PDB code 1C80), and E. coli MTAN (PDB code IJYS), with rms deviations of 0.9, 0.9, 2.5, and 2.6 Å, respectively, upon comparing Cα positions of 278, 279, 229 and 226 residues. The extended N-terminal region of 86 residues could not be built into the crystal structure due to lack of electron density. The lack of density might be due to disorder or proteolytic activity in the crystallization drop during crystallization. We know from gel analysis (results not shown) that the full-length protein was used for crystalization experiments. Analysis of mRNA isolated from mosquitoes demonstrated the transcript for this form of protein (Supporting Information).

The electron density of AgPNP-DADMe-ImmH·PO₄ shows a structurally conserved water near C2’ of bound DADMe-ImmH in human PNP, replacing the 2’-hydroxyl group found in nucleosides (PDB code 1RSZ). This water is missing in the AgPNP structure and may explain the lower K<sub>m</sub> values for ribonucleosides as substrates. However, the tighter binding of DADMe-ImH to AgPNP than to HsPNP is consistent with the close contacts to DADMe-ImH seen in the structures of AgPNP-DADMe-ImH·PO₄ compared to HsPNP-DADMe-ImH·SO₄. The number of close contacts, with cutoff values of 4.0 and 4.5 Å (by use of the program NCONT in the CCP4 program suite; 24), between DADMe-ImH and AgPNP in the complex are 25% and 15%, respectively, greater than for the same inhibitor bound to HsPNP. The ion pair between the N1’ cation of the inhibitor and the phosphate anion is also slightly shorter in the AgPNP-DADMe-ImH·PO₄ structure, 2.7 Å, than the distance of 3.0 Å in the HsPNP-DADMe-ImH·SO₄ structure. The presence of a sulfate instead of a phosphate ion in the HsPNP-DADMe-ImH·SO₄ structure does not change the interactions with the protein or the inhibitor. Similar results have been seen in other experiments (unpublished) where the structure of HsPNP has been solved with both SO₄ (PDB code 1RT9) and PO₄ (PDB code 1RR6) together with Immucillin-H, and these structures show no difference in coordination at the active site.

The Thr306 residues in all subunits of AgPNP fall into the disallowed region of the Ramachandran plot, also seen in the structure of human PNP where this residue corresponds to Thr221. The side-chain hydroxyl group of Thr306 forms hydrogen bonds to the side-chain amino group of Asn200 as well as to the hydroxyl group of Ser305, which coordinates the phosphate group in the active site. The same hydrogen-bond pattern is seen in HsPNP.

Active site of AgPNP. AgPNP-DADMe-ImmH·PO₄ has the catalytic sites located at the interface between a pair of monomers. Full catalytic site occupancy is suggested by the electron density of inhibitor and phosphate in the A, B, and C subunits. While the average B-factors for the A, B, and C chains are 24, 27, and 22 Å<sup>2</sup>, respectively, the bound ligands have B-factors below 20 Å<sup>2</sup>, consistent with full catalytic site occupancy. The distances of hydrogen bonds described in the text are averaged values for the A, B, and C catalytic sites. Cruickshank DPI coordinate error (39) was 0.17 for the structure complex and rms deviation between subunits was 0.13 to 0.15 Å; therefore, a 0.3 Å cutoff was applied for significant distance differences of interaction.

The active site can be divided into purine base, sugar, and phosphate binding sites. Deaza-hypoxanthine is bound in a hydrophobic region formed by Phe285, Ile345, Met304, and Ile302, with Phe285 and Ile302 in hydrophobic stacking interactions. The Thr306 residues in all subunits of AgPNP fall into the disallowed region of the Ramachandran plot, also seen in the structure of human PNP where this residue corresponds to Thr221. The side-chain hydroxyl group of Thr306 forms hydrogen bonds to the side-chain amino group of Asn200 as well as to the hydroxyl group of Ser305, which coordinates the phosphate group in the active site. The same hydrogen-bond pattern is seen in HsPNP.
FIGURE 4: X-ray crystal structure of AgPNP with DADMe-ImmH and PO₄ bound in the active site (deposited in the Protein Data Bank under ID code 2P4S). (a) Overall structure of AgPNP in complex with DADMe-ImmH; α-helices are shown in cyan and β-strands in yellow. (b) Stereoview of the active site of AgPNP with bound DADMe-ImmH and PO₄, with electron density shown as a $F_o - F_c$ map contoured at 3σ. (c) Schematic drawing of the active-site residues in AgPNP-DADMe-ImmH-PO₄ and HsPNP-DADMe-ImmH-SO₄. (d) Stereoview superposition of HsPNP-DADMe-ImmH-SO₄ (gray) and AgPNP-DADMe-ImmH-PO₄ (cyan) to show the difference in amino acids surrounding the C2' position of the 9-deazahypoxanthine group.
tions in mammalian PNPs, and its Asn to Asp mutation creates a nearly inactive PNP with altered base specificity (33). The close interaction of Asn328 and the base suggests transition-state stabilization by Asn328 through a H-bond with the N7 proton of the deaza hypoxanthine leaving group. Proton stabilization by the carbonyl oxygen of Asn328 to form the transition state requires proton donation from solvent water prior to reaching the transition state (40).

The 3′,4′-face of the ribosyl mimic is covered by Phe244* (the asterisk indicates a contact from the adjacent subunit), which may function to occlude solvent water from the catalytic site (Figure 4b). His342 is near the 5′-hydroxyl and Tyr173 is in a H-bond interaction with the 3′-hydroxyl (2.7 Å). The 5′-OH group of the DADMe-ImmH sugar forms a hydrogen bond to ND1 of His342 (2.8 Å), and the O3′ of the sugar forms a hydrogen bond with O4 of the phosphate (2.8 Å). The N1′ of the ribose forms a 2.7 Å ion pair with O4 of the phosphate group, reducing the overall charge in the active site.

The anionic phosphate binding site is formed by Arg169, His171, and H-bond donors from the amino groups of Ser118 and Ala201. The N1′ cation of DADMe-ImmH, together with Arg169 and the dianionic phosphate, form two ion pairs with a net zero charge. The PO4 moiety also hydrogen-bonds with the hydroxyl groups of Ser305 and Ser118. Phosphate O1 accepts hydrogen bonds from the amino groups of Ala201 (2.8 Å) and Ser118 (2.9 Å). The phosphate O2 interacts with the NE2 of His171 and the NE of Arg169, with distances of 2.7 and 3.1 Å, respectively. Phosphate O3 hydrogen-bonds to OG of Ser305 (2.8 Å) and to a structurally conserved water (2.6 Å), and phosphate O4 interacts with the 3′-OH of the DADMe-ImmH group (2.7 Å) as well as with OG of Ser118 (2.8 Å). The N1′ DADMe-ImmH cation makes an ion pair to the same PO4 oxygen with a distance of 2.7 Å. This distance is similar to the 3.0 Å predicted from transition-state analysis of human, bovine, and P. falciparum PNPs. Thus, the binding of DADMe-ImmH places the phosphate oxygen nucleophile close to the actual transition state distance proposed for related PNPs (38, 40, 41). The transition-state structure of AgPNP remains to be solved, but the preferred binding of DADMe-ImmH relative to ImmH supports a fully developed carbocation at its transition state (37).

Comparison of AgPNP−DADMe-ImmH−PO4 with HsPNP−DADMe-ImmH−SO4. The catalytic mechanism proposed for AgPNP involves Asn328 activation of the hypoxanthine leaving group. In the crystal structure of AgPNP−DADMe-ImmH−PO4, Asn328 hydrogen-bond distances are the same (within error limits) as the distances reported for HsPNP−DADMe-ImmH−SO4 (PDB code 1RSZ). As mentioned above, the phosphate O4 anion and the N1′ cation form an ion pair 0.3 Å shorter than seen in the HsPNP (3.0 Å). Ion-pair energy in a low dielectric is highly dependent on distance, and this difference is consistent with the differences in inhibitor affinity. Although there are many similarities between AgPNP−DADMe-ImmH−PO4 and HsPNP−DADMe-ImmH−SO4, the loss of a structural water close to C2′ of DADMe-ImmH is a significant difference. This water occupies a space similar to the 2′-OH group in the ribo sugars and may explain the lower affinity of AgPNP for nucleosides than in HsPNP. The inability of AgPNP to stabilize a water molecule at this site suggests an inability to stabilize the 2′-OH in the nucleoside substrates. The absence of this water in AgPNP might also allow for closer bonding interactions between N1′ and 3′-OH of the inhibitor to both the protein and the phosphate. His149 in AgPNP (His64 in the HsPNP) is positioned on a loop that interacts with phosphate (3.4 Å), similar to that in the BtPNP−ImmH−PO4 (40) and HsPNP−DADMe-ImmH−SO4 complexes but distinct from the HsPNP−DADMe-ImmH−SO4 complex (11 Å distant).

Studies of substrate and inhibitor binding indicate that AgPNP has a surprisingly low affinity for substrates or inhibitors with an amino group at the C2 position. Comparison of HsPNP and AgPNP near the substrate C2 position identifies two residues that differ. The more bulky Ile302 and Leu280 residues in AgPNP are substituted by values in human PNP and may explain the observed difference in substrate specificity.

CONCLUSIONS

AgPNP is unique in its substrate specificity. Its rapid pre-steady-state turnover number for guanosine supports high catalytic potential. The transition-state analogue DADMe-ImmH resembles a fully dissociated 2′-deoxyinosine ribo- acarbonium transition state and is a powerful, slow-onset, tight-binding inhibitor for AgPNP with a $K_{i}^*$ value of 3.5 pM and a $K_{i}/K_{i}^*$ value of 5.4 × 10$^{-7}$. 2′-Deoxyinosine is the most favorable substrate for AgPNP, and the inhibition pattern suggests that AgPNP forms a fully dissociated transition state in the phosphorolysis of 2′-deoxyinosine. AgPNP is highly selective for transition-state analogues, with DADMe-ImmH binding being preferred relative to other transition-state analogues. The structure of AgPNP is similar to other trimeric PNPs, although an 86 residue N-terminal region of AgPNP is disordered. The mRNA encoding this protein and an N-terminal splice variant are both present in mosquito extracts. Comparison with HsPNP, which binds DADMe-ImmH with a dissociation constant of 9 pM, allows observation of some differences in the structures, which might correspond to differences in the binding affinity of transition-state analogues. The structure of the AgPNP complex reveals that a conserved water molecule is missing in AgPNP, which may influence the substrate specificity and the tighter binding of DADMe-ImmH. The N1′ cation to phosphate-O4 anion distance is shorter (2.7 Å) in the AgPNP−DADMe-ImmH−PO4 compared to HsPNP−DADMe-ImmH−SO4, suggesting that the AgPNP transition state may include weak nucleophile participation by the phosphate. AgPNP has low $k_{cat}$ values for guanosine substrates and relatively weak binding to transition-state analogues of guanosine. For guanosine, burst kinetics indicate rate-limiting product release in the catalytic cycle. Larger residues (Ile and Leu) in proximity to the C2 position of DADMe-ImmH make the region sterically hindered for C2−NH$_2$ interactions.

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SUPPORTING INFORMATION AVAILABLE

Gene-specific oligonucleotide primers, PCR of genomic DNA and cDNA, and analysis of mRNA isolated from mosquitoes. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES


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