Four generations of transition-state analogues for human purine nucleoside phosphorylase

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Inhibition of human purine nucleoside phosphorylase (PNP) stops growth of activated T-cells and the formation of 6-oxypurine bases, making it a target for leukemia, autoimmune disorders, and gout. Four generations of ribocarbon transition-state mimics bound to PNP are structurally characterized. Immucillin-H (Ki = 58 pM, first-generation) contains an iminoribitol cation with four asymmetric carbons. DADMe-Immucillin-H (Ki = 9 pM, second-generation) uses a methylene-bridged dihydroxypyrrolidine cation with two asymmetric centers. DATMe-Immucillin-H (Ki = 9 pM, third-generation) contains an open-chain amino alcohol cation with two asymmetric carbons. SerMe-ImmH (Ki = 5 pM, fourth-generation) uses acetal dihydroxyminoalcohol serine as the ribocarbon mimic. Crystal structures of PNP analogues establish features of tight binding to be: 1) ion-pair formation between bound phosphate (or its mimic) and inhibitor cation, 2) leaving-group interactions to N1, O6, and N7 of 9-deazahypoxanthine, 3) interaction between phosphate and inhibitor hydroxyl groups, and 4) His257 interacting with the 5'-hydroxyl group. The first generation analogue is an imperfect fit to the catalytic site with a long ion pair distance between the iminoribitol and bound phosphate and weaker interactions to the leaving group. Increasing the ribocation to leaving-group distance in the second- to fourth-generation analogues provides powerful binding interactions and a facile synthetic route to powerful inhibitors. Despite chemical diversity in the four generations of transition-state analogues, the catalytic site geometry is almost the same for all analogues. Multiple solutions in transition-state analogue design are available to convert the energy of catalytic rate enhancement to binding energy in human PNP.

Human PNP catalyzes the phosphorolysis of 6-oxopurine nucleosides and deoxynucleosides to generate α-D-(deoxy) ribose 1-phosphate and the purine base. The purine is recycled or oxidized to uric acid for excretion. A rare genetic deficiency of PNP reveals that the enzyme is essential for recycling d-guanosine and formation of free purines leading to uric acid syntheses. PNP deficiency causes the presence of elevated concentrations of d-guanosine in the blood resulting in apopoptosis of dividing T-cells due to the metabolic accumulation of dGTP, an inhibitor of ribonucleotide reductase (1, 2). Inhibitors of PNP have been used for the treatment of T-cell cancers and autoimmune disorders where T-cell clones are misdirected against self-antigens causing disorders, including psoriasis, rheumatoid arthritis, and multiple sclerosis (2, 3). PNP inhibitors are also in clinical trials for gout because formation of purine base precursors for uric acid formation requires PNP in humans.

Knowledge of enzymatic transition-state structure is obtained from the experimental approach of kinetic isotope effects combined with quantum-chemical models (4). This analysis provides an atomic view of the difference in bond-vibrational environment between the reactants and the transition state for a given reaction. The quantum-chemical solution to the transition state provides geometry and electrostatic maps for comparison with those of the reactant molecules. These are the features needed for the design of analogues with geometric and electrostatic similarity to the transition state. Human PNP is known to have a fully-dissociated, classic Sₐ1 transition state and is the basis for design of the inhibitors described here (5). The first generation PNP transition-state analogue, Immucillin-H (ImmH, Fig. 1), was designed from the transition state of bovine PNP, which revealed an early transition state with ribocarbon character but a relatively close 1.8 Å distance between the leaving-group nitrogen and the anomeric carbon (6). An iminoribitol isostere of the ribose ring mimics the partial ribocation at its transition state and 9-deazahypoxanthine prevents phosphorylation and provides an elevated pKₐ at N7, another feature of the transition state (7). Bovine PNP was assumed to form a transition state the same as human PNP because of the 87% amino acid sequence identity between human and bovine PNP. However, this is not the case.

The transition states of human and bovine PNPs are distinct based on isotope effects and inhibitor specificity (5–9). Human PNP has a fully-dissociated purine leaving group with a fully-developed ribocation (5). A second-generation hPNP transition-state analogue inhibitor, 4’-deazah-1’-aza-2’-deoxy-1’-(9-methylamino)Immcillin-H (DADMe-ImmH, Fig. 1), was designed from this discovery (8). The 9-methylene bridge served to place the cationic N1’ nitrogen near the ribosyl C1’ position in the transition state because the most cationic atom is C1’ in this transition state (5). In addition, the 2’-hydroxyl group was removed to provide chemical stability. Because a physiological substrate for human PNP is d-guanosine, the 2’-deoxy configuration still mimics a physiologically relevant transition state. DADMe-ImmH is simpler to synthesize by virtue of its two asymmetric carbons. The modifications resulted in an 8-fold increased affinity for human PNP relative to ImmH and provided specificity for human relative to bovine PNP (8).


The Immucillins are in clinical development by BioCryst Pharmaceuticals Inc. under license from the Albert Einstein College of Medicine and Industrial Research Ltd. V.L.S. is a consultant to BioCryst Pharmaceuticals Inc.

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Data deposition: New atomic coordinates and structure factors deposited in the Protein Data Bank (www.pdb.org) are [PDB ID codes 3K8O (PNP–SO₄)·DATMe-ImmH complex] and 3K8Q (hPNP–PO₄·SerMe-ImmH complex)].

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The third-generation transition-state inhibitor of human PNP, 2'-deoxy-2'-amino-9-(9-methylene)-immucillin-H (DADMe-ImmH, Fig. 1), was discovered by exploring acyclic cationic di- and trihydroxy groups linked to 9-deazahypoxanthine via the 9-methylene bridge (9). DADMe-ImmH is similar to ImmH except for the open ring structure between C1' and C2' and the altered stereochemistry of the 3'-hydroxyl group. The more flexible ribocation analogue of DADMe-ImmH inhibits PNP better than ImmH and equivalent to DADMe-ImmH. Equal binding affinity for DADMe-ImmH and DADMe-ImmH was unexpected, considering the degrees of entropic freedom lost when DADMe-ImmH becomes immobilized at the catalytic site. However, increased dynamic motion of PNP bound to DADMe-ImmH has been proposed to compensate for the loss of rotational freedom in this complex (10). DADMe-ImmH retains two asymmetric carbon centers, making it similar to DADMe-ImmH as a synthetic chemistry challenge.

The fourth-generation transition-state inhibitor of human PNP is serinol-N(9-methylene)-immucillin-H (SerMe-ImmH; Fig. 1). This analogue eliminates the 2'-methylene carbon of ImmH. Two major advances are provided in SerMe-ImmH. Structurally, the compound is achiral, reducing the synthetic challenge. Despite this simple achiral structure, the dissociation constant (Kd) value is 5 pM, one of the most powerful human PNP transition-state analogues (11).

The inhibition kinetics and thermodynamics of binding are well-characterized for the four generations of human PNP transition-state, analogue inhibitors. In every case, full inhibition of the enzyme occurs when the first catalytic site of the homotrimer is bound. This fundamental question of how the flexible transition-state analogues capture more binding energy than the more rigid ImmH. Another important question is how the relatively more flexible second-, third-, and fourth-generation inhibitors compare in catalytic site alignment with the more rigid first-generation analogues. The crystal structures of ImmH, DADMe-ImmH, DATMe-ImmH, and SerMe-ImmH bound to human PNP were solved at 2.4 or 2.5 Å resolution to answer these questions. Key features of bound inhibitors are compared for the four generations of PNP inhibitors. The structural attributes are correlated with knowledge of the enthalpic and entropic parameters involved in binding these inhibitors.

**Results**

**Overall Structure of Human Purine Nucleoside Phosphorylase.** Crystal structures of human PNP in complex with four transition-state inhibitors and phosphate or sulfate were determined at 2.4 to 2.5 Å resolution (Table 1). Residues 4 to 284 of PNPs were ordered in the electron density maps with each of the four inhibitors bound. The crystallographic asymmetric units of PNP complexes with ImmH, DADMe-ImmH, and SerMe-ImmH contained one monomer of PNP. Functional PNP is a homotrimer and trimeric PNP complexes were generated from the monomers by using crystallographic symmetry operators corresponding to the space group of H3 (Fig. 2A). Diffraction data for DATMe-ImmH bound to PNP revealed two distinct homotrimers in the asymmetric unit. The monomers of inhibitor-bound PNP possess an αβ-fold consisting of a 4-stranded β-sheet, 8 α-helices, and a distorted β-barrel formed from a 6-stranded β-sheet (Fig. 2B). The active sites of PNP are located near the interface between two monomers of the trimer and each trimeric PNP forms three active sites (Fig. 2A).

**Inhibitor Binding to Human PNP.** Inhibitor binding sites of PNP are near the C-terminal region of the 6-stranded β-sheet. The purine base binding region consists primarily of hydrophobic residues including Phe200, Val217, Met219, Val245, and Val260. Purine base interactions also include the polar residues Glu201 and Asn243 as hydrogen bond acceptors from the NH1 and NH2 groups of 9-deazahypoxanthine (Fig. 2C and ref. 13). Asn243 also donates hydrogen bonds to O6 of the deazapurine, except in the case of ImmH (Fig. 3). ImmH bound to PNP has the 9-deazahypoxanthine group positioned closer to the phosphate binding region than in the complexes with the other inhibitors. This forces Asn243 into a single hydrogen bond interaction with ImmH rather than the bidentate interaction with 9-deazahypoxanthine in the more tightly bound second-, third-, and fourth-generation inhibitors (Fig. 3). The ribocation mimic is surrounded by Phe159, His257, Tyr88, and Met219. Phe159 is the only amino acid in the catalytic site contributed from the adjacent subunit. Its position over the catalytic site suggests a role in restricting the entry of substrate into the catalytic site from the direction of the ribosyl 5′-hydroxyl group (Fig. 2D). The 2′-, 3′-, and 5′-hydroxyl groups of ImmH are in H-bond distance from the amide of Met219, the hydrogen group of Tyr88 and the side chain of His257, respectively. His257 plays a significant role in catalysis by positioning the 5′-ribofuranose oxygen of substrate near the 4′-ribofuranosyl group that in turn is near the phosphate nucleophile. These unusual close interactions of electron-rich oxygens initiate electron donation from the ribosyl group to the purine and, thus, initiate formation of the C1′-phosphate (22).

**Table 1. Thermodynamics for inhibitors binding to hPNP***

<table>
<thead>
<tr>
<th>Inhibitor</th>
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</tr>
<tr>
<td>SerMe-ImmH</td>
<td>−15.5</td>
<td>−20.2</td>
<td>4.7</td>
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<tr>
<td>Hypoxanthine</td>
<td>−7.4</td>
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*The data was obtained from ref. 10.

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The inhibition kinetics and thermodynamics of binding are well-characterized for the four generations of human PNP transition-state, analogue inhibitors. In every case, full inhibition of the enzyme occurs when the first catalytic site of the homotrimer is bound. The inhibitors bind to the first catalytic site with large favorable enthalpic interactions (up to −22 kcal/mol) and smaller, unfavorable entropic penalties (up to 7 kcal/mol). The second and third sites also bind these analogues but with reduced affinity and progressive negative cooperativity (10). The crystal structures of ImmH bound to bovine and human PNPs have been described previously (11–13). However, the high affinity of the second-, third-, and fourth-generation transition-state analogue inhibitors raises fundamental questions of how the flexible transition-state analogues capture more binding energy than the more rigid ImmH. Another important question is how the relatively more flexible second-, third-, and fourth-generation inhibitors compare in catalytic site alignment with the more rigid first-generation analogues. The crystal structures of ImmH, DADMe-ImmH, DATMe-ImmH, and SerMe-ImmH bound to human PNP were solved at 2.4 or 2.5 Å resolution to answer these questions. Key features of bound inhibitors are compared for the four generations of PNP inhibitors. The structural attributes are correlated with knowledge of the enthalpic and entropic parameters involved in binding these inhibitors.

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of the transition state (14). All four generations of inhibitors demonstrate interactions between bound phosphate and the ribocation mimic. These include hydrogen bonding between phosphate oxygen and hydroxyl groups of the inhibitors as well as ion pair formation between the amino cations and the phosphoanion of the incipient nucleophile (Figs. 2 and 3).

The analogous phosphate binding site is lined with the side chain residues of Arg84, His86 and Ser220, the amide nitrogen of Ala116 and Ser33, and the hydroxyl group of Tyr192 interacting through a structural water molecule (Fig. 2E). The phosphate and sulfate groups are in the same position for each of the four inhibitors bound to PNP and this same position is also found in the complex of human PNP with phosphate alone (PDB ID: 1M73). The numerous interactions between phosphate and PNP anchor it firmly in the phosphate binding site independent of nucleoside analogue binding.

**Immuclillin-H Binding to Human PNP.** A first-generation transition-state analogue, ImmH, is isosteric with inosine, a preferred substrate for the enzyme, with two atomic replacements. Carbon replaces nitrogen at N9 and nitrogen replaces oxygen at the O4' of inosine (Fig. 1) (7). The 4'-imino group of ImmH has a pKₐ 6.9, but when bound to PNP at neutral pH, the inhibitor exists as a cationic mimic of the ribocation transition state (15). Replacing the N9 nitrogen of hypoxanthine with carbon creates a chemically stable C-C bond and increases the pKₐ value of the NH7 nitrogen to >10 to mimic the protonated N7 of the purine leaving group at the enzymatic transition state of PNP.

The 9-deaza hypoxanthine of ImmH forms one less hydrogen bond to PNP than the other transition state inhibitors. ImmH was designed for the transition state of bovine PNP where the N-ribosidic bond at the transition state is 1.8 Å (6). But at the transition state of human PNP this distance is 3.0 Å. ImmH bound in the catalytic site of human PNP finds itself upon the Procrustean bed where it is too short to span the distance between the leaving-group and ribocation centers and, thus, does not achieve a perfect fit for either. Despite these imperfections, the dissociation constant for this complex is 58 pM. Leaving-group interactions include the NH1, O6, and NH7 of the deazapurine and these are all closer than in structures of purine nucleosides bound to the enzyme (ref. 16, Figs. 3A and 4A). The iminoribitol group of ImmH is bound in the C3'-exo conformation. The C1' of the iminoribitol is 3.5 Å away from the nucleophilic phosphate oxygen and the cationic N4' imine is 3.3 Å from the anionic phosphate. The iminoribitol hydroxyl groups are in hydrogen bond distance to His257, Tyr88, Met219, and the phosphate. The 5'-hydroxyl group interacts with an imidazole nitrogen of His257 (2.8 Å) to form an internal hydrogen bond with the 4'-NH2 amine (2.8 Å). The 3'-hydroxyl group is in weak hydrogen bond distance to the hydroxyl group of Tyr88 (3.2 Å) and a stronger interaction with phosphate (2.7 Å). The 2'-hydroxyl group interacts with the amide of Met219 (3.0 Å) and the phosphate (2.9 Å). Bound phosphate is virtually immobilized and interacts through an extensive hydrogen bonding network with the surrounding residues including the side chains of His86 (3.1 Å), Arg84 (3.0 Å) and Ser220 (2.8 Å), the amides of Ser33 (2.8 Å) and Ala116 (2.9 Å), and a structurally observed water molecule held in position by the side chain of Tyr192 (2.8 Å). A sum of the interactions for ImmH includes one ion-pair interaction at a distance of 3.3 Å and 15 potential hydrogen bonds, including those to two structural water molecules. Among those hydrogen bonds, 12 of them are 3.0 Å or shorter (Table 2).

Several crystal structures of apo and liganded human PNP are available in the Protein Data Bank (PDB ID: 1M73, IPWY, 1RCT, 1V3Q, 1V2H, and 3D1V). These structures lack electron density for peptide backbone atoms for amino acid residues 250 to 260, an important region that contains the catalytic His257. In our structure, continuous electron density is present in this region, providing structural evidence for the proposed neighboring-group participation of His257 in the catalytic mechanism of PNP (11).

**DADMe-ImmH Binding to Human PNP.** The transition state for inosine arenoslysis catalyzed by human PNP is a fully-dissociated ribocation with the N-ribosidic bond increased to 3.0 Å without significant bond participation by the anionic nucleophile (5). DADMe-ImmH is a second-generation transition-state inhibitor of hPNP designed to increase the distance between ribose and purine by placing a methylene bridge spacer between the ribocation mimic and the purine leaving group. In this transition state, the cationic center develops at C1' of the ribosyl group. Therefore, a cationic nitrogen is used to replace the anomeric carbon and the 9-deaza hypoxanthine provides the elevated pKₐ at N7 (5, 8). These changes require the 2'-C of the hydroxypropyridine to be deoxy for chemical stability. Thus, DADMe-ImmH mimics the transition-state ribooxacarbonyl ion for PNP with 2'-deoxynosine as the substrate. 2'-Deoxyinosine and 2'-deoxyguanosine...
are good substrates for PNP, thus the absence of the 2′-hydroxyl group is well tolerated in DADMe-ImmH.

The methylene bridge in DADMe-ImmH extends the distance between ribocation and purine groups to a distance of 2.6 Å. The increased distance and geometric freedom permits an altered orientation of the purine leaving group compared to ImmH binding. An additional hydrogen bond forms between the purine O6 and Asn243 (3.0 Å) and the hydrogen bond between N7 and Asn243 is shorter (2.8 Å) (Fig. 3B). Hydrogen bonds between Glu201, N1 (2.7 Å), and O6 (via a water molecule) remain similar to those in the complex with ImmH (Figs. 3A and B). The hydroxypyrrolidine group of DADMe-ImmH is in a C2′-endo conformation. The methylene bridge allows the 1′-aza cation to form a more favorable 3.0 Å ion pair with sulfate as the phosphate mimic. The 3′-hydroxyl group forms a hydrogen bond to the imidazole of His257 (2.8 Å) and the 3′-hydroxyl is positioned between the hydroxyl group of Tyr88 (3.1 Å) and sulfate (3.1 Å). The position occupied by the 2′-hydroxyl in the ImmH structure is occupied by a water molecule when DADMe-ImmH is bound. This structural water replaces the missing 2′-hydroxyl group and interacts with the Met219 amide (3.2 Å), sulfate (2.8 Å) and the 3′-hydroxyl group of DADMe-ImmH (3.2 Å). Sulfate is positioned similar to phosphate in the ImmH complex and interacts with the side chains of His86 (3.2 Å), Arg84 (3.2 Å) and Ser220 (2.8 Å), the amide of Ser33 (2.9 Å) and Ala116 (2.9 Å), and the side chain of Tyr192 through a water molecule (2.8 Å). Bound DADMe-ImmH is stabilized by an ion-pair (3.0 Å) and 18 potential hydrogen bonds, involving three structural water molecules. Twelve of the potential hydrogen bonds are 3.0 Å or shorter (Table 2).

DATMe-ImmH Bound to Human PNP. DATMe-ImmH is a third-generation transition-state analogue of human PNP and binds with a slow-onset dissociation constant of 9 pM (Fig. 1) (9). DATMe-ImmH differs from ImmH by its lack of a ribosyl ring mimic and the inverted stereochemistry of the 3′-hydroxyl group but, like ImmH, has three hydroxyl groups (Fig. 1; numbered as in ImmH for consistency). The acyclic amino alcohol is bound with the cationic nitrogen 3.0 Å from the sulfate anion (average distance of the six monomers in the asymmetric unit), similar to the geometry of bound DADMe-ImmH (Figs. 3 and 4). The C1′ methylene bridge allows the 9-deazahypoxanthine of DATMe-ImmH to bind with the same favorable interactions found for DADMe-ImmH (see above and Fig. 3B and C). Compared to the binding geometry of ImmH and DADMe-ImmH, DATMe-ImmH binding occurs by placing the 2′-hydroxy group

Table 2. The summary of interaction between hPNP and inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>The distance of the ion-pair interaction (Å)</th>
<th>The number of hydrogen bonds shorter than 3 Å</th>
<th>The number of hydrogen bonds greater than 3 Å</th>
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<tbody>
<tr>
<td>ImmH</td>
<td>3.3</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>DADMe-ImmH</td>
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<td>12</td>
<td>6</td>
</tr>
<tr>
<td>DATMe-ImmH</td>
<td>3.0</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>SerMe-ImmH</td>
<td>3.1</td>
<td>11</td>
<td>5</td>
</tr>
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</table>
in the 5′-hydroxyl group position and the 5′-hydroxyl group in the 2′-hydroxy binding pocket. This geometry permits the 2′-hydroxyl group to form a hydrogen bond with His257 (2.9 Å). The 5′-hydroxyl group captures the 2′-hydroxyl interactions also found in ImmH binding and is in a hydrogen bond with the amide of Met219 (2.9 Å) and sulfate (3.3 Å and 2.6 Å). Sulfate interacts with the side chains of His86 (2.8 Å), Arg84 (3.1 Å), Ser220 (2.6 Å) and Ser33 (2.6 Å), the amides of Ser33 (3.1 Å) and Ala116 (2.8 Å), and the hydroxyl group of Tyr192 through a structural water molecule (2.7 Å). DATMe-ImmH binding involves one 3.0 Å ion-pair interaction and 16 hydrogen bonds, involving two structural water molecules. Twelve of the hydrogen bonds are 3.0 Å or shorter (Table 2).

SerMe-ImmH Binding to Human PNP. SerMe-ImmH is a fourth-generation PNP transition-state analogue distinguished by its achiral chemistry, two hydroxyl groups in the acyclic ribocation-mimic, and extraordinarily slow-onset tight binding to give a 5 pM dissociation constant (Fig. 1). As DADMe-ImmH also has two hydroxyl groups, we compare its binding geometry to DADMe-ImmH. Bound SerMe-ImmH has the nitrogen ribocation mimic 3.1 Å from the phosphate anion, similar to DADMe-ImmH and DATMe-ImmH. The bond rotational freedom of the SerMe group allows the 9-deazahypoxanthine to form hydrogen bonds with the same favorable interactions of the second- and third-generation Immcultins. Both hydroxyl groups of SerMe-ImmH are bound in similar positions as the two hydroxyl groups of DADMe-ImmH. The truncated structure of the SerMe group causes the hydroxyl group interacting with His257 to result in a weak interaction of 3.5 Å compared to 2.8 Å between His257 and the 5′-hydroxyl groups of other inhibitors. The 3′-hydroxyl group of SerMe-ImmH forms a hydrogen bond with phosphate (2.8 Å). A water molecule replaces the missing 2′-hydroxyl group and forms a network with the phosphate nucleophile (2.7 Å and 2.9 Å), the 3′-hydroxyl group (2.7 Å), and the amide of Met219 (3.2 Å). The phosphate in the SerMe-ImmH complex is bound in a similar manner to the other PNP-inhibitor complexes. SerMe-ImmH interactions with PNP include the ion-pair interaction (3.1 Å) and 16 hydrogen bonds, including two structural water molecules. Eleven of the hydrogen bonds are 3.0 Å or shorter, easily accounting for the −15.5 kcal/mol binding energy for SerMe-ImmH to the PNP-phosphate complex (Tables 1 and 2).

PNP Conformations with Different Inhibitors. The Cα backbone residues 3–58 and 66–278 of PNP with all inhibitors bound reveal closely related conformations with a r.m.s. deviation of 0.3–0.8 Å. The electron density map of residues 250–263 is well defined with inhibitors bound while earlier human PNP structures are disordered in this region (13, 16). Residues 250–263 are ordered by the interaction of His257 with the 5′-hydroxyls (ribosyl numbering) of bound inhibitors. The conformations of residues 250–263 for three of the four complexes of transition-state inhibitors are the same within crystallographic error. The exception is the complex with DATMe-ImmH where its 5′-hydroxyl group is positioned 1.3 Å away from the position of hydroxyl groups of other inhibitors. To maintain the hydrogen bond contact, His257 moves 1.4 Å relative to its position in the other complexes. This movement causes a minor shift of the 250–260 loop and a 23° rotation of the Phe200 side chain that is adjacent to the loop. This motion prevents a steric clash with Ala255.

Loop 59–65 also shows conformational differences in complexes with the four analogues. In the DATMe-ImmH complex, loop 59–65 moves toward the active site and His64 forms hydrogen bonds with Ser33 and Arg84, interactions not seen in the complexes with ImmH and DADMe-ImmH. In the SerMe-ImmH complex, loop 59–65 shifts away from the active site. The conformation of loop 59–65 may also respond to the difference in sulfate or phosphate binding.

Discussion

Ion-pair Interactions with Transition-State Analogues. The transition state of human PNP is characterized by a fully developed ribocation with N9 of the the purine leaving group and the phosphate oxygen nucleophile both located approximately 3 Å from the C1′ cation (5). All four generations of transition-state analogues take advantage of ion-pair formation to bound phosphate (or sulfate) anions in mimicry of the transition state. ImmH forms the weakest ion pair with an ion-pair separation of 3.3 Å between the cationic 4′-nitrogen and phosphate. With the cationic nitrogen as a ribosyl 1′-analogue, these analogues more closely resemble the transition state and reduce the ion-pair distance to approximately 3.0 Å. The acyclic analogues DATMe-ImmH and Ser-ImmH incorporate molecular flexibility in the orientation of the nitrogen cation, and both inhibitors form 3.0 Å ion-pair distances resembling bound DADMe-ImmH rather than ImmH (Fig. 4). Although ImmH has hydrogen bonds to the 2′-hydroxyl that are missing in DADMe-ImmH, the closer ion pair more than compensates for the loss of these hydrogen bonds. The 3.0 Å ion-pair distances with the best inhibitors reproduce the distance in the transition state.

Purine Leaving Group. DADMe-ImmH binds tighter than ImmH as a consequence of improved PNP-purine and ribocation-mimic to anion interactions. Transition-state geometry for human PNP indicated a fully-dissociated bond (>2.5 Å) between the ribocation C1′ and N9 of the protonated purine leaving group (5). ImmH is constrained to 1.5 Å in this dimension but the methylene bridge in DADMe-ImmH spaces the 1′-aza nitrogen and the C9 of 9-deazahypoxanthine at 2.6 Å. This geometry permits DADMe-ImmH to place the purine leaving group for favorable leaving-group interactions and simultaneously form a favorable ion pair with sulfate. The methylene bridge between the ribocation mimics and 9-deazahypoxanthine is also present in the DATMe-ImmH and SerMe-ImmH complexes, permitting the cationic amines of DATMe-ImmH and SerMe-ImmH to form 3.0 Å ion pairs. The added distance between 9-deazahypoxanthine and the ribocation mimic allows the leaving-group interactions to be optimal and different from those with bound ImmH (Fig. 5).

Structural Correlation to Binding Enthalpy. The enthalpy of inhibitor binding arises from ion-pair interactions, hydrogen bonding, and hydrophobic (van der Waals) interactions, primarily between the deazapurine and surrounding hydrophobic residues. Surprisingly, ImmH binding has the most favorable enthalpy (∆H) of binding (Table 1, ref. 10) even though ImmH has the weakest ion-pair interaction (3.3 Å, Table 2). The suboptimal positions of both 9-deazahypoxanthine and the ribocation mimic in ImmH presumably cause the protein to be stretched taut, thereby paying a high price (7 kcal/mol) in the entropic penalty arising from loss of dynamic motion of the PNP protein. The sum of these forces makes ImmH the least tightly bound of the inhibitors. Binding of hypoxanthine alone to the enzyme-phosphate complex eliminated the energetically coupled interactions of the ribocation mimics. The remarkable ∆H value of ~30.5 kcal/mol (Table 1), is likely to exceed the enthalpy available from the three modest hydrogen bonds observed to 9-deazahypoxanthine in ImmH binding or the four hydrogen bonds with DADMe-ImmH, DATMe-ImmH, and SerMe-ImmH binding. The source of the favorable enthalpy may be the extensive van der Waals interactions of the deazapurine moiety with its surrounding hydrophobic residues (10). This hydrophobic interaction at the catalytic site organizes protein structure not only at the local binding site but also in the neighboring structure, creating the large entropic penalty of 23.1 kcal/mol (Fig. 5).
Structural Correlation of Binding Entropy. The four generations of PNP inhibitors bind within 1.4 kcal/mol ΔΔG (Table 1, ref 10). However the enthalpic and entropic contributions to ΔG vary by as much as 3.7 and 4.8 kcal/mol resp. for these inhibitors. The entropic penalty is related to altered solvent order and the losses of dynamic flexibility in both the inhibitor and protein molecules. In these structures, neither the number of ordered water molecules at the active sites nor the loss of inhibitor geometric freedom (DATMe-ImmH and SerMe-ImmH contain the most rotatable bonds) correlates with the thermodynamic data (Tables 1 and 2). Thus, the variable entropic penalty dominates the difference in energetic of the inhibitor binding and is most likely contributed from the changes in global protein dynamics, although solvent order cannot be eliminated. The large entropic penalty

Fig. 5. Stereoview comparisons of the PNP protein conformations in contact with four generations of transition-state analogue inhibitors. (A) ImmH and the surrounding catalytic site residues are shown in Cyan. (B) DADMe-ImmH and its surrounding catalytic site residues are shown in Yellow. Overlaid in Cyan are the 9-deazahypoxanthine, Phe200, and His257 from the ImmH structure (A) to show the relative difference in conformations. (C) DATMe-ImmH and its surrounding catalytic site residues are shown in Magenta. Overlaid in Cyan are the 9-deazahypoxanthine, Phe200 and His257 from the ImmH structure (A) to show the relative difference in conformations. (D) SerMe-ImmH and the surrounding catalytic site residues are shown in Gray. Overlaid in Cyan are the 9-deazahypoxanthine, Phe200, and His257 from the ImmH structure (A) to show the relative difference in conformations.
from hypoxanthine binding ($-\Delta S = 23.1$ kcal/mol) suggest major contributions from PNP dynamics linked to the purine binding pocket. Structural analysis revealed a rotational change of Phe200 in the complex of DATMe-ImmH with PNP, the inhibitor causing the least entropic penalty. Organization of loop 250–260 in the inhibitor complexes caused a change of Phe200 and its adjacent Ala255 to also interact with the 250–260 loop. Altered geometry of loop 250–260 is implicated in an altered dynamic structure at the trimeric interface, including the loop 158–164 from the adjacent subunit. Human PNP is strongly cooperative in inhibitor binding, thus subunit-linked conformational changes may contribute to protein dynamics and the variable entropic penalties for inhibitor binding. Because dynamic motion is required to find transition-state geometry in PNP (17), inhibitors closely resembling the transition state are postulated to permit transition-state motions and cause smaller entropic penalties and increased binding affinity.

**PNP Transition-State Inhibitor Design.** Powerful transition-state inhibitors for PNP mimic the transition-state features: 1) a ribocation mimic in a geometry to permit favorable ion pairing with the highly immobilized phosphate anion, 2) a 6-oxypurine leaving group with an elevated $pK_a$ in a geometry to permit full interaction with the enzymatic leaving-group contacts, 3) di- or tri-hydroxyl groups to capture the di- and tri-hydroxyl group interactions engaged by 2′-deoxy- and ribonucleosides, and 4) covariant incorporation of a phosphate mimic is not a design element in inhibitors because the enzyme immobilizes phosphate at a geometry similar to the transition-state distance. Inhibitors with these features capture much of the Δ$\Delta G$ for PNP catalysis as binding energy from the PNP transition state. Thus, ImmH, DADMe-ImmH and SerMe-ImmH give $K_m/K_i$ values of 739,000, 4,300,000, and 7,700,000 relative to the PNP substrates they mimic. The geometry of PNP-bound di- and tri-primary alcohols in the ribocation mimic is highly constrained to the ion pair and the groups stabilizing the 3′- and 5′-hydroxyls. Different geometry is poorly tolerated. Thus, in a family of eight diols related to DADMe-ImmH but with the open ring flexibility of DATMe-ImmH, all bound more weakly by factors of 50 to 35,000 (9). The 2′-hydroxyl of analogues is not critical for tight binding. This site on PNP can be filled with a structural water, presumably the mechanism evolved to permit PNP efficient catalysis of both 6-oxynucleosides and 6-oxo-2′-deoxynu-

**Lessons from ImmH Structures with Human and Bovine PNPs.** The catalytic site residues of bovine and human PNPs are completely conserved in amino acid sequence and the proteins are 87% identical overall (Fig. 6). Despite this similarity, their transition states are distinct. In bovine PNP, the N9-C1′ distance at the transition state is 1.8 Å whereas for human PNP this distance is greater than 2.6 Å (5). ImmH, therefore, more closely resembles the bovine transition state and shows more favorable interactions with bovine PNP. Specifically, the major binding force of the phosphate anion–nitrogen cation differs in that the distance is 2.8 Å in bovine PNP but is 3.3 Å in human PNP. Likewise, the leaving group interactions to Asn243 are more favorable in the bovine enzyme (Fig. 6; 12).

How can it be that PNPs with near-identical amino acid sequences can form distinct transition states and complexes with ImmH? The answer lies in the dynamic nature of transition states linked to short time scale protein dynamic vibrational modes, in contrast to the thermodynamic nature of the complexes with bound transition-state analogues (19, 20). In catalysis, the transition-state barrier is crossed by coincident dynamic interactions, all of which contribute to barrier crossing. These thermal, local motions of catalytic site residues operate on the fsec to psec timescale (17). In PNPs, the interactions contributing to barrier crossing include: 1) Asn243 and Glu201 distances are dynamically minimized to form leaving-group interactions; 2) the compression mode of His257, the 5′-oxygen, the 4′-ribosyl ring oxygen, and the nucelophilic phosphate oxygen destabilize electrons from O4′ to form the ribocation; and 3) the ribocation stabilizing interaction of the phosphate oxygen nucleophile, a highly polarized anion in its binding site (21). Transition-state formation is achieved by the simultaneous minimization of these distances.

What do the complexes of human and bovine PNPs with bound ImmH represent in the enzymatic reaction coordinate? The transition-state analogue is proposed to captures the distance minima of dynamic promoting vibrational modes contributing to
barrier crossing. These minima are frozen in a thermodynamically stable complex that exists only on the fsec time scale in catalysis (17). Evidence for this conformational stabilization comes from the relative ease of crystallizing proteins in the presence of transition-state analogues. The similar geometry of catalytic site residues with all four generations of transition-state analogues bound to human PNP also indicates a common protein conformational limit optimized by evolution for barrier passage and captured with four different classes of transition-state analogues with different chemical architecture (Fig. 5).

Within this interpretation, it is of interest to see that bovine PNP folds more closely around ImmH and does human PNP. We interpret this to be a direct reflection of distinct dynamic modes enforced by the protein scaffolds of human and bovine PNPs linked to their distinct transition-state structures.

Methods and Materials

Inhibitor Synthesis. Immucillin-H [7-((2S,3S,4R,5R)-3,4-dihydroxy-4-(hydroxymethyl)pyrrolidin-1-yl)methyl]-3H-pyrrolo[3,2-d]pyrimidin-4(5H)-one] (9) were synthesized as described.

Human Purine Nucleoside Phosphorylase/Inhibitor Complex Crystallization and Data Collection. Recombinant human purine nucleoside phosphorylase (hPNP) was expressed and purified as described previously (11). Purified hPNP was reconstituted in 50 mM Tris pH 8.0, concentrated to 20 mg/ml and, following the addition of 1 mM inhibitor and 1–3 mM K$_3$PO$_4$, incubated on ice for 10 min. The hPNP/inhibitor complexes presented in this report were crystallized and prepared for data collection as detailed in Table S1.

Crystals of hPNP/ImmH, hPNP/DADMe-ImmH, and hPNP/DATMe-ImmH were briefly transferred to the reservoir solution supplemented with 20% glycerol prior to flash cooling at ~178 °C. hPNP/DATMe-ImmH crystals were additionally covered with Al$_2$O$_3$ oil (Hampton Research) prior to flash cooling at ~178 °C to limit evaporation. Crystals of hPNP/SerMe-ImmH were transferred to a solution of 50% glucose in mother liquor prior to freezing in liquid nitrogen. X-ray diffraction data for the inhibitor-bound hPNP crystal complexes were collected at the beamlines X9A (hPNP/ImmH, hPNP/DADMe-ImmH) and X29A (hPNP/DATMe-ImmH, hPNP/SerMe-ImmH) at the National Synchrotron Light Source, Brookhaven National Laboratory. Outcomes from the statistical analysis of the diffraction data, all of which were processed using the HKL2000 suite, are summarized in Table S2 (24). The resolution limit of 2.5 Å for hPNP-SerMe was determined on an Σ cutoff greater than two. The electron density was improved when the highest resolution shell was included.

Structure Determination and Refinement. The structures of hPNP in complex with ImmH and DADMe-ImmH were solved by molecular replacement using the published structure of hPNP (PDB: 1M73) as a search model. Molecular replacement, refinement and model building tasks were conducted in AMoRe, CNS and COOT (25–27). Molecular replacement solutions were obtained for hPNP/DATMe-ImmH and hPNP/SerMe-ImmH structures by using the published hPNP structure without the ligand (PDB: 3BGS) as the search model using the program MOLREP (28). These models were iteratively built and refined by using COOT and REFMAC5 (27, 29). Last stage refinement of Translation/Libration/Screw parameters were also introduced for the hPNP/DATMe-ImmH models that feature six monomers in the crystallographic asymmetric unit. Manual ligand building was initiated only after the R$_{free}$ decreased below 30% and was guided by clear ligand density in Fo–Fc electron density maps contoured at 5σ.

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