Purine nucleoside phosphorylase catalyzes reversible phosphorolysis of purine nucleosides and 2′-deoxypurine nucleosides to the free base and ribose (or 2′-deoxyribose) 1-phosphate. Whereas the human enzyme is specific for 6-oxopurine ribonucleosides, the Escherichia coli enzyme accepts additional substrates including 6-oxopurine ribonucleosides, 6-aminopurine ribonucleosides, and to a lesser extent purine arabinosides. These differences have been exploited in a potential suicide gene therapy treatment for solid tumors. In an effort to optimize this suicide gene therapy approach, we have determined the three-dimensional structure of the E. coli enzyme in complex with 10 nucleoside analogs and correlated the structures with kinetic measurements and computer modeling. These studies explain the preference of the enzyme for ribose sugars, show increased flexibility for active site residues Asp204 and Arg24, and suggest that interactions involving the 1- and 6-positions of the purine and the 4′- and 5′-positions of the ribose provide the best opportunities to increase prodrug specificity and enzyme efficiency.

Purine nucleoside phosphorylase (PNP) reversibly catalyzes the phosphorolysic cleavage of glycosidic bonds to generate ribose 1-phosphate (R 1-P) and a free purine base. PNPs can be divided into two classes, trimeric and hexameric. Trimeric PNPs are specific for 6-oxopurine nucleosides and are found both in higher organisms and in procaryotes. Trimeric PNPs usually have a molecular mass of about 100 kDa. The structures of several trimeric PNPs have been determined (1–4), and the catalytic mechanism has been well studied; the cleavage reaction is believed to proceed through an S$_{N}$1 mechanism with an oxocarbenium nucleoside intermediate (5–7), with or without substrate binding, and with product release as the rate-limiting step (8, 9). Hexameric PNPs are found only in lower organisms and have broader substrate specificity, accepting both 6-amino and 6-oxopurine nucleosides. Hexameric PNPs have a molecular mass of about 150 kDa, and two such structures have been determined (10, 11). Although the active site residues of trimeric and hexameric PNPs show considerable differences, the mechanisms are thought to be similar, with kinetic isotope effects consistent with an S$_{N}$1 mechanism (12, 13) (Scheme 1).

The differences in substrate specificity between the hexameric and trimeric PNPs suggest a strategy for anticancer suicide gene therapy (14) in which nontoxic nucleoside prodrugs are cleaved to cytotoxic purine analogs (15). In this approach, tumor cells transfected with the gene for hexameric E. coli PNP activate the prodrug, whereas normal cells do not. Excellent in vivo activity against tumors that express E. coli PNP (16–19) has been demonstrated with the analogs 9-β-D-[2-deoxycytidinofuranosyl]-6-methylpurine (MeP-dR), 9-β-D-arabinofuranosyl-2-fluoroadenine (F-araA), and 2-fluoro-2′-deoxyadenosine (F-Ado), which are not substrates for trimeric PNPs. The toxic purine analogs (6-methylpurine (MeP) and 2-fluoroadenine (F-Ado)) generated from these prodrugs readily diffuse across cell membranes, have high bystander activity not requiring cell-to-cell contact, and can kill the complete cell population even with gene expression in only 0.1–1% of the cells (15, 20, 21). In addition, MeP and F-Ado are toxic to both proliferating and nonproliferating tumor cells (22). These characteristics distinguish PNP anticancer gene therapy from the well studied herpes simplex virus thymidine kinase anti-cancer gene therapy strategy (23–29) and suggest that the selective generation of MeP or F-Ado could elicit considerable activity against solid tumors that have a low growth fraction.

To optimize PNP anticancer gene therapy, the prodrug should be completely inert with respect to human enzymes and efficiently cleaved only by tumor cells expressing E. coli PNP. But whereas excellent in vivo antitumor activity has been demonstrated with MeP-dR, F-araA, and F-Ado, these compounds all have dose-limiting toxicities. A better understanding of the structural basis for substrate specificity of the enzyme would allow modification of the prodrugs coupled with enzyme redesign to maintain activity levels while decreasing toxicity. Therefore, we have used crystallography to study the binding of MeP-dR, F-Ado, and other nucleoside analogs to E. coli PNP.

In our previous work (10), the purine binding site was ident-
tified in complexes with R1-P and 6-iodopurine, whereas Koeller et al. determined the structure of complexes with the inhibitors formycin B (FMB; 8-aza-7-deazainosine) (30) and formycin A (8-aza-7-deazaadenosine) (31). We report here the structure of *E. coli* PNP in association with 10 purine nucleosides (Fig. 1), which vary in both the base (hypoxanthine, 8-aza-7-deazahypoxanthine, adenine, 7-deazaadenine, 2-fluoroadenine, 6-methylpurine, and 6-methylthiopurine) and sugar (ribose, 2'-deoxyribose, arabinose, and xylose) portion of the molecule. We have also performed kinetic studies on the substrates and carried out computer modeling to examine the role of sugar modifications on reactivity. This information, together with knowledge about the structure of mammalian PNPAs (32, 33), should aid in the design of more effective produgs that can be readily cleaved by *E. coli* PNP. Furthermore, an understanding of substrate analog binding should allow us to rationally change the enzyme to cleave novel produgs that are not cleaved by either human or wild type bacterial PNPAs.

### EXPERIMENTAL PROCEDURES

**Crystallization—**Purified *E. coli* PNP was a gift from Dr. George W. Koszalka of Wellcome Research Laboratories. Crystals have been reported in hexagonal (34) and monoclinic (10) forms. Soaks using monoclinic crystals gave poor ligand electron density. Therefore, the hexagonal crystal form (space group P622) with three monomers designated A, B, and C per asymmetric unit) was used. Crystals were grown by hanging drop vapor diffusion at room temperature. Drops contained 3 μl of 60 mg/ml *E. coli* PNP and 3 μl of well solution consisting of 1 ml of 30% ammonium sulfate and 50 mM citrate buffer, pH 5.4. Needle-like crystals appeared in about 1 week, with approximate dimensions of 1 mm in length × 0.1 mm in thickness. Typical unit cell dimensions were a = 122.0 Å and c = 242.8 Å with 56% solvent content.

**X-ray Intensity Measurements—**Ligands were soaked into the crystals for 24–36 h, with concentrations ranging from 5 to 50 mM depending on solubility. Crystals were flash frozen at 100 K with liquid nitrogen, using 30% glycerol as a cryoprotectant, and typically diffracted to about 2.2 Å resolution. Data were collected at CHESS stations A1, F1, and F2. Various CCD X-ray detectors were used, including Area Detector Systems Quantum 1 single module and Quantum 4 mosaic CCD detectors and a Princeton Scientific Instruments 2k CCD detector. Exposure times ranged from 30 to 50 s per frame with a frame typically consisting of 1° of rotation. A total of 50–120° of data were collected depending on beam time constraints and the type of detector used. The data were processed using the programs DENZO (35) and SCALEPACK (36). Table I summarizes the data collection statistics.

**Structure Determination and Refinement—**The structures were solved by molecular replacement using the program X-PLOR (37). The *E. coli* PNP structure from the monoclinic crystal form (Protein Data Bank code 1ECP) was used as a search model. A set of 3-fold related monomers was positioned in the unit cell and used to calculate a difference Fourier map using the PHASER software (38). The structure was refined by simulated annealing with restricted, isotropic individual B-factors. A final round of crystallographic energy minimization and B-factor refinement, including all reflections, was performed using CNS (39). Starting R-factors ranged from 38 to 42%. Most of the protein backbone required only minor changes. Manual side chain adjustments were performed using the programs CHAIN (40) and O (41). The loop region from residue 205 to 220 showed varying degrees of disorder, with the best electron density generated using a weighted (2mFo - DFc) map based on SIGMAA (42) from the CCP4 package (43). Some loops could be built at the 0.8 σ level, but others have missing residues in the final model. Temperature factors in this region were much higher than for the rest of the molecule. Models of inosine (Ino), FMB, 7-deazaadenosine (TBN), 2-fluoroadenosine (F-Ado), 9-β-D-ribofuranosyl-6-methylthiopurine (MTP-R), MeP-DR, and F-Ado were directly constructed into the corresponding difference electron density. The ligand densities for adenosine (Ado), 9-β-D-arabino furanosyl-adenine (AraA), and 9-β-D-xylofuranosyl-adenine (XylA) were less clear. These maps were improved by 3-fold averaging using the program RAVE (44).

FIG. 1. Structures of the nucleoside ligands bound to *E. coli* PNP. IUPAC numbering conventions differ for the purine and formycin ring systems. The purine numbering system, shown for inosine, is used throughout the paper for consistency. Abbreviations used throughout are shown in parentheses. *R*, ribose.
Monte Carlo simulations and two genetic algorithm runs were performed. The Monte Carlo simulation gave reasonable results. These calculations were performed using the Cornell Theory Center work station cluster. Four Monte Carlo simulations and two genetic algorithm runs were performed with a distance cut-off of 8.0 Å. For the four Monte Carlo simulations, the numbers of trials were set at 10,000, 20,000, 40,000, and 20,000, and dielectric constants were set at 1, 2, and 4, respectively. For genetic algorithm runs, the number of generations was set at 10,000, and the dielectric constant was set at 2 or 4. The results of the genetic algorithm runs were inconsistent with no convergence.

TABLE I
Data collection and refinement statistics

| Experiment | K<sub>m</sub> (µM) | V<sub>max</sub> (k<sub>cat</sub>/K<sub>m</sub>) |
|------------|------------------|-------------------------------|
| F-Ado      | 2                | 12 ± 1                        |
| F-dAdo     | 4                | 23 ± 9                        |
| MeP-dR     | 2                | 136 ± 20                      |
| F-araA     | 2                | 958 ± 243                     |
| XylA       | —                | —                             |

<sup>a</sup> Numbers in parentheses are for the highest resolution shell.
<sup>b</sup> r.m.s., root mean square.

RESULTS AND DISCUSSION

Structure of E. coli PNP—The structures of unliganded E. coli PNP (10) and complexes with formycin B (30) and a formycin A analog (31) have been previously reported. The PNP hexamer is a disc 60 Å thick and 100 Å in diameter, with D<sub>1</sub> symmetry and a noncrystallographic 3-fold axis in the crystal form utilized for these studies (Fig. 2). The monomers alternate in an up/down fashion around the disc, with three of the active sites near the top and three near the bottom. Each of the six active sites utilizes residues from a pair of 2-fold related monomers. In the present work, the structures of the three crystallographically independent monomers vary slightly within the hexamer and from complex to complex. The largest difference is the conformation of the loop region from residues 205–220, which follows the catalytically important residue Asp<sup>204</sup>. The other three subunits of the hexamer are generated by crystallographic symmetry. No global differences in domain orientation are observed for the previously reported structures and the 10 complexes reported here.

Each monomer consists of a mixed β-sheet core flanked by eight α-helices. The core can be divided into a large eight-stranded β-sheet and a smaller five-stranded β-sheet that pack together to form a distorted β-barrel, an arrangement also found in human PNP (2), bovine PNP (32, 33), and human 5′-deoxy-5′-methylthioadenosine phosphorylase (48), although there is little sequence homology between the E. coli enzyme and the trimeric class mammalian enzymes (10).

Active Site of E. coli PNP—The previously determined structures of E. coli PNP (10, 30, 31) identified the residues involved in substrate binding and catalysis (Fig. 3). The purine binding...
site consists of Ala\textsuperscript{156}, Phe\textsuperscript{159}, Val\textsuperscript{178}, Met\textsuperscript{180}, Ile\textsuperscript{206}, and Asp\textsuperscript{204}. The first four of these residues form a hydrophobic pocket around the purine base. Phe\textsuperscript{159} is located between the purine base and the hydrophobic face of the sugar and makes an angle of \(-60^\circ\) with the plane of the purine ring. Met\textsuperscript{180} also lies between the purine base and the hydrophobic face of the ribose group. It has been proposed that in \textit{E. coli} PNP, a protonated Asp\textsuperscript{204} stabilizes the transition state, in which electron density from the weakening bond is transferred to the purine ring, by donating a hydrogen bond to the purine N-7 atom (10). Evidence for protonation of Asp\textsuperscript{204} comes from fluorescence studies on the inhibitor FMB, which exists in tautomeric equilibrium between N-7–H and N-8–H forms (IUPAC conventions for ring numbering systems vary among bases; for consistency, we use only the purine ring numbering as shown for inosine in Fig. 1). Although the N-8–H form (which could hydrogen-bond to a protonated Asp\textsuperscript{204}) is the minor (\(-20\%\)) tautomer in solution (30, 49), fluorescence studies have detected an equilibrium shift in favor of this tautomer upon binding to \textit{E. coli} PNP (49). Also consistent with the proposed role for Asp\textsuperscript{204}, mutating this residue to Ala results in a \(-100\)-fold reduction in activity on MeP-dR and F-araA.\textsuperscript{2}

The ribose binding site consists primarily of interactions with Glu\textsuperscript{181} and His\textsuperscript{4}\# (residues from an adjacent monomer are designated by \# throughout). The Glu\textsuperscript{181} side chain accepts hydrogen bonds from the 2’- and 3’-hydroxyl groups, and His\textsuperscript{4}\# accepts a hydrogen bond from the 5’-hydroxyl group. The phosphate group hydrogen bonds with the 3’-hydroxyl and, in some structures, the 2’-hydroxyl.

\textbf{Structures of \textit{E. coli} PNP Complexes—}Unlike mammalian PNP\textsubscript{s}, which are highly specific for 6-oxopurine nucleosides (or 2’-deoxynucleosides) (50, 51), \textit{E. coli} PNP accepts 6-aminopurine nucleosides as substrates and shows some activity for purine nucleosides with ribosyl modifications (52, 53). The structures of the nucleosides used here (Fig. 1) include a variety of modifications to the purine bases and ribosyl group, but all are \(\beta\)-d-furanosides. The ligands bind to the active site in the high \textit{syn} conformation about the glycosidic bond and generally adopt a C-4’-endo sugar pucker (Fig. 4A). Strong electron density shows the binding of each ligand, except AraA and Ado, where only weak density with poor sugar/base connectivity is visible. The AraA complex also shows extremely high ligand B-factors (>75 Å\textsuperscript{2} in each monomer), suggesting low ligand occupancy. Table II shows the kinetic constants for seven substrates (no cleavage of XylA by \textit{E. coli} PNP was detected). The \(K_v\) values for FMB and TBN were previously determined to be 5 \(\mu\text{M}\) (54) and 120 \(\mu\text{M}\) (55), respectively. The collection of structures along with the kinetic data allows us to examine the roles of substituents at positions 1, 2, 6, and 7 of the purine base and the effects of changing stereochemistry at positions 2’ and 3’ of the sugar.

\textbf{Structural Basis for Ribose Selectivity—}PNPs prefer ribose as the sugar. \textit{E. coli} PNP shows some activity for arabinose sugars and no detectable activity for xylose sugars. The structures of AraA and XylA complexed with PNP suggest a possible explanation. In the case of ribonucleosides, bond breaking occurs well ahead of bond formation in the phosphorolytic direction (5, 6), leading to the proposal of an oxocarbenium intermediate (7). Similar mechanisms are also suggested for other enzymes catalyzing reactions similar to PNP (56). For \textit{E. coli} PNP, the leaving group (the purine base) is stabilized by a hydrogen bond from protonated Asp\textsuperscript{204} to purine N7 (31, 49). As the glycosidic bond begins to cleave, the resulting oxocarbenium ion is probably stabilized by the negatively charged phosphate group, since no amino acid side chain is available for this purpose (7). The reaction is completed by bond formation when the phosphate ion captures the oxocarbenium intermediate. Glycosidic bond cleavage requires lengthening of the glycosidic bond and flattening of the C-4’–O-4’–C-1’–C-2’ torsion angle of

\textsuperscript{2} E. M. Bennett, C. Li, P. W. Allan, W. B. Parker, and S. E. Ealick, unpublished data.
the ribose so that those atoms are coplanar in the oxocarbenium ion intermediate (Scheme 1).

Ribose normally makes two hydrogen bonds to Glu181, one from the O-2’-hydroxy group to one of the carboxylate oxygen atoms and one from the O-3’-hydroxy group to the other carboxylate oxygen atom. The 3’-hydroxyl group of 2’-deoxy ligands can interact with both carboxylate oxygen atoms, as in MeP-dR (at 3.1 Å), or maintain only the usual single (but stronger, 2.6-Å) hydrogen bond, as in F-dAdo. Similar B-factors were observed for both compounds, and the $K_m$ for F-dAdo was only marginally lower than for F-dAdo, suggesting that loss of the O-2’-hydroxy group may not significantly affect substrate binding. When the glycosidic bond lengthens during the reaction, flattening of the C-4’-O-4’-C-1’-C-2’ torsion requires that either the C-1’ atom or the base or both must move. We investigated this motion in ribose substrates by energy-minimizing the crystal structures of Ado and Ino using a modified AMBER force field with parameters forcing the nucleosides to adopt a conformation mimicking the transition state between the substrate and oxocarbenium intermediate. For Ado, the crystallographically determined torsion angles in the three monomers range from 20 to 23°. Restricted energy minimization with parameters requiring a 50% increase in the glycosidic bond length and 90° O-4’–C-1’–N-9 and C-2’–C-1’–N-9 angles caused the C-1’ atom to move toward the plane of the other atoms, giving a final torsion of 7°, even without explicitly changing this torsion parameter to require flattening. With Ino, the torsion value decreased from 14–16° to 8°, confirming that flattening of the torsion is a natural response to lengthening the glycosidic bond.

In the case of XylA, the crystallographically determined sugar pucker is C-3’-exo (Figs. 4C and 5B), which allows XylA to maintain hydrogen bonding between Glu181 and the C-2’- and C-3’-hydroxyl groups (Fig. 6E). The C-3’-exo pucker means that C-4’, O-4’, C-1’, and C-2’ are nearly coplanar before the reaction begins (with a torsion angle of 2°), making glycosidic bond lengthening incompatible with the required planar geometry. Upon AMBER energy minimization, the torsion value increased to 22°, suggesting that XylA is not a substrate because its pucker makes the oxocarbenium intermediate energetically inaccessible.

The crystallographically determined AraA pucker of C-1’-endo (Fig. 4B) in all three monomers is compatible with simultaneously flattening the required torsion angle while lengthening the glycosidic bond, but AMBER energy minimization showed that the resulting intermediate ring was flat, with all five atoms nearly coplanar. This sterically unfavorable conformation contrasts with the C-3’-exo pucker adopted by the Ino and Ado sugars after AMBER minimization and may be one reason for the poor catalysis of arabinosides. However, because the electron density used to establish the original pucker was poor, this result must be interpreted with caution. Another factor in the low activity of PNP with AraA may be an energetic penalty for desolvating O-2’; the crystal structure showed no possible hydrogen bonding partners for this atom. It may be possible to mutate PNP to bind AraA and XylA with an appropriate geometry for catalysis, but the redesign required to accomplish this change would probably be extensive.

Role of Asp204 in Binding and Catalysis—The purine binding site is largely hydrophobic, but N-1, N-7, and O-6 or N-6 typically form hydrogen bonding interactions with protein side chains or water molecules. The proposed catalytic mechanism suggests that a hydrogen bond between Asp204 and N-7 should be observed in complexes with substrates. This hydrogen bond was not observed in the previous study with the inhibitor FMB (30) (but in our FMB complex, one subunit has a 2.9-Å hydrogen bond), but it was present in a complex with a formycin A analog (31). These structures left open the question of how closely purine base binding matches the binding of formycins. A comparison of the 10 present complexes shows that the overall ligand binding geometry is similar for both 6-oxo and 6-amino

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**Fig. 4. Observed puckers of different sugars.** The proposed mechanism for PNP requires that the C-1’ atom be above the plane defined by C-4’, O-4’, and C-2’ (see “Results and Discussion”). This condition is met by the C-4’-endo pucker of ribose (A) and the C-1’-endo pucker of arabinose (B) but not by the C-3’-exo pucker of xylene (C).

**Fig. 5. Stereodiagram of the relative orientation of E. coli PNP ligands from the C monomer (except MeP-dR, B monomer) after superposition on the Ino C monomer.** A, ligands with changes in the base: Green, Ino; dark blue, Ado; purple, FMB; red, F-dAdo; black, MeP-dR; light blue, MTP-R; yellow, TBN. B, ligands with changes in the sugar. Green, Ino; purple, F-dAdo; light blue, XylA. Final 2F_o - F, density is shown contoured at 2.0 σ for XylA to show the differing pucker relative to ribose compounds. This figure was created with Bobscript (61) and Raster3D (60).
FIG. 6. Stereodiagrams for ligands bound to the active site of *E. coli* PNP. A, MeP-dR; B, MTP-R; C, F-Ado; D, F-dAdo; E, XylA. This figure was created with Molscript (59) and Raster3D (60).
bases (Fig. 5), including formycin B. Although there is clearly only one major mode of base binding, the minor variations are enough to affect some contacts made by the base.

Most significantly, good substrates do not necessarily show an Asp204/N-7 hydrogen bond, despite the evidence from mutation studies that Asp204 is critical for catalysis. Although the natural substrates Ado and Ino show a hydrogen bond in one subunit each, no hydrogen bond is observed in any subunit for MeP-dR and MTP-R, both of which have similar catalytic efficiencies with PNP (Table II). In the C monomer, MeP-dR is displaced deeper into the active site pocket, farther from the active site opening and phosphate group. The 4.9-Å distance between the phosphate and sugar C-1/H1 suggests that this conformation would not lead to catalysis. In the A monomer and B monomer (Fig. 6A), MeP-dR binds with the general conformation observed in all monomers of all of the other complexes.

A significant difference relative to other complexes is observed in all three monomers of the MTP-R complex, where the Asp204 side chain rotates toward the active site opening and cannot interact with the purine N7 (Fig. 6B). The change in position is probably due to steric conflicts between the normal Asp204 rotamer and the MTP-R sulfur atom. A similar displacement of Asp204 may be envisioned for binding 7-methyl analogs, which have previously been confirmed as substrates (57). However, these analogs already carry a positive charge at N-7, whereas MTP-R would presumably still require a nearby proton donor for catalysis. Although a water molecule is observed hydrogen-bonding with N-7 and the side chain of Ser203, Asp204 is still required, since mutations to Asn and Ala both prevent cleavage of MTP-R.2 Taken together, the structures and mutation data suggest that Asp204 is not critical for initial ligand binding but must move in concert with the substrate during catalysis to donate a proton to N-7 as the glycosidic bond breaks. Movement of Asp204 during the reaction is consistent with the work of Koellner, who proposed that Asp204 also changes conformation after protonation of N-7 (31).

Structurally Conserved Waters at N-1—In 6-oxo-purines, the N-1 position is protonated, whereas in 6-aminopurines the N-1 position is unprotonated. In the PNP complexes, the N-1 position interacts with a series of water molecules, the first of which can be either a donor or acceptor for the purine N-1 position and for the second water molecule (Fig. 7). In complexes with substrates, the first water molecule must be a hydrogen bond donor to the backbone carbonyl group of Phe159, whereas the first water molecule in some of the inhibitor complex subunits is too far from this oxygen atom to form a hydrogen bond. The second water molecule must donate a hydrogen bond to the backbone carbonyl of Leu158 but can be either a donor or acceptor to a third water molecule, which is observed 50% of the time. In some cases, an additional water molecule bridges to the side chain of Asp112. The residues lining the water channel are highly conserved.

Consistent with their 6-oxo substrate requirement, mammalian PNP's instead utilize a conserved glutamate as hydrogen bond acceptor for the protonated N-1 atom (2). In human 5'-deoxy-5'-methylthioadenosine, a water molecule hydrogen-bonds to the N-1 atom, but a network of highly conserved residues requires that this water molecule donate a hydrogen bond to the unprotonated N-1 atom (48). In addition to acting either as a hydrogen bond acceptor or donor for N-1, E. coli PNP tolerates changes that would displace the water molecule altogether; 1-methyladenosine is a good substrate for E. coli
NP, and both 1-methylformycin A and 1-methylformycin B are inhibitors (54).

Effect of C-2 Substituents—Substitutions at C-2 can be made without affecting the water channel, as observed in both the F-Ado (Fig. 6C) and F-dAdo (Fig. 6D) complexes. coli PNP also accepts 2-amino substrates such as guanosine. The environment for the C-2 substituent is hydrophilic, consisting of Ala156, Val178, Met180, and Phe159. With 2-fluoro compounds, the fluorine atom packs against the Met180 γ-carbon and sulfur, providing more favorable van der Waals interactions than the hydrogen atom of Ado. The improved steric fit may account for the stronger electron density and significantly lower B-factors observed for F-Ado and F-dAdo relative to Ado.

Role of Arg24—Because the crystals were grown from high concentrations of ammonium sulfate, and because sulfate is a known inhibitor of PNP, a sulfate ion probably occupies the phosphate binding site in the coli PNP complexes. However, because PNP has two binding affinities for phosphate, one of which has a Kₚ much lower than that for sulfate (58), the identity of the bound ion cannot be conclusively determined. The phosphate binding site consists mainly of two arginine residues (87 and 43#), backbone interactions with Gly20, and side chain interactions with Ser90, Arg24 sometimes participates as well. This positively charged cavity is buried within the active site. Upon ligand binding, the phosphate site is further shielded by the nucleoside sugar.

Arg24 may provide an explanation for the existence of two difference binding states for phosphate. Koellmer observed small differences around Arg24 in open and closed protein conformations, with higher side chain B-factors and more diffuse density in the open form and stabilizing interactions between Arg24 and a moved helix in the closed form, and suggested that these differences accounted for the two different phosphate affinities (31). We consistently observe a more significant variation; in all of the complexes except AraA (where electron density indicates very poor nucleoside binding), the Arg24 guanidinium group interacts with the phosphate in only one of the three independent active sites and is far away (up to 8 Å, with clear density indicating the different conformation) in the other two. Although movement of Arg24 is the most promising structural explanation for observed phosphate affinities, note that sulfate (present at high concentrations for all coli PNP complexes published to date) shows only a single binding affinity (58).

Modeling Studies of Ribose 1-Phosphate—In earlier studies of coli PNP using monoclinic crystals (10), attempts to observe bound R 1-P were unsuccessful because of low occupancy. In the current work, high sulfate concentrations prevent R 1-P binding. Therefore, we have used modeling studies to better understand R 1-P binding.

The docking studies predict that R 1-P adopts a C-2′-exo conformation. This is the most common ribose pucker and was observed in the bovine PNP/hypoxanthine/R 1-P crystal structure (33). The C-3′–C-4′–C-5′–O-5′ torsion angle is 174.3°, the C4′–O4′–C1′–O torsion angle is 102.8°, and the O-4′–C-1′–O–P torsion angle is 63.9°. Comparison with the Ino complex shows that the ribose ring conformation flips after cleavage such that the C-1′ atom moves away from the purine base and about 0.9 Å toward the phosphate binding site (Fig. 8). The phosphate group moves 1.0 Å toward the sugar ring. The purine base moves 1.1 Å along the line of the glycosidic bond but retains the same general interactions. R 1-P maintains hydrogen bonds with Gly20, Arg57, Ser200, Arg53#, and Glu181, but the O-5′ contact with His5# is lost. The major movement of the ribosyl group is a rotation about a line drawn from O-4′ to the midpoint of the C-2′–C-3′ bond. In contrast to trimeric bovine PNP, in which almost all movement is in the sugar (33), the modeling predicts that structural changes necessary to break the bond in the coli enzyme are shared roughly equally by the sugar, base, and phosphate. Based on the present structures, the movement of the base would be required for some substrates (such as MTP-R) to accept a proton from Asp204.

Implications for Substrate Design and Protein Engineering—The goal of this project at the outset was to identify coli PNP substrates that, upon cleavage, would release a cytotoxic agent such as 6-methylpurine. Activity measurements show that modifications to the ribosyl group are generally detrimental, and modifications to the purine are much more easily tolerated. However, the gene therapy strategy requires a nontoxic nucleoside prodrug and a toxic purine analog, greatly restricting the choice of purine base. Interactions between the ribosyl group and the enzyme are important for orienting the substrate and for maintaining a pucker compatible with oxocarbenium ion formation. This leaves few possibilities for substrate modification and suggests that compounds such as MeP-dR may already be close to optimal for the wild type enzyme. However, further improvement may be possible by combining multiple modifications that individually enhance binding or catalysis.

Because the enzyme active site allows little room for modification of the sugar, we have also begun to turn our attention to protein engineering as a way of improving catalytic efficiency. By matching a given substrate to an engineered PNP, it may be possible to optimize the conversion of the prodrug. This series of 10 complexes suggests possible strategies for achieving this goal. Residues near the sugar C-5′ position (Met180, Met180, and Ile71#) are believed to be important primarily for hydrophobic packing rather than catalysis and might be mutated to smaller residues and tested with modified versions of MeP-dR, F-dAdo, or F-AraA with bulky substituents added to C-3′ or C-5′. In addition, the structure of PNP complexed with nonstandard sugars suggests residues that could be modified to improve the cleavage of arabinonucleosides. Finally, combinations of the above approaches may be considered. Modeling and crystallographic studies are currently under way to explore possible PNP/prodrug pairs based on these ideas.
Structural Basis for Substrate Specificity of *Escherichia coli* Purine Nucleoside Phosphorylase
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