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Calf spleen purine nucleoside phosphorylase: structure of its ternary complex with an N(7)-acycloguanosine inhibitor and a phosphate anion

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The calf spleen purine nucleoside phosphorylase (PNP) ternary complex with an N(7)-acycloguanosine inhibitor and a phosphate ion has been crystallized in the cubic space group $P2_13$, with unit-cell parameter a = 94.11 Å and one monomer per asymmetric unit. X-ray diffraction data were collected using synchrotron radiation (Station X31, EMBL Outstation, DESY, Hamburg). The crystal structure was refined to a resolution of 2.2 Å and R and $R_{\rm free}$ values of 17.5 and 24.5%, respectively. The acyclonucleoside inhibitor is bound in the active site in an inverted ('upside-down') orientation of the purine base compared with natural substrates. The side chain of Asp243 forms two hydrogen bonds with the base ring: N^{δ} donates a hydrogen to N(3) and O^{δ} accepts a hydrogen from the guanine N(2)-amino group. N(1)—H of the base is hydrogen bonded to O^{ε} of Glu201, while N(9) accepts a hydrogen bond from Thr242 O^{γ}. In addition, a water molecule (W417) bridges the N(2)-amino group of the base and O^{ε} of Glu201. In the phosphate-binding site, a phosphate ion is bound to Ser33, His64, Arg84, His86, Ala116 and Ser220. The acyclic chain of the N(7)-acycloguanosine inhibitor is in a folded conformation and together with a water molecule (W388) occupies the pentose-binding site, with possible hydrogen bonds to Tyr88 O^{η} and His257 $N^{\delta1}$. This new binding mode fully accounts for the previously observed substrate properties of 7-β-D-ribofuranosides of hypoxanthine and guanine. It also provides a new starting point for the design of inhibitors of PNP for therapeutic and other applications.

1. Introduction

Purine nucleoside phosphorylase (PNP; purine nucleoside: orthophosphate ribosyl transferase; E.C. 2.4.2.1) is the key enzyme of the purine-salvage pathway (Stoeckler, 1984; Montgomery, 1993). In mammals, 'low molecular mass' ($M_r \simeq 90\,000$) homotrimeric PNPs catalyse the reversible phosphorolytic cleavage of the glycosidic bond of 6-oxopurine nucleosides (ribo- and 2′-deoxyribo) and some analogues,

$$\text{purine nucleoside} + \text{orthophosphate} \longleftrightarrow \text{purine base} + \text{ribose-1-phosphate}.$$

The specificity of prokaryotic PNPs is usually not restricted to 6-oxo purine nucleosides: the genomes of several prokaryotic organisms, *e.g. Escherichia coli* and *Bacillus subtilis*, encode two distinct PNPs, one with specificity similar to mammalian enzymes; the second, 'high molecular mass' mainly homo-hexameric phosphorylases ($M_{\rm r} \simeq 110\,000$–$150\,000$), with broader specificity, also accept 6-aminopurine nucleosides as a substrate (*e.g.* Jensen & Nygaard, 1975; Jensen, 1978).

For many fundamental and practical reasons, considerable attention is devoted to PNPs isolated from both eukaryotic and prokaryotic organisms. With some substrates, PNPs show complex non-Michaelis kinetic behaviour pointing to possible cooperativity between subunits or non-identity of active sites (*e.g.* Jensen & Nygaard, 1975; Stoeckler, 1984; Ropp & Traut, 1991*a*,*b*; Wielgus-Kutrowska *et al.*, 1997). Therefore, PNPs are interesting models for studies of enzyme catalysis. Potent inhibitors of human and parasitic PNPs are potential chemotherapeutic agents (*e.g.* Stoeckler, 1984; Daddona *et al.*, 1986; Montgomery, 1993). Non-specific bacterial phosphorylases are useful tools for enyzmatic synthesis of purine nucleosides, including some with chemotherapeutic activity (*e.g.* Krenitsky *et al.*, 1981; Utagawa *et al.*, 1985; Hennen & Wong, 1989; Ling *et al.*, 1990, 1994; Shirae & Yokozeki, 1991). Recently, *E. coli* PNP was shown to be a promising candidate for tumour-directed gene therapy (Sorscher *et al.*, 1994; Hughes *et al.*, 1995; Puhlmann *et al.*, 1999).

Calf spleen PNP, a 'low-molecular mass' PNP, is very interesting from both academic and practical aspects. Its specificity and response to inhibitors are similar to those of the human enzyme (*e.g.* Agarwal *et al.*, 1975; Stoeckler *et al.*, 1978; Bzowska *et al.*, 1990, 1991), but in contrast to the latter it is more stable and has no isoelectric variants (Edwards *et al.*, 1971; Zannis *et al.*, 1978). Therefore, it is more convenient than its human counterpart for studies of the mechanism of phosphorolysis and the design of potent inhibitors. Both enzymes show conservation of active-site amino acids (Koellner *et al.*, 1997; Narayana *et al.*, 1997; Mao *et al.*, 1998). The purine ring N(7) and O^6 are hydrogen bonded to Asn243 O^{δ} and Asn243 N^{δ}, respectively. Glu201 $O^{\varepsilon 1}$ accepts the hydrogen of the purine-ring N(1) and, in the case of guanine, also from the amino group at C(2). The best inhibitors identified to date are either 'bisubstrate' analogue inhibitors, such as acyclovir diphosphate (Tuttle & Krenitsky, 1984), or the 9-cyclo- and 9-acyclonucleosides of 9-deazaguanine and 9-deazahypoxanthine. The latter utilize the above binding mode for potent binding (*e.g.* Montgomery *et al.*, 1993; Niwas *et al.*, 1994; Bantia *et al.*, 1996; Miles *et al.*, 1998). Since these 9-deaza analogues have a hydrogen at N(7) of the imidazole ring, like the N(7) tautomers of hypoxanthine and guanine, they are able to form a strong hydrogen bond with Asn243 O^{δ}. The transition-state analogue inhibitors immucillins H and G, showing inhibition constants in the picomolar range for mammalian PNPs (Miles *et al.*, 1998), also belong to the 9-deaza class of inhibitors.

It was previously shown (Bzowska *et al.*, 1994) that mammalian PNPs are also able to catalyze phosphorolysis of purine nucleosides with the ribose attached to N(7) of the base instead of the 'normal' N(9) and that inhibitors that are non-cleavable analogues of 7-β-D-ribofuranosylguanine may have a higher affinity for the enyzme than their N(9)-counterparts. Preliminary X-ray studies of the calf spleen PNP binary complex with the inhibitor N(7)-acycloguanosine demonstrated that such inhibitors have a different purine-base binding mode, with the base bound in an inverted ('upside-down') orientation and one of the H atoms of the N(2) amino group of guanine bonded to Asn243 (Bzowska *et al.*, 1995). We now describe the high-resolution (2.2 Å) X-ray crystal structure of calf spleen PNP in a ternary complex with N(7)-acycloguanosine and a phosphate anion.

2. Experimental

2.1. Crystallization

Calf spleen purine nucleoside phosphorylase was purchased from Sigma; PEG 4000, Tris buffer, citric acid, sodium citrate and magnesium chloride were purchased from Merck. The N(7)-acycloguanosine inhibitor was synthesized as described previously (Bzowska *et al.*, 1994).

The commercially available enzyme has a specific activity of about 20 U mg^{-1}. 1 U of PNP is the amount of enzyme which leads at 298 K to phosphorolysis of 1 µmol of inosine per minute to hypoxanthine and ribose-1-phosphate (0.5 m*M* inosine as substrate, 50 m*M* phosphate pH 7 as both buffer and substrate). The enzyme preparation shows a single band on SDS–PAGE. It was used for crystallization of the ternary complex without further purification. Prior to crystallization, the enzyme was washed four times on a 30 kDa cutoff Centricon filter (Amicon) with 10 m*M* citrate buffer pH 7 and 1 m*M* β-mercaptoethanol in order to remove the large excess of ammonium sulfate (3.2 *M*) present in the commercial preparation. The protein sample was tested for phosphate using the malachite green method (Lanzetta *et al.*, 1979), which showed its presence. Even after the washing procedure, a binary complex of PNP with phosphate remained. To this binary complex (protein at 13 mg ml^{-1}, about 400 µ*M* subunit concentration), a solution of N(7)-acycloguanosine inhibitor was added to a final ligand concentration of 1.8 m*M*. The complex was then crystallized as described previously, using the vapour-diffusion hanging-drop method (Bzowska *et al.*, 1995; Koellner *et al.*, 1997).

2.2. X-ray data collection and processing

The ternary complex of calf spleen PNP with N(7)-acycloguanosine and phosphate crystallized in the cubic space group $P2_13$, with unit-cell parameter a = 94.11 Å (V = 833 500 $Å^3$) and one monomer per asymmetric unit (Z = 12). A complete set of X-ray diffraction data was collected at 277 K from one crystal (dome-shaped, 0.15 mm diameter) at the EMBL Outstation at DESY (Hamburg) on beamline X31 with monochromatic X-ray synchrotron radiation (λ = 0.908 Å) using a MAR Research imaging-plate detector. Data were processed using the programs *DENZO* and *SCALEPACK* (Minor, 1993; Otwinowski, 1993). Measured intensities were converted to structure-factor amplitudes using the program *TRUNCATE* incorporated in the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Data-collection and data-processing statistics are shown in Table 1.

2.3. Refinement

Restrained least-squares refinement was carried out with the *CNS* program package version 1.0 (Brunger *et al.*, 1998) using atomic coordinates of the isomorphous complex of calf

spleen PNP with hypoxanthine (Protein Data Bank code 1vfn; starting R value 0.30). Omit and difference electron-density maps ($\big||2F_o| - |F_c|\big|$ and $\big||F_o| - |F_c|\big|$) were calculated with the same program. Model building was performed on an ESV station using the graphics program *FRODO* (Jones, 1978). 113 water molecules with good hydrogen-bonding geometry were included in the model ($O_W\cdots O/N$ distances in the range 2.3–3.5 Å). The electron density of amino acids Ser59–Ala65 from a peripheral loop, which were not well defined in the binary complex with hypoxanthine (Koellner *et al.*, 1997), could now be clearly interpreted. The three N-terminal amino acids as well as the sequence Glu250–Gln252 could not be modelled owing to poor electron density. One Mg^{2+} and one unidentified metal atom were found with the same geometry as in the binary complex of PNP with hypoxanthine (Koellner *et al.*, 1997). The final model has an r.m.s. deviation of 0.26 Å from the starting model. The final crystallographic R value is 17.5% and R_{free} is 24.5%. Fig. 1 shows the omit difference electron density in the ternary complex at the 2.5σ level, where inhibitor, phosphate ion and four neighbouring water molecules were not included for several refinement cycles. Relevant numerical data for the refinement are given in Table 1.

Table 1
Data collection and refinement statistics for the ternary complex of PNP with N(7)-acycloguanosine and phosphate ion.

Data collection	
Space group	Cubic, $P2_13$
Unit-cell parameter (Å)	a = 94.11
Detector	MAR Research imaging plate, 180 mm
X-ray source	Synchrotron radiation, X31 EMBL Hamburg
Temperature (K)	277
Wavelength (Å)	0.908
Measured reflections	38538
Unique reflections	12801
R_{sym}† (%)	9.7
Resolution range (Å)	22.2–2.2
Completeness (%)	89.0
In last resolution shell (2.28–2.20 Å) (%)	87.2
Refinement statistics	
R factor (%)	17.5
R_{free} (10.3% reflections) (%)	24.5
No. of protein atoms	2167
No. of substrate atoms	24
No. of water molecules	113
No. of metal atoms	2
R.m.s. deviations from ideal distances (Å)	0.011
R.m.s. deviation from ideal bond angles (°)	1.7
R.m.s. deviation of torsion angles (°)	24.5
R.m.s. deviation of improper angles (°)	1.04
Mean B factor for main-chain residues (Å^2)	21.7
Mean B factor for side-chain residues and water molecules (Å^2)	25.5
Mean B factor for substrate atoms (Å^2)	29.2
B factor for Mg^{2+} (Å^2)	17.8
B factor for unidentified metal-binding site, refined as Zn^{2+} (Å^2)	16.3
Estimated coordinate error (after Luzzati, 1952) (Å)	0.22
Estimated coordinate error (from σ_A; Read, 1986) (Å)	0.21

† $R_{sym} = \sum_h \sum_i \big||I| - I(h)_i\big| \big/ \sum_h \sum_i I(h)_i$ for symmetry-related observations.

3. Results and discussion

3.1. The overall structure of the enzyme

The overall structure of calf spleen PNP complexed with N(7)-acycloguanosine and phosphate is similar to the binary complexes of the enyzme with N(7)-acycloguanosine (Bzowska *et al.*, 1995) and with hypoxanthine (Koellner *et al.*, 1997) and to various binary and ternary complexes presented by Mao *et al.* (1998). The core of the enzyme is formed by a nine-stranded mixed β-barrel flanked by nine α-helices and two short 3_{10} helices. The core carries several extended loops. One of the longest (15 amino acids) is the loop between helix H2 and β-strand S3 (see Koellner *et al.*, 1997). Part of this loop, residues 59–65, not previously modelled in the binary complex with hypoxanthine, is now well defined, revealing a type II β-turn (Hutchinson & Thornton, 1996) between Val61 and His64. Two metal cations, magnesium and most probably zinc (Koellner *et al.*, 1997), participate in crystal packing.

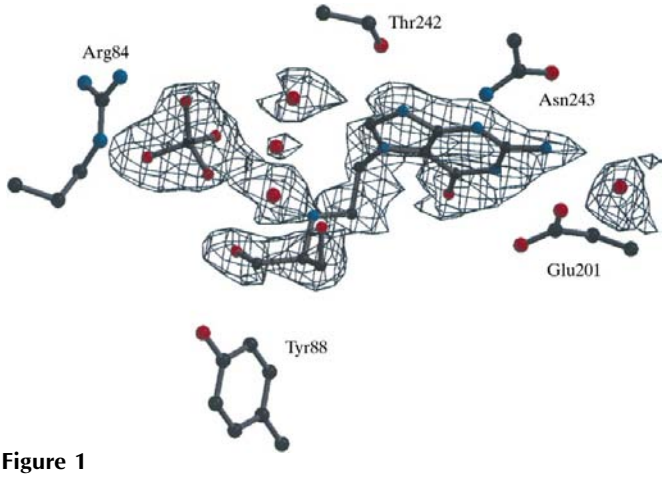

Figure 1
Omit difference electron-density map at the 2.5σ contour level superimposed on the final model of N(7)-acycloguanosine, phosphate ion and four water molecules. The acyclonucleoside inhibitor is bound in the inverted ('upside-down') orientation of the purine base when compared with natural substrates (see also Fig. 4*b*), but the same amino acids participate in binding of the base, *i.e.* Asn243, Glu201 and in addition Thr242 (see Fig. 2 for possible hydrogen bonds). The acyclic chain occupies, together with water molecules, the ribose-binding site. This figure was drawn with *BOBSCRIPT* (Kraulis, 1991; Esnouf, 1997).

3.2. The active site of the ternary complex

The active site contains a phosphate ion, one molecule of N(7)-acycloguanosine and four water molecules. The putative hydrogen bonds in the active site are listed in Table 2 and shown in Fig. 2(*a*). The acyclonucleoside inhibitor is bound in the active site in an inverted ('upside-down') orientation of the purine base compared with natural substrates such as hypoxanthine (Koellner *et al.*, 1997) and inosine (Mao *et al.*, 1998; Fig. 2*b*). The side chain of Asn243 forms two hydrogen bonds with the base ring: N^{δ} donates a hydrogen to N(3) and O^{δ} accepts a hydrogen from N(2). N(1)—H of the base is

hydrogen bonded to $O^{\varepsilon 1}$ of Glu201 and N(9) to Thr242 O^{γ}. In addition, a water molecule (W417) bridges N(2) of the base and $O^{\varepsilon 1}$ of Glu201.

The acyclic chain of the inhibitor is located in the ribose-binding site and has two direct contacts with the protein. One terminal hydroxyl group of the chain, O(17), forms a hydrogen bond with Tyr88 O^{η} and a phosphate O atom. The other terminal OH group, O(15), is directed towards the base-binding site, where it is hydrogen bonded to the side chain of His257. The N—H group of the acyclic chain is bridged *via* a water molecule (W388) to Ala116 O of the phosphate-binding site and to the phosphate ion *via* the water molecules W388 and W399. The acyclic chain is in a folded form and the distances from N(7) of the base to the O atoms of the terminal hydroxyl groups O(15) and O(17) are 4 and 5.8 Å, respectively.

The phosphate ion is hydrogen bonded to the side chains of Ser33, His64, Arg84, His86 and Ser220 and to Ser33 N and Ala116 N of the backbone. In addition, three water molecules are in contact with the ion (W387, W388 and W399). Each phosphate O atom is linked by at least one direct hydrogen bond to the protein.

The side chain of His64 forms a non-conventional C—H···O hydrogen bond through the acidic $C^{\varepsilon 1}$—H with the phosphate ion O(4)—P (3.4 Å). Notably, $N^{\delta 1}$ of His64 accepts a hydrogen bond from Arg84 N (2.95 Å) and this defines the rotameric state of the histidine side chain. Rotation by 180° would lead to a 'bad contact' $C^{\delta 1}$—H···H—N Arg84. Formation of the hydrogen bond Arg84 NH···N^{δ} His64 also shows unambiguously that the side chain of His64 is uncharged.

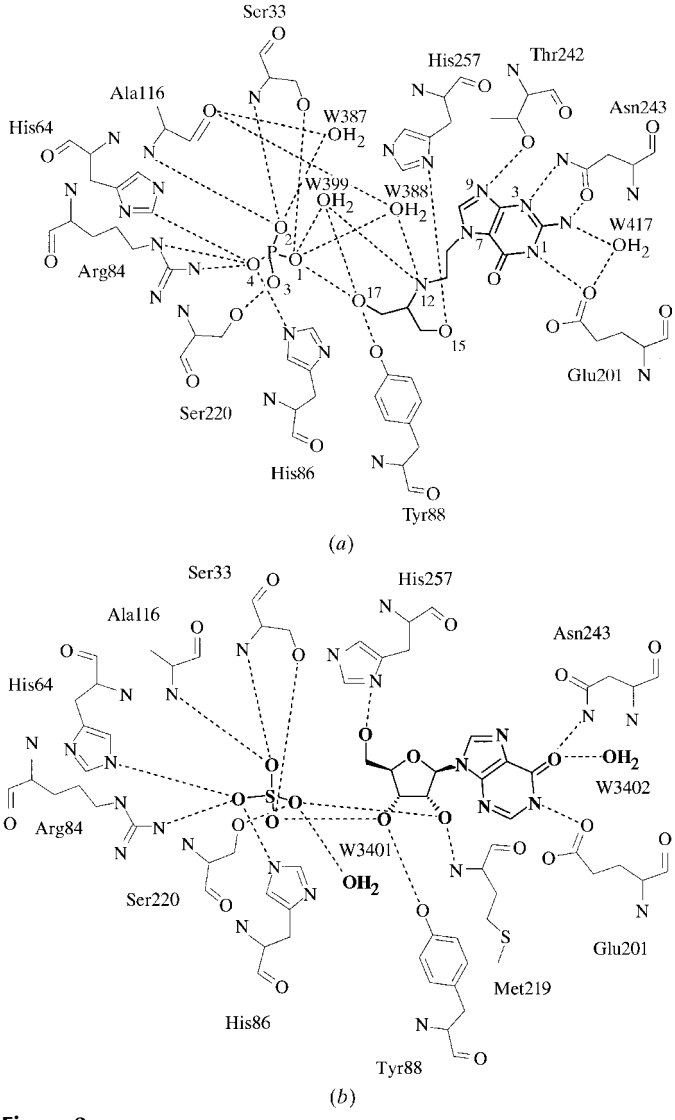

Figure 2
(*a*) Mode of binding of the N(7)-acycloguanosine inhibitor and a phosphate ion in the active centre of calf spleen PNP. Possible hydrogen bonds are indicated by broken lines. The N(7)-acycloguanosine inhibitor is in an inverted ('upside-down') orientation such that the acyclic chain, although located at N(7) of the base, occupies the ribose-binding site. The chain is in the folded conformation, with distances from N(7) to the terminal hydroxyl groups O(15) and O(17) of 4.0 and 5.8 Å, respectively. This conformation of the chain is observed in the ternary complex; in the binary complex, with no phosphate, the acyclic chain adopts an elongated conformation such that the terminal hydroxyl groups O(15) and O(17) extend to the phosphate-binding site (see Fig. 4*b*). All donor and acceptor sites of the guanine base, except oxygen O^6, are involved in hydrogen bonds with protein residues. This novel binding mode opens up new possibilities for design of inhibitors of the enzyme. (*b*) Mode of binding of inosine and sulfate in the active site of bovine spleen PNP, derived from the three-dimensional structure of the PNP–inosine–sulfate ternary complex (PDB entry 1a9s; Mao *et al.*, 1998).

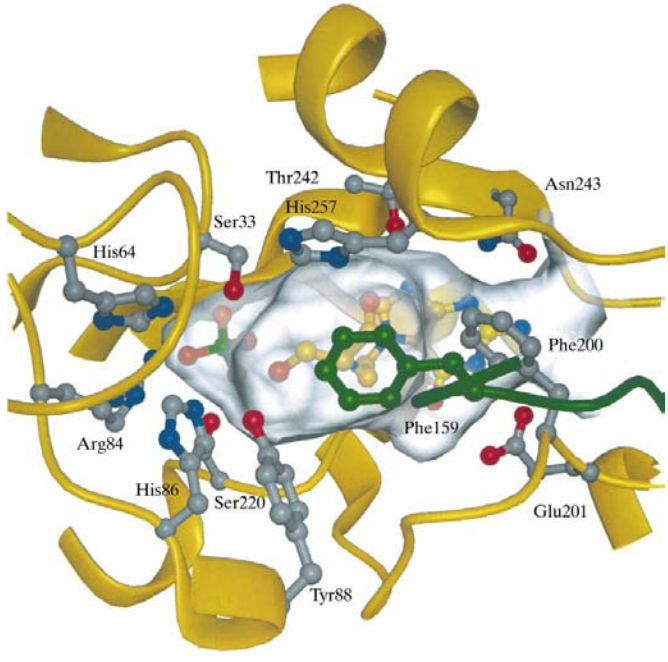

Figure 3
Binding pocket of calf spleen PNP described through the solvent-accessible surface calculated without phosphate and inhibitor (MSMS; Sanner *et al.*, 1996). The biologically active form of the enzyme is a homotrimer, with active sites located at the interfaces of two monomers. The neighbouring subunit contributes the side chain of Phe159 to the pocket (drawn in green), which stacks flat against the ribose-binding site, here occupied by the acyclic chain of the N(7)-acycloguanosine inhibitor. The active site is buried in the protein structure and the walls of the binding pocket appear to be fairly compact. However, some flexibility must occur to enable binding and release of inhibitor and phosphate (see text for discussion). This figure was produced using *DINO* (Philippsen, 2000).

The biologically active form of the enzyme is considered to be the homotrimer, with the active sites located at the interfaces between two monomers. The surfaces of the active sites comprise residues from both contacting subunits, leading to a tight pocket as shown in Fig. 3. A long and solid part of the binding pocket is located at the C-terminal end of the extended nine-stranded β-sheet core (below the plane of the paper in Fig. 3). The front side of the phosphate-binding site (above the plane of the paper) consists of several loops, three of them containing the active-site residues Ser33, His64, His86 and Arg84. In addition, there is a contribution from a tight γ-turn with Ser220 and the sterically strained residue Thr221 with unfavourable φ/ψ angles according to the Ramachandran diagram (not shown). Apart from the hydrogen bonds, there is an edge-to-face π-stacking interaction between the inhibitor base and Phe200. The neighbouring monomer contributes the side chain of Phe159 to the pocket (shown in green) which stacks flat against the acyclic chain of the inhibitor (ribose binding site). The walls of the pocket seem to be fairly compact, but flexibility must occur to enable inhibitor binding and release. This is supported by two observations. Firstly, the electron density for three residues of the loop preceding the active-site residue Asn243 is poorly defined, suggesting either flexibility or disorder in this part of the protein molecule. Secondly, the loop containing His64 could only be located in complexes with PNP and phosphate and appears to be disordered in the binary complexes of PNP with bases or nucleosides (Koellner *et al.*, 1997; Mao *et al.*, 1998).

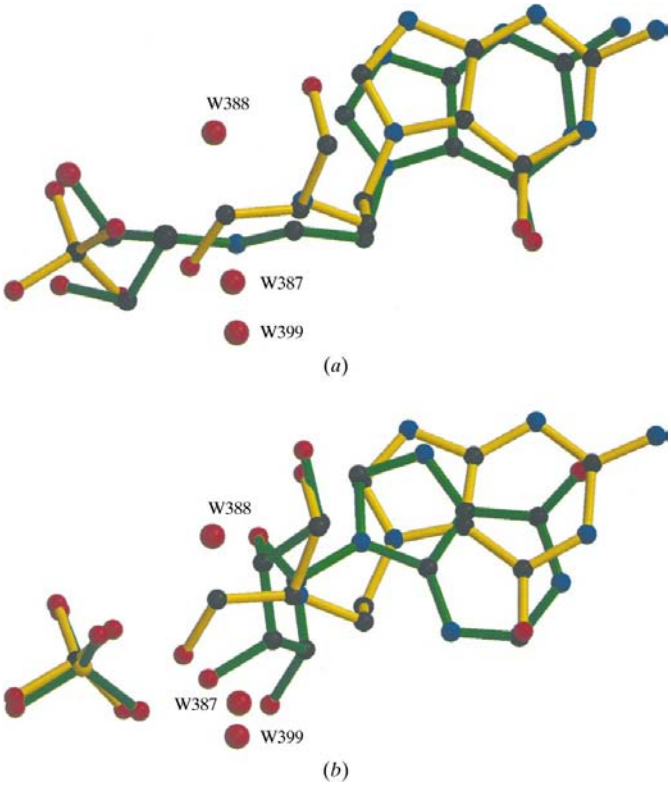

Figure 4
(*a*) Superposition of the conformations of the N(7)-acycloguanosine inhibitor in a ternary complex with phosphate (yellow) and with the binary complex (green; Bzowska *et al.*, 1995). Three water molecules (W387, W388, W399) are from the crystal structure of the ternary complex. The position of the base is in principle similar in the ternary and binary complexes. The acyclic chain in the binary complex adopts an extended conformation so that both terminal CH_2OH groups reach the phosphate-binding site, in contrast to the ternary complex in which the acyclic chain is in a folded conformation and occupies, together with the water molecules, the ribose-binding site. (*b*) Superposition of the ternary complexes of PNP with the N(7)-acycloguanosine inhibitor and phosphate (yellow) and with inosine and sulfate (green, Mao *et al.*, 1998). Sulfate and phosphate ions occupy the same position in both structures. The orientation of the purine base guanine is inverted relative to that of hypoxanthine, so that N(7) of the acyclonucleoside inhibitor and N(9) of inosine are located in almost the same position. Consequently, the acyclic chain of the inhibitor occupies the same part of the binding pocket as the ribose in the inosine/sulfate complex, with the terminal hydroxyl groups O(15) and O(17) of the acyclic chain superimposed with O atoms O(3′) and O(5′) of the ribose. This figure was drawn using *MOLSCRIPT* (Kraulis, 1991).

Table 2
Possible hydrogen-bonding interactions in the active site of the ternary complex of PNP with N(7)-acycloguanosine and phosphate ion.

	Residue	$D\cdots A$ (Å)	$D-H\cdots A$ (°)
Inhibitor			
N(1)	Glu201 $O^{\varepsilon1}$	2.80	174
N(2)	Asn243 $O^{\delta1}$	3.39	152
N(2)	W417	3.39	
N(3)	Asn243 $N^{\delta2}$	2.62	166
N(9)	Thr242 $O^{\gamma1}$	2.83	136
N(12)	W388	2.60	
N(12)	W399	3.20	
O(15)	His257 $N^{\delta1}$	3.05	
O(17)	Tyr88 O^{η}	2.91	
O(17)	O-1P (phosphate)	2.63	
O(17)	W399	3.17	
Phosphate			
O-1P	Ser33 O^{γ}	2.48	
O-1P	O(17) (inhibitor)	2.63	
O-1P	W388	2.74	
O-1P	W399	3.13	
O-2P	Ser33 N	2.90	160
O-2P	Ala116 N	3.04	157
O-3P	Ser220 O^{γ}	2.49	
O-3P	W387	2.85	
O-3P	W399	2.67	
O-4P	His64 $C^{\varepsilon1}$	3.40	152
O-4P	Arg84 N^{ε}	2.86	166
O-4P	Arg84 $N^{\zeta2}$	3.18	141
O-4P	His86 $N^{\varepsilon2}$	2.96	153

3.3. Comparison with the binary complex of PNP with N(7)-acycloguanosine

The binary complex of PNP with N(7)-acycloguanosine (Bzowska *et al.*, 1995) overlaid with the ternary complex with N(7)-acycloguanosine and phosphate ion is shown in Fig. 4(*a*). The base-binding mode is, in principle, similar to that in the ternary complex, but fewer contacts between the base and protein are observed. The acyclic chain in the binary complex adopts an extended conformation to reach the phosphate-binding site with both terminal CH_2-OH groups (see Fig. 4*a*), in contrast to the ternary complex where the acyclic chain of the inhibitor is in a folded conformation and occupies the

pentose-binding site. Owing to the limited resolution of the binary complex structure (2.9 Å), a more detailed comparison of interaction contacts is not feasible.

3.4. Comparison with the ternary complex of PNP with inosine and sulfate

Possible hydrogen-bond contacts in the ternary complex of the enzyme with inosine and sulfate ion (PDB entry 1a9s) are shown in Fig. 2(*b*). The superimposed ligands present in both ternary complexes [N(7)-acycloguanosine and phosphate, and inosine and sulfate, respectively] are shown in Fig. 4(*b*).

The binding mode of the purine base is different in both complexes, since the N(7)-acycloguanosine inhibitor is bound in the inverted ('upside-down') orientation compared with inosine. However, in both ternary complexes the same amino acids are involved in binding of the base, Asn243 and Glu201. The O^6 atom of inosine forms two hydrogen bonds with Asn243 N^{δ} and the water molecule W3402. One inosine N atom hydrogen bonds to the protein, N(1)—H with Glu201 O^{ε}. Thr242 has no direct contact with the base in the inosine/sulfate complex, while in N(7)-acycloguanosine/phosphate it donates a hydrogen bond to base nitrogen N(9).

In the inosine/sulfate complex, the phosphate-binding site is occupied by a sulfate ion in the same position as the phosphate in the N(7)-acycloguanosine/phosphate complex (Fig. 4*b*). The acyclic chain of the inhibitor is in a folded form and occupies the space filled by the ribose moiety in the inosine/sulfate complex. The positions of the two terminal O atoms of the acyclic chain, O(17) and O(15), superimpose well with O atoms O(3′) and O(5′) of the ribose. The position of the ether O atom O(4′) of the ribose in the inosine/sulfate complex corresponds to the position of water molecule W388 in the N(7)-acycloguanosine/phosphate complex.

It is clear that the N(7)-acycloguanosine inhibitor in a ternary complex with PNP and phosphate occupies the major binding positions of the nucleoside in the corresponding ternary complex PNP–inosine–sulfate.

3.5. Substrate properties of 7-β-D-ribosides

The present study of the crystal structure of the ternary complex of N(7)-acycloguanosine and phosphate with calf spleen PNP, together with the previous crystal structure of the binary complex of calf spleen PNP with the same inhibitor (Bzowska *et al.*, 1995), verifies the existence of a binding mode of the purine base other than that observed with natural substrates of the enzyme (Koellner *et al.*, 1997; Mao *et al.*, 1998), *i.e.* with an inverted orientation of the purine base. This had been predicted previously from the finding that the 7-β-D-ribosides of guanine and hypoxanthine are substrates, albeit weak ones, of PNP from various sources and that the N(7)-acycloguanosine inhibitor has a higher affinity for the human and rabbit PNPs than its 'natural' N(9) counterpart (Bzowska *et al.*, 1994).

On the basis of their newly found binding mode, the substrate properties of 7-β-D-ribosides can be easily understood since, when bound in the inverted orientation of the purine base, these nucleosides structurally mimic natural substrates of the enzyme, *i.e.* the 9-β-D-ribosides of guanine and hypoxanthine. The guanine base of N(7)-acycloguanosine bound in the inverted orientation in the ternary complex is almost fully saturated with hydrogen bonds *via* ring N atoms N(1), N(3) and N(9) and the amino group $-N(2)H_2$. Only O^6 of the base is not involved in hydrogen bonding. The multiple possible contacts observed for the inverted orientation of the base account for the good inhibitory properties of N(7)-acycloguanosine observed in kinetic studies (Bzowska *et al.*, 1994).

3.6. 'Bisubstrate' analogue inhibitors and implications for drug design

The differences in observed binding modes of the acyclic chains in the binary and ternary complexes of N(7)-acycloguanosine confirm that this analogue acts as a so-called bisubstrate analogue inhibitor, *i.e.* it interacts with the binding sites of both PNP substrates (purine base and phosphate) *via* the guanine base and the terminal part of the acyclic chain, respectively, if the phosphate-binding site is not occupied by phosphate. Although the acyclic chain does not carry a terminal phosphate or phosphonate, like typical bisubstrate analogue inhibitors such as acyclovir diphosphate (Tuttle & Krenitsky, 1984), it is still capable of competing with phosphate for the phosphate-binding site. This observation explains why inhibition constants determined for such inhibitors are dependent on the phosphate concentration. The inhibition constants are typically lower with lower phosphate concentrations (*e.g.* Bzowska *et al.*, 1991; Montgomery *et al.*, 1993), indicating a higher affinity of such analogues for the enzyme in binary, relative to ternary, complexes.

In the ternary complex of the enzyme with the N(7)-acycloguanosine inhibitor, all donor and acceptor sites of the guanine base, except oxygen O^6, are involved in hydrogen bonds with protein residues. Replacement of O^6 with a hydrogen donor such as an amino group may result in an additional hydrogen bond with Glu201 $O^{\varepsilon 2}$, which is appropriately located for this purpose (see Fig. 2*a*).

The new binding mode identified for calf spleen PNP offers new possibilities for drug design. Since both the structure and the kinetic properties of the calf enzyme are very similar to those of human erythrocyte PNP and both enzymes show similar responses to many inhibitors (*e.g.* Bzowska *et al.*, 1990, 1991; Koellner *et al.*, 1997; Narayana *et al.*, 1997; Mao *et al.*, 1998; Miles *et al.*, 1998), the information obtained with the calf spleen enzyme may be used to design inhibitors of the human enzyme, a key target for chemotherapeutic intervention (Stoeckler, 1984; Montgomery, 1993). Although many potent inhibitors are presently available and new ones have been synthesized (*e.g.* Morris *et al.*, 2000), only one, 9-(3-piridynylmethyl)-9-deazaguanine (BCX-34; Peldesine; Bantia *et al.*, 1996; Iwata *et al.*, 1998) is currently undergoing clinical trials, with as yet unknown results. Development of new membrane-permeable inhibitors is a desirable goal.

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