Crystal structure of *Escherichia coli* purine nucleoside phosphorylase in complex with 7-deazahypoxanthine

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Purine nucleoside phosphorylases (EC 2.4.2.1; PNPs) reversibly catalyze the phosphorolytic cleavage of glycosidic bonds in purine nucleosides to generate ribose 1-phosphate and a free purine base, and are key enzymes in the salvage pathway of purine biosynthesis. They also catalyze the transfer of pentosyl groups between purine bases (the transglycosylation reaction) and are widely used for the synthesis of biologically important analogues of natural nucleosides, including a number of anticancer and antiviral drugs. Potent inhibitors of PNPs are used in chemotherapeutic applications. The detailed study of the binding of purine bases and their derivatives in the active site of PNPs is of particular interest in order to understand the mechanism of enzyme action and for the development of new enzyme inhibitors. Here, it is shown that 7-deazahypoxanthine (7DHX) is a noncompetitive inhibitor of the phosphorolysis of inosine by recombinant *Escherichia coli* PNP (*Ec*PNP) with an inhibition constant K_i of 0.13 mM. A crystal of *Ec*PNP in complex with 7DHX was obtained in microgravity by the counter-diffusion technique and the three-dimensional structure of the *Ec*PNP–7DHX complex was solved by molecular replacement at 2.51 Å resolution using an X-ray data set collected at the SPring-8 synchrotron-radiation facility, Japan. The crystals belonged to space group $P6_122$, with unit-cell parameters $a = b = 120.370$, $c = 238.971$ Å, and contained three subunits of the hexameric enzyme molecule in the asymmetric unit. The 7DHX molecule was located with full occupancy in the active site of each of the three crystallographically independent enzyme subunits. The position of 7DHX overlapped with the positions occupied by purine bases in similar PNP complexes. However, the orientation of the 7DHX molecule differs from those of other bases: it is rotated by $\sim 180^\circ$ relative to other bases. The peculiarities of the arrangement of 7DHX in the *Ec*PNP active site are discussed.

1. Introduction

Purine nucleoside phosphorylases (PNPs) catalyze the reversible phosphorolysis of the β -D-glycosidic bond in purine nucleosides, deoxynucleosides and a large number of nucleoside derivatives to the free base and ribose (or 2-deoxyribose) 1-phosphate and are key enzymes in nucleoside metabolism (Kalckar, 1947; Bennett *et al.*, 2003). During the course of the reverse reaction, PNPs catalyze the synthesis of a glycosidic bond from a purine base and ribose 1-phosphate.

PNPs from different sources are divided into two classes in accordance with their quaternary structure and specificity (Bennett *et al.*, 2003). The majority of bacterial PNPs, which

are characterized by a hexameric quaternary structure and a broad substrate specificity, belong to class I, whereas mammalian PNPs, which belong to the other class, have a trimeric quaternary structure and a narrow specificity (Tahirov *et al.*, 2004; Schnick *et al.*, 2005). Despite the very low sequence homology between them, PNPs of both classes have a similar polypeptide fold (Ealick *et al.*, 1990; Mao *et al.*, 1997, 1998; Koellner *et al.*, 1997, 1998, 2002; Appleby *et al.*, 2001; Shi *et al.*, 2004; Tahirov *et al.*, 2004; Grenha *et al.*, 2005; Schnick *et al.*, 2005; Zang *et al.*, 2005; Dessanti *et al.*, 2012).

PNPs of both classes are of particular interest in medicine and biotechnology owing to their catalysis of the transglycosylation reaction. The ability to transfer a ribosyl moiety from one base to another (transglycosylation) plays an essential role in the salvage pathway of nucleoside biosynthesis and is widely exploited in biotechnology as a tool for the synthesis of nucleoside analogues with potential antiviral and antitumour activity (Mikhailopulo, 2007; Lewkowicz & Iribarren, 2006; Canduri *et al.*, 2004; Galmarini, 2006).

The difference in the specificity of the mammalian and bacterial enzymes allows the use of bacterial enzymes for the treatment of some diseases as well as for the activation of antitumour prodrugs (Dranoff, 1998). Mammalian PNPs are involved in the process of T-cell development, and compromised PNP activity leads to T-cell immunodeficiency (Gelfand *et al.*, 1978). Inhibitors of human PNP are used for therapy after the transplantation of organs and tissues. The PNPs of parasitic organisms in which *de novo* synthesis of purine is absent serve as targets for the design of antiparasitic drugs (Bzowska *et al.*, 2000).

The application of PNPs in different fields of medicine and biotechnology has attracted attention to the detailed study of their structures in complexes with nucleosides as well as with nucleobase derivatives, which are potential leading compounds for drug design.

Here, the interaction of 7-deazahypoxanthine (7DHX) with *Escherichia coli* PNP (*EcPNP*) has been studied using kinetic and X-ray techniques. It is shown that 7DHX inhibits the phosphorolysis of inosine by *EcPNP* with a K_i of 0.13 mM. A crystal of the complex of *EcPNP* with 7DHX was prepared and its three-dimensional structure was solved at 2.51 Å resolution. The position of 7DHX in the nucleoside-binding pocket of *EcPNP* was compared with the positions of purine bases and their derivatives in PNPs from several other sources. It was found that the positions of 7DHX and purine derivatives in other PNPs overlap. However, the 7DHX molecule is bound in an inverted orientation relative to the orientations of other purine derivatives. The peculiarities of the arrangement of 7DHX are discussed.

2. Materials and methods

2.1. Macromolecule production

EcPNP was produced using the producer strain *E. coli* BL21(DE3)/pERPUPHOI (Esipov *et al.*, 2002). The producer strain was cultured at 310 K in YT medium supplemented with ampicillin (100 µg ml⁻¹) until an absorbance (A_{595}) of 0.8

Table 1

Macromolecule-production information.

Source organism	<i>E. coli</i>
DNA source	<i>E. coli</i>
Forward primer	AAACCATGGCTACCCACACATTAATGC
Reverse primer	CGGAATTCTATTACTCTTTATCGCCAGCA GAAC
Cloning vector	pET-23d(+)
Expression vector	pET-23d(+)
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	ATPHINAEMGDFADVVLMPGDPLRAKYIAE TFLEDAREVNNVRGMLGFTGTYKGRKIS VMGHGMGIPSCSIYTKELITDFGVKKII RVGSCGAVLPHVKLRDVGVMGACTDSK VNRIKFKDHDFAAIAADFMDVRNAVDAK ALGIDARVGNLFSADLFYSPDGMFDMV EKYILGVEMEAAGIYGVAEEFGAKALT ICTVSDHIRTHERQTAAERQTTFNMDIK IALESVLLGDK

was achieved. The culture was supplemented with 0.4 mM isopropyl β-D-1-thiogalactopyranoside to induce expression of the recombinant enzyme and grown for a further 4 h at 210 K. The cells were separated by centrifugation (5180g, 20 min, 277 K). The wet weight of the cells was 4 g per litre of culture. The biomass containing the target protein was resuspended in lysis buffer (25 mM Tris-HCl pH 7.7, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and disintegrated by ultrasonication for 10 min at 283 K. The cell debris was pelleted by centrifugation at 21 044g for 40 min at 283 K. The supernatant was applied onto an XK 16/20 column packed with Q Sepharose XL (GE Healthcare) and pre-equilibrated with buffer consisting of 20 mM Tris-HCl pH 7.7, 2 mM EDTA; the target enzyme was eluted using a gradient from 0 to 0.5 M NaCl at 296 K. The pooled fractions were concentrated by ultrafiltration using a 200 ml stirred ultrafiltration cell (Amicon 8200; Millipore, USA) with a regenerated YM30 cellulose membrane (Millipore) at 283 K. The final purification was performed on a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with buffer consisting of 20 mM Tris-HCl pH 7.7, 100 mM NaCl, 0.04% Na₂S₂O₃ at 296 K. After size-exclusion chromatography, the protein was concentrated to 32 mg ml⁻¹ by ultrafiltration at 283 K and stored at -193 K.

These techniques provided a yield of *EcPNP* of not less than 96 mg per litre of culture medium, with an electrophoretic purity of at least 97%. The protein concentration was determined by the Bradford method using BSA as a standard (Bradford, 1976). Protein purity was determined by polyacrylamide gel electrophoresis under denaturing conditions (Laemmli, 1970). Macromolecule-production information is summarized in Table 1.

The kinetic parameters for the phosphorolysis of inosine by *EcPNP* in the presence of 7DHX were measured in 50 mM potassium phosphate buffer pH 7 containing 0.02–0.7 mM inosine at an *EcPNP* concentration of 0.288 mg ml⁻¹ and a 7DHX concentration from 0.1 to 0.5 mM. The reaction was performed at room temperature for 2 min. Each reaction was replicated three times. The concentrations of inosine and 7DHX were determined by isocratic high-performance liquid chromatography using 0.1% trifluoroacetic acid as the eluent at a wavelength of 254 nm (Hitachi Chromaster 5410 detector;

Table 2

Kinetic parameters for the phosphorolysis of inosine by *Ec*PNP at different concentrations of 7DHX.

7DHX concentration (mM)	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_i (mM)
0	0.035 ± 0.005	25 ± 4	
0.1	0.034 ± 0.005	16 ± 2	0.19 ± 0.03
0.2	0.031 ± 0.004	7.3 ± 0.9	0.084 ± 0.011
0.5	0.039 ± 0.008	4.5 ± 0.9	0.11 ± 0.02

YMC-Triart C18 50 $\times$ 3.0 mm 5 μm column). The kinetic parameters were determined by nonlinear regression analysis using *SciDAVis* v.1.D013. It was found that the maximum reaction rate V_{max} decreases when the concentration of 7DHX is increased, while the Michaelis constant K_m remains constant. Such a dependence is typical of noncompetitive inhibition. The inhibition constant K_i for 7DHX was calculated according to the equation for noncompetitive inhibition for each experiment on the basis of the observed maximum reaction rate at a given concentration of the inhibitor and the maximum rate in the absence of the inhibitor (Table 2). The average K_i value is $0.13 \pm 0.04 \text{ mmol l}^{-1}$. These results are illustrated by Lineweaver–Burk plots (Fig. 1).

2.2. Crystallization

The crystallization conditions for *Ec*PNP obtained using the vapour-diffusion technique were adapted and optimized in order to apply the capillary counter-diffusion technique. The experiments were performed as described elsewhere (Kurayama *et al.*, 2011; Tanaka *et al.*, 2004; Takahashi *et al.*, 2010). Protein solution (7 μl) was placed in a glass capillary of 0.5 mm in diameter. One end of the capillary was hermetically sealed and the other end was plugged with a 0.5 mm silicone tube filled with 1% agarose gel. The silicone tube was dipped into a cylinder containing the reservoir solution and sealed. Crystal growth was performed in microgravity. The protein solution consisted of protein at a concentration of 21.6 mg ml^{-1} , 0.02 M Tris–HCl pH 7.5, 0.1 M NaCl, 0.04% NaN_3 , 5 mM 7DHX. The reservoir solution was composed of 25% ammonium sulfate, 0.05 M sodium citrate pH 5.0, 0.02 M Tris–HCl pH 7.5, 0.1 M NaCl, 0.04% NaN_3 , 5 mM 7DHX. Crystallization information is summarized in Table 3.

Figure 1

Lineweaver–Burk plots for the phosphorolysis of inosine at 7DHX concentrations of 0, 0.10, 0.20 and 0.5 mM.

Table 3

Crystallization.

Method	Liquid diffusion
Plate type	Capillary
Temperature (K)	294
Protein concentration (mg ml^{-1})	21.6
Buffer composition of protein solution	0.02 M Tris–HCl pH 7.5
Composition of reservoir solution	25% ammonium sulfate, 0.05 M sodium citrate pH 5.0, 0.02 M Tris–HCl pH 7.5, 0.1 M NaCl, 0.04% NaN_3 , 5 mM 7DHX
Volume of drop (μl)	7
Volume of reservoir (μl)	180

Table 4

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL41XU, SPring-8
Wavelength ($\text{\AA}$)	0.8
Temperature (K)	100
Detector	PILATUS3 6M
Crystal-to-detector distance (mm)	100
Rotation range per image ($^\circ$)	0.5
Total rotation range ($^\circ$)	180
Exposure time per image (s)	0.1
Space group	$P6_122$
a, b, c ($\text{\AA}$)	120.37, 120.37, 238.97
α, β, γ ($^\circ$)	90, 90, 120
Mosaicity ($^\circ$)	0.57
Resolution range ($\text{\AA}$)	29.87–2.51 (2.65–2.51)
No. of unique reflections	35075
Completeness (%)	98.12
Multiplicity	4.47
$\langle I/\sigma(I) \rangle$	10.44 (4.3)
$R_{\text{r.i.m.}}$	0.074
Overall B factor from Wilson plot ($\text{\AA}^2$)	32.7

2.3. Data collection and processing

Before the collection of the X-ray diffraction data set, the crystals were transferred into cryoprotectant solution, which contained the same components as the reservoir solution with the addition of 15% glycerol, using a cryoloop. Diffraction data were collected on the BL41XU station at the SPring-8 synchrotron, Japan at a temperature of 100 K. A Dectris PILATUS3 6M detector was used. The diffraction data were obtained by rotation using a single crystal. The wavelength was 0.8 $\text{\AA}$, the crystal-to-detector distance was 100 mm, the oscillation angle was 0.5° and the angle of rotation was 180° . The experimental intensities were processed to 2.51 $\text{\AA}$ resolution using *iMosflm* (Battye *et al.*, 2011). Data-collection and processing statistics are summarized in Table 4.

2.4. Structure solution and refinement

The crystal structure was solved by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007) with the coordinates of *Ec*PNP at 0.99 $\text{\AA}$ resolution (PDB entry 4rj2; V. I. Timofeev, Y. A. Abramchik, R. S. Esipov & I. P. Kurayama, unpublished work) as the search model. Structure refinement was carried out using *REFMAC5* (Murshudov *et al.*, 2011). Manual rebuilding of the model was performed using the *Coot* interactive graphics program (Emsley *et al.*, 2010) and electron-density maps were calculated with

Table 5

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	29.86–2.51 (2.575–2.510)
Completeness (%)	97.9
No. of reflections, working set	33258 (2290)
No. of reflections, test set	1762 (114)
Final R_{cryst}	0.155 (0.176)
Final R_{free}	0.201 (0.248)
No. of non-H atoms	
Protein	5391
Ion	15
Ligand	30
Water	137
Total	5573
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.428
Average B factors (Å ²)	
Protein	27.9
Ion	31.9
Ligand	30.2
Water	25.9
Ramachandran plot	
Most favoured (%)	98
Allowed (%)	2

$2|F_o| - |F_c|$ and $|F_o| - |F_c|$ coefficients. In the electron-density map calculated with $|F_o| - |F_c|$ coefficients at the 2.0σ level sharp electron-density peaks were found in the active site of each subunit, which were interpreted as the 7DHX molecule (Fig. 2). Clear electron densities for sulfate ions, which are present at high concentration in the crystallization solution, were also found. A number of water molecules were located in the difference electron-density maps. The refinement statistics are summarized in Table 5. The atomic coordinates of the PNP–7DHX complex have been deposited in the Protein Data Bank as PDB entry 5iu6.

3. Results and discussion

The interactions of purine derivatives with the amino-acid residues of the active site of PNPs are of particular interest in order to understand the mechanism of the reaction catalyzed by PNPs in the salvage pathway of purine biosynthesis and for the rational design of PNP inhibitors.

X-ray studies of complexes of PNPs with nucleosides and their derivatives have revealed the surroundings of the purine bases in the active sites of the enzymes (Bennett *et al.*, 2003; Bzowska *et al.*, 1990, 1995; Koellner *et al.*, 1997; Lulić *et al.*, 2001; Caceres *et al.*, 2010). On the basis of X-ray and kinetic studies, a mechanism for the phosphorolytic cleavage and formation of the glycosidic bond in purine nucleosides catalyzed by PNP has been proposed (Erion *et al.*, 1997; Bzowska *et al.*, 1990, 2000; Wielgus-Kutrowska *et al.*, 2002; Koellner *et al.*, 2002; Mikhailopulo & Miroshnikov, 2010; Pugmire & Ealick, 2002). It is suggested that the forward reaction of cleavage of the glycosidic bond proceeds *via* nucleophilic attack of the phosphate O atom on the electrophilic C1' atom of the sugar ring, with the formation of an oxocarbenium nucleoside intermediate. The attack of the N9 atom of the

purine ring on the electrophilic C1' atom of α -D-pentafuranose 1-phosphate provides the synthesis of the glycosidic bond during the course of the reverse reaction (Wielgus-Kutrowska *et al.*, 2002). A key role in the suggested catalytic mechanism is played by the N7 and N9 atoms of the purine base (Mao *et al.*, 1997). The purine base in the nucleoside-binding site of PNP is arranged in such a way that a hydrogen bond is present between the N7 atom of the purine ring and the carboxylic group of the catalytically essential aspartic acid residue (or asparagine in trimeric PNPs). Proton transfer between the carboxyl group of the catalytically essential aspartic acid (or amido group of asparagine) and the N7 atom of the purine base changes the nucleophilicity of the N9 atom and promotes the forward or reverse course of the reaction. The N7 atom is replaced by a C atom in 7DHX, so 7DHX is not a substrate of PNP. However, it is known that 7DHX binds strongly to PNP, while 7-deazainosine is only able to bind weakly (Mikhailopulo & Miroshnikov, 2010; Bzowska *et al.*, 2000; Doskočil & Holý, 1977).

We have studied the kinetic parameters for the phosphorolysis of inosine by *Ec*PNP in the absence and the presence of the inhibitor 7DHX (Table 2). It was found that the Michaelis constant remains constant during the reaction, while the maximum reaction rate is decreased. If it is assumed that 7DHX is a noncompetitive inhibitor of the enzyme, the average K_i value is 0.13 mmol l^{-1} . These results are illustrated by Lineweaver–Burk plots (Fig. 1).

The crystals of the *Ec*PNP–7DHX complex used for X-ray study were prepared by co-crystallization of the enzyme with an excess of 7DHX in microgravity using the counter-diffusion technique, as described previously (Kuranova *et al.*, 2011). The crystals of the enzyme belonged to space group $P6_122$ and contained three subunits (*A*, *B*, *C*) of the hexameric enzyme molecule in the asymmetric unit related by a local threefold axis. The polypeptide chain in the subunit has the typical fold of this enzyme family (Pugmire & Ealick, 2002; Tahirov *et al.*,

Figure 2

7DHX in the active site of the *Ec*PNP subunit. The electron-density map is calculated without ligands with $2|F_o| - |F_c|$ coefficients and is contoured at 2σ . This figure was created using PyMOL (<https://pymol.org/2/>).

2004). Each monomer contains a central core consisting of five β -strands ($\beta 5$ – $\beta 9$) that form a barrel surrounded by α -helices $\alpha 2$, $\alpha 4$, $\alpha 6$ and $\alpha 7$. The active site comprising the nucleoside- and phosphate-binding sites is situated close to the dimer interface between the subunits and includes two amino-acid residues, Arg43 and His4, from the neighbouring subunit. The molecule of 7DHX is located with full occupancy in the nucleoside-binding site of each subunit (Fig. 3). It occupies the pocket formed by Met180, Cys91 and Asp204 and the hydrophobic side chains of Phe159, Ile206 and Val178. The phosphate-binding site is occupied by a sulfate ion, which is present in a large excess in the precipitant solution. The sulfate ion is surrounded by Arg24, Gly20, Arg87 and Ser90 and by Arg43 from the neighbouring subunit.

Superposition of the *A* subunits of the PNP–7DHX complex and of the *Ec*PNP apoenzyme at 0.99 Å resolution (PDB entry 4rj2; Timofeev *et al.*, 2016) based on C^α atoms reveals a remarkable shift of amino-acid residues in the region 205–220, where Gln211 is displaced 4.293 Å; the mean r.m.s.d. value for the structural alignment is 0.406 Å. There are small shifts in the positions of Phe159 and Ile206 near the purine base and the side chains of Arg24 and Ser90 in the phosphate-binding site. The position of the catalytically important Asp204 coincides with its average position in the apoenzyme. Superposition of the three independent subunits of the PNP–7DHX complex on C^α atoms shows that the 7DHX molecule occupies a similar position in the *A* and *C* subunits, whereas in subunit *B* it is slightly rotated and shifted relative to the other subunits (Fig. 4). The most striking finding is the orientation of the 7DHX molecule in the nucleoside-binding pocket of *Ec*PNP. 7DHX is accommodated in such a way that the N3 and N9 atoms of the base ring are directed towards the catalytically essential Asp204, while the C7 and O6 atoms face the phosphate-binding site (Figs. 3 and 4). It is clear that the orientation of 7DHX differs from the normal orientation of purine bases in the active site of PNPs. Only in subunit *C* is the N9 atom of the base within hydrogen-bonding distance of Asp204,

which supplies the carboxylate group essential for catalysis. The 7DHX N9...Asp204 OD1 and 7DHX N9...Asp204 OD2 distances are 3.34 and 3.10 Å, respectively, while these distances are longer in subunits *A* and *B*. The O6 atom of the base does not form a hydrogen bond to Glu179, which usually interacts with the ribose ring of the bound nucleoside. The position of 7DHX in the nucleoside-binding pocket practically overlaps with the position of the substrate. Nevertheless, according to the results of the kinetic measurements 7DHX acts as a noncompetitive inhibitor. A similar situation was previously found for the phosphorolysis of inosine catalyzed by *Ec*PNP in the presence of heterocyclic bases (Jensen, 1976; Krenitsky *et al.*, 1968). The authors explained this result as being a feature of the kinetics when the heterocyclic base only binds to the enzyme–phosphate complex and not to the enzyme–nucleoside complex. Thus, the inhibition is not really noncompetitive but appears to be so.

In order to understand the reasons for the special arrangement of 7DHX, the structure of *Ec*PNP–7DHX was compared with the structures of hypoxanthine (HX) complexed with hexameric *Bacillus subtilis* PNP (PDB entry 4dab; de Giuseppe *et al.*, 2012) and with trimeric PNP from calf spleen (PDB entry 1vfn; Koellner *et al.*, 1997). The ligands 7DHX and hypoxanthine (HX) differ only in the replacement of the N7 atom by a C atom. The subunits of the corresponding complexes were superimposed on several homologous amino-acid residues of the active site (Fig. 5). The hypoxanthine molecules in hexameric *B. subtilis* and trimeric calf spleen PNP are arranged in the normal way. The N7 and O6 atoms of hypoxanthine in *B. subtilis* PNP face the essential Asp203 (Asn243 in calf spleen PNP). HX in *B. subtilis* PNP is bound to Asp203 *via* a water molecule and makes hydrophobic interactions with Phe159 and Val17 (de Giuseppe *et al.*, 2012). The HX molecule in the calf spleen PNP–HX complex (Koellner *et al.*, 1997) is fixed not only by hydrogen bonds between the O6 and N7 atoms of the base and Asn243 but also

Figure 3

The active site of *Ec*PNP with a bound molecule of 7DHX in the nucleoside-binding site and a sulfate ion in the phosphate-binding site. This figure was created using PyMOL (<https://pymol.org/2/>).

Figure 4

Comparison of the positions of 7DHX in the three crystallographically independent subunits *A* (red), *C* (blue) and *B* (green) of *Ec*PNP. The structures of the subunits are superimposed on C^α atoms. This figure was created using PyMOL (<https://pymol.org/2/>).

by a hydrogen bond between the N1 atom of the base and the COOH group of Glu201. There is also interaction between the O6 atom of the base and Glu201 through a water molecule.

Figure 5
Comparison of the positions of (a) 7DHX (red) in the *Ec*PNP–7DHX complex, (b) HX in the *B. subtilis* PNP–HX complex and (c) HX in the human PNP–HX (yellow) complex. The subunits are superimposed on the C α atoms of Ser90, Val178 and Asp204 in *Ec*PNP, Ser90, Val177 and Asp203 in *B. subtilis* PNP and Ala116, Val217 and Asn243 in human PNP. This figure was created using PyMOL (<https://pymol.org/2/>).

The interaction of the heterobase N atom with the side chain of the glutamic acid (Glu201 in calf spleen PNP) is observed only in mammalian PNP and is absent in bacterial enzymes.

It is possible to see that the base planes of 7DHX and HX in these complexes are partly overlapped. However, the 7DHX molecule is rotated relative to other two by about $\sim 180^\circ$. The rotation occurs around the line that intersects the C8 and N3 atoms of the base ring. Rotation of 7DHX in the nucleotide-binding site of *Ec*PNP is apparently triggered by the opportunity to form hydrogen bonds between the essential aspartic acid residue and the N9 and N3 atoms of the base, while the normal hydrogen bond between atom 7 of the base and the enzyme is lost in PNP–7DHX because of the replacement of N7 by C7. It is probable that the formation of hydrogen bonds to the aspartic acid residue stabilizes the inverted position of 7DHX in the *Ec*PNP–7DHX complex. 7-Deazainosine in complex with PNP cannot rotate because of the presence of the ribosyl group and binds to PNP more weakly than 7DHX.

Figure 6
Comparison of the positions of (a) 7DHX in the *Ec*PNP–7DHX complex and (b) 7-acycloguanosine in the calf spleen PNP–7-acycloguanosine complex. The structures were superimposed on the C α atoms of Ser90, Glu92, Val178, Met180, Ser203 and Asp204 in *Ec*PNP and the homologous residues Ala116, Glu118, Val217, Met219, Thr242 and Asn243 in calf spleen PNP. This figure was created using PyMOL (<https://pymol.org/2/>).

This suggests that the affinity of the acyclic part of guanine derivatives for the ribose-binding site is the trigger that leads to the change in the orientation of the base.

An inverted position of the base was previously found in the complex of calf spleen PNP with 7-acycloguanosine (PDB entry 1fxu; Luić *et al.*, 2001; Fig. 6). The inverted orientation of this ligand is determined by the presence of the acyclo fragment at N7 instead of at N9. The acyclo group is complementary to the position usually occupied by the ribose ring of the nucleoside and causes inversion of the ligand (Luić *et al.*, 2001; dos Santos *et al.*, 2003). It is remarkable that 7-deazaguanine, which differs from 7DHX by the addition of an amino group at C2, retains the normal orientation without rotation in complex with human PNP, which is probably owing to both the N1 and the NH2 groups acting as hydrogen-bond donors to the carboxyl group of Glu201, as well as a hydrogen bond between O6 and Asn243 (PDB entry 3iny; Caceres *et al.*, 2010; Fig. 7). The special orientation of 7DHX in the active site of *Ec*PNP indicates the possibility of using 7DHX as a lead compound for the development of new PNP inhibitors.

Figure 7
Comparison of the positions of (a) 7DHX in the *Ec*PNP–7DHX complex (red) and (b) 7-deazaguanine in the human PNP–7-deazaguanine complex (blue). This figure was created using PyMOL (<https://pymol.org/2/>).

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