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Calf spleen purine nucleoside phosphorylase: purification, sequence and crystal structure of its complex with an $N(7)$-acycloguanosine inhibitor

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Abstract Calf spleen purine nucleoside phosphorylase was purified to homogeneity and its amino acid sequence was determined. The complex of the enzyme with an $N(7)$-acycloguanosine inhibitor crystallized in the cubic space group $P2_13$, with unit cell dimension $a = 94.02$ Å and one monomer in the asymmetric crystal unit. The biologically active trimer is formed by the crystallographic three-fold axis. The structure was solved by molecular replacement methods, using the model of the human erythrocyte enzyme, and refined at a resolution of 2.9 Å to an R-factor of 0.21. The orientation of the inhibitor at the active site is examined in relation to the catalytic activity of the enzyme in the phosphorolysis of $N(7)$-β-D-purine nucleosides.

Key words: Purine nucleoside phosphorylase; Amino acid sequence; Crystallization; X-ray crystallography; Molecular replacement; $N(7)$-β-D-Purine nucleoside

1. Introduction

Purine nucleoside phosphorylase (PNP, purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1) catalyzes the reversible phosphorolysis of purine ribo and deoxyribo nucleosides, such as inosine and guanosine in eucaryotes, as well as adenosine in some procaryotes, e.g. *E. coli*, as follows:

purine nucleoside + phosphate ⇌ purine base + ribose-1-phosphate

The high cellular level of this ubiquitous enzyme, and its propensity to cleave and inactivate chemotherapeutically active nucleoside analogues, has led to an intensive search for potent inhibitors of the enzyme from various sources [1–9]. Furthermore, patients with PNP deficiency exhibit marked T-cell immunodeficiency [10,11], and it is considered that PNP inhibitors could be useful in treatment of T-cell proliferative diseases, in suppression of host versus graft response in organ transplantation, and in treatment of T-cell-mediated autoimmune diseases [10–12].

The enzyme from human erythrocytes has been crystallized, and its structure was determined [13]. Cocrystallization with various inhibitors, together with molecular modelling, were employed in attempts to develop more potent inhibitors [14–16]. These studies suggested that binding of guanine analogue inhibitors involves the purine ring $N(7)$ as a hydrogen bond acceptor [15]. This is in striking contrast to our finding that a variety of $N(7)$-alkylguanosines, in which the ring $N(7)$ is blocked and the imidazole ring carries a positive charge, are good substrates, some even superior to the parent guanosine [17–19]. Furthermore, the isomeric $N(7)$-β-D-ribosides of hypoxanthine and guanine are substrates, albeit weak ones, of mammalian and bacterial PNPs [20].

Of further importance are differences in the behaviour of PNP from various sources. For example the structural requirements for substrates are quite different for the PNP from *E. coli* or *S. typhimurium* as compared to that from mammalian sources [21–23]. This led to the development of potent inhibitors of the *E. coli* enzyme which are inactive against calf spleen and human erythrocyte PNPs [24]. Differences within mammalian PNPs are less pronounced, but sufficient to warrant detailed investigations of their mechanisms of action and behaviour towards inhibitors.

Here we describe the purification to homogeneity of calf spleen PNP, its amino acid sequence, and the crystal structure of the enzyme complexed with an acycloguanosine inhibitor in which the acyclic chain is located not at the 'natural' conventional $N(9)$ position, but at the ring $N(7)$.

2. Experimental and results

2.1. Materials

Inosine, Tris, citric acid, sodium citrate, sodium phosphate, sodium chloride, ammonium carbonate, cyanobromide, trifluoroacetic acid and acetonitrile were products of Merck (Darmstadt, Germany). Sepharose CL-6B was from Pharmacia (Uppsala, Sweden); buttermilk xanthine oxidase (1 U/mg), trypsin and partially purified calf spleen purine nucleoside phosphorylase (~20 U/mg) from Sigma Chemical Co. (St. Louis, MO, USA); endoproteinase GLU-C from Boehringer Mannheim. The inhibitor 7-[(1,3-dihydroxypropyl-2)-amino]-ethylguanine was synthesized as earlier described [20].

2.2. Purification of calf spleen PNP

The commercial enzyme was purified by affinity chromatog-

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Abbreviations: PNP, purine nucleoside phosphorylase; TFA, trifluoroacetic acid; N^7Guo, $N(7)$-β-D-ribofuranosylguanine; N^7Ino, $N(7)$-β-D-ribofuranosylhypoxanthine; $N(7)$-acycloguanosine, 7-[(1,3-dihydroxypropyl-2)amino]ethylguanine; PEG, polyethylene glycol.

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raphy as first described for human erythrocyte PNP [25], with all operations at 4°C. The affinity gel consisted of the substrate analogue 6-hydroxy-9-*p*-aminobenzylpurine coupled to Sepharose CL-6B via trichloro-*s*-triazine [25]. Following chromatography, PNP substrates (inosine and phosphate) that were used to elute the enzyme from the affinity column, as well as the products of phosphorolysis (hypoxanthine and ribose-1-phosphate) formed during elution, were removed from the enzymatically active fractions by washing on 30 kDa cut-off Amicon filters with 10 mM citrate buffer pH 6.9 + 1 mM β-mercaptoethanol. The washing was continued until the absorbance of the effluent was less than 0.01 at 249 nm (maximum of absorbance of inosine and hypoxanthine). In the final wash, the enzyme solution was concentrated to ~20 mg/ml, with specific activity 34 U/mg. Gel electrophoresis (Phast system, Pharmacia) exhibited a single band (silver staining), and gel filtration (FPLC, Superose 12 Hr 10/20 column, Pharmacia) showed one symmetric peak.

Protein concentration was measured spectrophotometrically in 50 mM Tris-HCl buffer pH 7.6, with $\varepsilon_{280}^{1\%} = 9.64$, similar to that for human erythrocyte PNP [26,27].

2.3. Sequencing of calf spleen PNP

Sequencing of PNP from solution or blotted on polyvinylidene difluoride membrane via SDS-PAGE revealed that the major part of the protein was N-terminal blocked, probably by acetylated or acylated methionine. A minor amount of the protein had an unblocked N-terminus. The analysis of this minor amount of PNP indicated methionine and glutamine in positions 1 and 2. Position 3 could not be determined.

The enzyme was digested with trypsin in 100 mM Tris-HCl buffer, pH 8.5 at 37°C over night. Digestion with endoproteinase GLU-C was performed in 25 mM ammonium carbonate buffer, pH 8.0, at 25°C for 24 h. PNP was cleaved with cyanobromide according to [28]. Digestions were checked by analytical SDS-PAGE. Peptides were separated on a Vydac C 18 column, 0.46 × 15 cm, 5 μm, 300 A (type 218 TP 5415) using a gradient system (buffer A: 0.1% TFA/H_2O; buffer B: 0.1% TFA/acetonitrile), gradient t_0: 5% buffer B, t_{15min}: 5% buffer B, t_{180min}: 60% buffer B, t_{240min}: 80% buffer B; flow rate: 0.5 ml/min; 37°C (HPLC from Shimadzu/Kyoto; LC-10AD; SCL-10A).

Sequence analysis of peptides was performed by automatic Edman-degradation on an Applied-Biosystem (Foster-City, CA, USA) gas phase sequencer. Data were analyzed using the software 610A Data Analysis Program, Version 1.2. Sequencing of peptides from different digests revealed the sequence shown in Fig. 1. Relative to the sequence of human PNP, there are some amino acid exchanges, and a truncation of 5 amino acids at the C-terminus.

2.4. Enzyme assay

PNP activity was monitored spectrophotometrically with inosine as substrate and coupling with xanthine oxidase [27,29]. With 0.5 mM inosine as substrate in 50 mM phosphate buffer pH 7.0, one unit of PNP is the amount of enzyme which leads to phosphorolysis of one μmol of inosine to hypoxanthine and ribose-1-phosphate per minute at 25°C.

2.5. Binding constant of the inhibitor

The inhibition constant K_i for 7-[(1,3-dihydroxypropyl-2)amino]ethylguanine with the calf spleen enzyme was obtained as described for the human erythrocyte PNP [20]. Inhibition was competitive with respect to the substrate inosine, with $K_i = 5.6 \pm 0.5$ μM as compared to the value of 5 ± 2 μM with the human enzyme [20]. The structures of both inhibitors are shown in Fig. 2A.

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(M E N)              (K N)   (E)          (K H)          (I)
(M Q ?)G Y T Y E D Y Q D T A K W L L S H T E Q R P Q V A V I
                     10                   20                  30

                 (T D)           (I)                   (R)
C G S G L G G L V N K L T Q A Q T F D Y S E I P N F P E S T
                     40                   50                  60

                      (F)
V P G H A G R L V F G I L N G R A C V M M Q G R F H M Y E G
                     70                   80                  90

   (L)                    (H)       (D)
Y P F W K V T F P V R V F R L L G V E T L V V T N A A G G L
                     100                  110                 120

   (K)                                              (Q)
N P N F E V G D I M L I R D H I N L P G F S G E N P L R G P
                     130                  140                 150

 (D)       (D)                        (T)      (R) (L)
N E E R F G V R F P A M S D A Y D R D M R Q K A H S T W K Q
                     160                  170                 180

                              (V A)   (S)              (V) (Q)
M G E Q R E L Q E G T Y V M L G G P N F E T V A E C R L L R
                     190                  200                 210

(K).
N L G A D A V G M S T V P E V I V A R H C G L R V F G F S L
                     220                  230                 240

                         (L E)                (A)
I T N K V I M D Y E S Q G K A N H E E V L E A G K Q A A Q K
                     250                  260                 270

            (I)          (L P D K A S)
L E Q F V S L L M A S I P V
                     280
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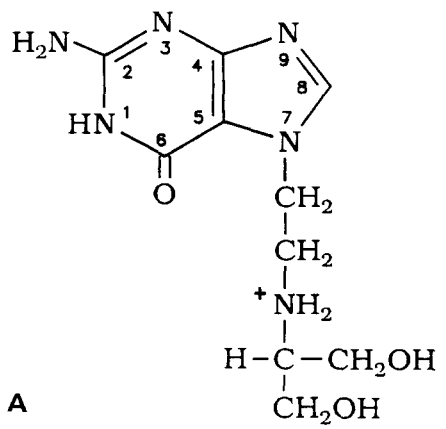
Fig. 1. Amino acid sequence of calf spleen PNP. Respective amino acids in the human enzyme are given above in parentheses. Relative to human PNP, the calf spleen enzyme is truncated at the carboxy terminus. Active site residues are underlined.

2.6. Crystallization

The complex of purified PNP with 7-[(1,3-dihydroxypropyl-2)amino]ethylguanine was prepared by addition of an appropriate volume of PNP (20 mg/ml) to a solution of 8 mM inhibitor, both in 10 mM citric buffer pH 6.9 + 1 mM β-mercaptoethanol. The resulting solution, containing 12 mg/ml PNP (0.3 mM in terms of monomer) and 2.7 mM inhibitor, was then incubated for 30 min at 18°C.

Crystallization of the complex was achieved by the hanging drop vapour diffusion technique at 18°C using 24-well tissue culture Linbro plates. The reservoir solution consisted of 11–16% (w/v) of PEG 4000, 40 mM Tris-HCl buffer pH 8.2–8.5 and 80 mM $MgCl_2$. The 4 μl hanging drops consisted of 2 μl of protein–inhibitor complex solution and 2 μl of the reservoir solution. Dome shaped crystals, with dimensions up to 0.25 mm, appeared after two weeks.



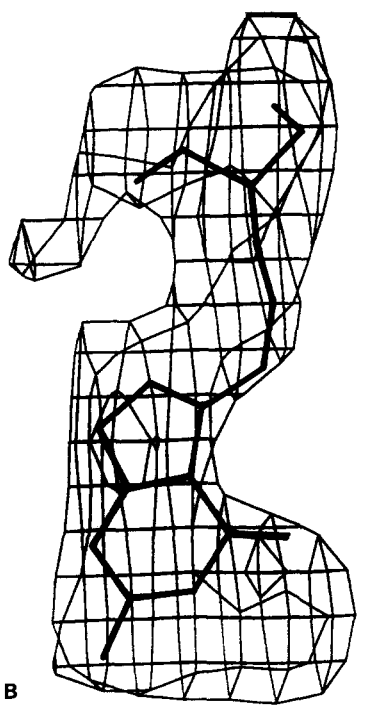


Fig. 2. (A) Chemical structure of 7-[(1,3-dihydroxypropyl-2)amino]ethylguanine. (B) Omit difference electron density superimposed with the 7-[(1,3-dihydroxypropyl-2)amino]ethylguanine model ($\|F_o| - |F_c\|$ map at $2 \times$ rms). Inhibitor model atoms were not included in the structure factor calculation.

2.7. Data collection and processing

Diffraction data were collected at 4°C on a MAR-imaging plate system on an Enraf-Nonius-rotating anode X-ray generator FR 571 (40 kV, 80 mA, 3×0.3 mm^2 focus, graphite monochromator, CuKα radiation), 30 frames of 1.0° oscillation. Data were processed using DENZO and SCALEPACK [30], R-merge = 0.11 for 6351 unique reflections for $|F_o| \geq 1\sigma|F_o|$ (99.7% of unique data in the resolution range of 38–2.9 Å). The intensities were converted to structure factor amplitudes using the program TRUNCATE in the CCP4 suite [31]. The estimated $B_{overall}$ from Wilson statistics is 16 Å^2. The space group is cubic $P2_13$ with a = 94.02 Å, V = 831910 Å^3 and one monomer in the asymmetric unit. PNP forms a trimer in solution [26]; in the crystal structure, the 3-fold crystallographic axis relates monomers to trimers.

2.8. Structure solution by molecular replacement

The crystal structure was solved by molecular replacement using the program AMoRe [31]. The rotation search was undertaken with the trimer of human PNP [14] in a space group of lower symmetry, $P2_12_12_1$ (resolution range 9–5 Å), and showed one prominent maximum (correlation coefficient 23.1, approximately twice that of the next highest maximum). This orientation was used to calculate the translation function for the monomer in the correct space group $P2_13$ (resolution range 8–4 Å, correlation coefficient 43.3). Rigid body refinement of the positioned monomer resulted in an R-value of 42%.

2.9. Refinement

One refinement cycle was done employing XPLOR's [32] slowcooling and Powell minimization steps. Restrained least-squares refinement was carried out with the TNT program package [33] in conjunction with omit- and difference electron density maps ($\|2F_o| - |F_c\|$ and $\|F_o| - |F_c\|$), which were interpreted on an ESV-station using the graphical program FRODO [34]. 31 water sites with good hydrogen bonding geometry were included in the model.

Refinement converged with an R-value of 0.21. Final geometrical quality (Å, degr.): bond distance rms deviation 0.013; bond angle rms deviation 2.54; torsion angle rms deviation 21.5 (not restrained in the refinement). Fig. 2B shows the omit difference electron density for the excluded inhibitor, superimposed with the final model of the inhibitor.

3. Discussion

The sequence of the calf spleen enzyme corresponds closely to that reported for human erythrocyte PNP [13], but is truncated at the carboxy terminus by 5 amino acids. The first three amino acids could not be conclusively sequenced for reasons described above. The N-terminus is neither involved in, nor located near, the active center.

None of the 32 amino acid residues which differ from those in the human enzyme is located in the region of the active site (see Fig. 1). This is in line with the sequence reported for a bovine PNP (origin not specified and no details given), which exhibits 87% indentity with the human PNP and conservation of all amino acids in the catalytic region [35]; and is consistent with the similar physico-chemical and kinetic properties of the calf and human enzymes [12,20,26] and their similar responses to inhibitors [8].

The crystal structure of human PNP was determined with crystals obtained from ammonium sulphate at pH 5.5, where the enzyme activity is low [36]; subsequent investigations on the binding of inhibitors by this enzyme were severely hampered by the fact that the phosphate binding site was fully occupied by sulphate [13]. The studies on human PNP show that guanine O^6 hydrogen bonds to Lys$^{244}N\eta$. Asn$^{243}N\delta$ bridges O^6, while N(1)-H and one of the N(2) amino hydrogens bind to Glu$^{201}O\varepsilon$'s (Fig. 3) [13,14,35].

The calf spleen PNP complexed with $N(7)$-acycloguanosine crystallizes at pH 8.2–8.5 from PEG, which is almost optimal for enzyme activity [17]. It has the advantage that the crystals are free of sulphate, which should facilitate studies on the role

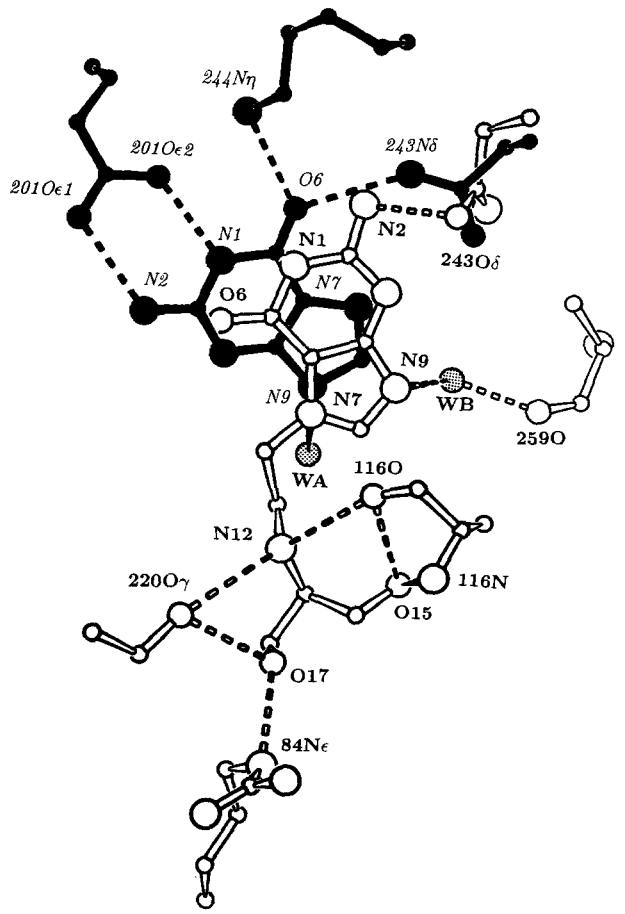


Fig. 3. Mode of binding of 7-[(1,3-dihydroxy-propyl-2)amino] ethylguanine in the active site of calf spleen PNP (white). Possible hydrogen bond lengths and angles (hydrogen positions Donor H···Acceptor were calculated where possible) are: Inhibitor O^{17}–Ser^{220} *Oγ* 2.65 Å; Inhibitor O^{17}–Arg^{84} *Nε* 2.79 Å (106°); Inhibitor O^{15}–Ala^{116} *N* 2.66 Å (138°); Inhibitor O^{15}–Ala^{116} *O* 3.10 Å; Inhibitor N^{12}–Ala^{116} *O* 3.11 Å (152°); Inhibitor N^{12}–Ser^{220} *Oγ* 3.33 Å (151°); Inhibitor N^{7}–Water A 3.41 Å; Inhibitor N^{9}–Water B 3.00 Å; Water B–Glu^{259} *O* 2.32 Å; Inhibitor N^{2}–Asn^{243} *Oδ* 2.41 Å. For comparison, mode of binding of guanine in the active site of human erythrocyte PNP is given (black, labelled in italic) [14]. The figure drawn with SCHAKAL 88 [39].

of the phosphate binding site in binding of inhibitors/substrates. Fig. 3 depicts the binding mode of the *N*(7)-acycloguanosine inhibitor in the active site of calf spleen PNP. As previously predicted [20] on the basis of kinetic studies on *N*(7)-nucleoside substrates (N^7Guo and N^7Ino), the inhibitor binds in an inverted ('upside down') orientation with respect to guanine in the human PNP [14]. One of the N(2) amino hydrogens is bonded to Asn^{243}*Oδ* (Fig. 3), N(9) is bonded via a water molecule (W B in Fig. 3) to the peptide O of Glu^{259}, and N(7) is within bonding distance of a water molecule W A.

The acyclic chain, which is located in the region normally occupied by the ribose, may be engaged in several hydrogen bonds with the CH_2–OH hydroxyls (O^{15} and O^{17}) and the nitrogen N^{12} (Fig. 3). Since the crystals were grown at pH 8.2–8.5, this secondary nitrogen (pKa~9.5) should be protonated, and can form hydrogen bonds to Ser^{220}*Oγ* and to Ala^{116}*O*. It follows that it is the acyclic chain which is predominantly responsible for binding of the inhibitor.

These findings may have additional far reaching implications. We have elsewhere drawn attention to the natural occurrence, albeit uncommon, of purine *N*(7)-nucleosides and their substrate/inhibitor properties vs. PNP-like enzymes from various sources [20]. Also relevant are recent reports that 7-riboside derivatives of 1,3-dialkylxanthines are active antagonists in the A_3 receptor-mediated inhibition of adenylate cyclases, and in some instances are more potent and/or more specific than the corresponding *N*(9)-nucleosides [37]. The present results provide at least a good lead to the possible binding mode of these agonists to the A_3 receptors. Furthermore, it is worth noting that *N*(7)-acyclonucleosides of guanine exhibit significant antiherpes activities [38], the mechanisms of which remain to be elucidated.

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