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Crystallization and preliminary X-ray studies of purine nucleoside phosphorylase from *Cellulomonas sp.*

AGNIESZKA BZOWSKA,[a] JAN TEBBE,[b] MARIJA LUIĆ,[c] BEATA WIELGUS-KUTROWSKA,[a] WERNER SCHRÖDER,[b] DAVID SHUGAR,[a] WOLFRAM SAENGER[b] AND GERTRAUD KOELLNER[b]* *at* [a]*Department of Biophysics, Institute of Experimental Physics, University of Warsaw, Zwirki i Wigury 93, 02-089 Warsaw, Poland,* [b]*Institut für Kristallographie, Freie Universität Berlin, Takustrasse 6, D-14195 Berlin, Germany, and* [c]*Rudjer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia. E-mail: koellner@chemie.fu-berlin.de*

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Abstract

The commercially available enzyme purine nucleoside phosphorylase (PNP) from *Cellulomonas sp.* was purified by ion-exchange chromatography, partially sequenced and crystallized in two different crystal forms using the hanging-drop vapour-diffusion technique. Crystal form *A* grows as polyeders and/or cubes in the cubic space group $P4_232$ with unit-cell dimension $a = 162.5$ Å. Crystal form *B* appears as thick plates in the space group $P2_12_12_1$ with unit-cell dimensions $a = 63.2$, $b = 108.3$ and $c = 117.4$ Å. Both crystal forms contain three monomers (one trimer) in the asymmetric unit.

1. Abbreviations

PNP, purine nucleoside phosphorylase; BIS/TRIS-propane buffer, 1,3-bis[tris-(hydroxymethyl)methylamino]propane; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

2. Introduction

Purine nucleoside phosphorylase (PNP, purine nucleoside: orthophosphate transferase E.C. 2.4.2.1) is a ubiquitous enzyme of the purine salvage pathway (Stoeckler, 1984; Montgomery, 1993). It catalyzes the reversible phosphorolysis of the glycosidic bond of ribo- and 2′-deoxyribo-purine nucleosides and some analogues, to generate the respective purine base and ribose- and deoxyribose-1-phosphate.

In mammals 'low-molecular weight' (M_r about 90 kDa, subunit M_r about 30 kDa) trimeric PNPs are found that are specific, in both binding and catalysis, for 6-ketopurine nucleosides. Some prokaryotes contain a second class of PNPs characterized as so-called 'high-molecular weight', with M_r in the range 110–150 kDa. These are mainly hexameric enzymes with broader specificities, in that they accept as substrates all three naturally occurring purine nucleosides, *i.e.* inosine, guanosine and adenosine (*e.g.* Jensen & Nygaard, 1975; Jensen, 1978).

Enzymes from both classes show simple Michaelis kinetics with some of their substrates, *e.g.* PNP from *E. coli* with purine nucleosides (Jensen & Nygaard, 1975) and PNP from calf spleen with some ribonucleosides (Agarwal *et al.*, 1975; Ropp & Traut. 1991*a*,*b*). For PNPs from other sources, kinetic data for one or more substrates indicates cooperativity between subunits *e.g.* for phosphate as substrate of *E. coli* PNP, and both phosphate and inosine as substrates of human erythrocyte PNP (Jensen & Nygaard, 1975; Stoeckler, 1984). The mechanism of enzymatic catalysis by PNPs is not fully characterized. There is some evidence, however, that the N(7) of the purine base is protonated during the course of the reaction in the case of mammalian PNPs (Kline & Schramm, 1993; Bzowska *et al.*, 1993).

Both classes of PNPs are considered promising targets for the design of inhibitors. There is a need for potent selective inhibitors of PNPs from various sources because of their potential utility as immunosuppresive or antiparasitic agents (Giblett *et al.*, 1975; Stoeckler, 1984; Montgomery, 1993; Daddona *et al.*, 1986) and their ability to potentiate the activities of nucleoside analogues as antiviral and antitumor agents. Some 'high-molecular weight' non-specific PNPs have been employed for enzymatic synthesis of purine nucleoside analogues, including those with chemotherapeutic value like virazole and ribovirin (*e.g.* Krenitsky *et al.*, 1981; Utagawa *et al.*, 1985; Hennen & Wong, 1989; Ling *et al.*, 1990, 1994; Shirae & Yokozeki, 1991). Moreover the *E. coli* PNP gene was recently shown to be useful in gene therapy of human tumors (Sorscher *et al.*, 1994; Hughes *et al.*, 1995). High-resolution three-dimensional structures of the calf spleen and *E. coli* enzymes have recently been made available in the Brookhaven Protein Data Bank (1VFN: Koellner *et al.*, 1997; 1A9T: Mao *et al.*, 1998; 1ECP: Mao & Ealick, 1997; 1A69: Koellner *et al.*, 1998) and these should prove useful in studies on the mechanism of the reaction and the design of potent inhibitors.

The PNP from *Cellulomonas sp.* exhibits an interesting combination of properties of both classes of PNPs, *e.g.* the molecular weight estimated from gel filtration is 114 kDa (Tebbe *et al.*, 1997), indicating that it could belong to the 'high-molecular weight' class. However, adenosine is not a substrate, but is a competitive inhibitor of the enzyme with a K_i of 160 μ*M* (Wielgus-Kutrowska *et al.*, 1997). One other PNP that does not phosphorylate adenosine, but is able to bind it, was isolated from *Proteus vulgaris* (Surette *et al.*, 1990). Similarities between PNP from *Cellulomonas sp.* and mammalian PNP are reflected in the subunit molecular weight, which is about 30 kDa (Stoeckler *et al.*, 1978); but the molecular weight of 114 kDa obtained from gel filtration does not exclude that the enzyme from *Cellulomonas sp.* is a tetramer in solution, while for all mammalian PNPs a trimer is the active form. *Cellulomonas sp.* PNP is thermostable up to 323 K, similar to PNP from *E. coli*, while the mammalian enzymes are much more sensitive to thermal inactivation (Krenitsky *et al.*, 1981). Furthermore, as for *E. coli* PNP, it shows cooperativity in kinetic data for orthophosphate, but not for nucleoside substrates (Jensen & Nygaard, 1975; Wielgus-Kutrowska *et al.*, 1997). Hence, the enzyme from *Cellulomonas sp.* is not a member of either of the two main classes of PNP, since some of its properties are common to both classes while others are unique (especially binding, but not phosphorolysis, of adenosine).

This prompted us to undertake a detailed X-ray diffraction analysis of the crystal structure of PNP from *Cellulomonas sp*. A comparison of its three-dimensional structure with those from calf spleen and *E. coli*, representing the two main classes of PNPs, should assist in further elucidation of the mechanism of phosphorolysis and the design of specific inhibitors, and provide an additional tool for enzymatic synthesis of purine nucleosides. We here describe crystallization of PNP from *Cellulomonas sp*. in two different crystal forms, both suitable for X-ray structural analysis.

3. Crystallization and partial sequencing

Commercially available purine nucleoside phosphorylase (Toyobo, Japan), with specific activity of approximately 8 U mg^{-1}, was purified on a BioCad station (PerSeptive Biosystems), using a strong ion-exchange POROS 20HQ (quaternized polyethyleneimine) column, followed by a weak ion-exchange POROS 20PI (polyethyleneimine) column (both columns 4.6 mm diameter, 100 mm long). The columns were run with BIS/TRIS-propane buffer pH 6.0 and 9.0, respectively. The enzyme was removed from the columns with an NaCl gradient and finally concentrated and rebuffered by Amicon ultrafiltration devices (Centricon and Centriprep). The final specific activity was 107 U mg^{-1} (*versus* inosine, at pH 7, 298 K). The enzyme was better than 90% pure, with a 29 kDa subunit from mass spectra (MALDI-MS).

An aliquot of purified protein was subjected to N-terminal automatic Edman degradation on an Applied Biosystems gas-phase sequencer. For further internal sequence information, peptides were generated by digestion with various proteases and cyanobromide cleavage. Sequencing of overlapping peptides led to an almost complete amino-acid sequence alignment with two links remaining to be identified. Comparison of this (almost complete) amino-acid sequence with those available in data banks (*e.g.* human, calf, mouse, *B. subtilis*, *Mycobacterium leprae*) showed no sequence homology of any of the latter to the N-terminal region of the PNP from *Cellulomonas sp*. In other regions some similarities to mammalian PNP's were noted (Bzowska *et al.*, 1995; Williams *et al.*, 1984; Jenuth & Snyder, 1991); in particular, most amino acids involved in the purine and phosphate binding sites (Koellner *et al.*, 1997; Narayana *et al.*, 1997; Mao *et al.*, 1998) appear to be conserved in the *Cellulomonas* PNP. Closest sequence homology was found to the putative PNP from *M. leprae* (Smith & Robison, 1994; Robison *et al.*, 1994).

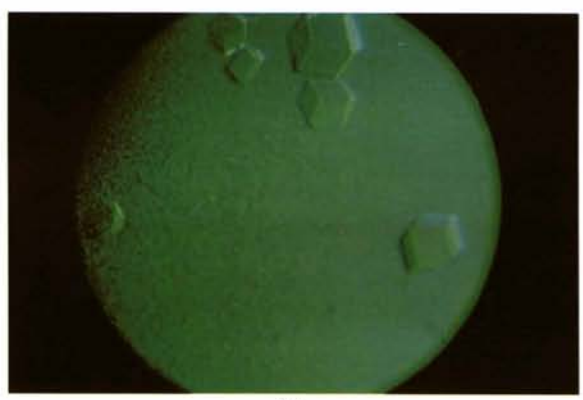
Single crystals of purified PNP were obtained by the vapour-diffusion hanging-drop method in two crystal forms. The reservoir for crystal form *A* contained 100 m*M* Hepes buffer, pH 7–8 and 1.2–1.5 *M* ammonium sulfate. The protein drop was prepared by mixing 2 µl of reservoir with 2 µl of protein (11 mg cm^{-3} in 10 m*M* TRIS–buffer pH 7.6 plus 1 m*M* β-mercaptoethanol). Crystals appeared after few days, in Linbro plates kept in either 277 or 291 K surroundings. Crystals obtained at 277 K were more stable and better diffracting. At both temperatures crystals grew up to 0.6 mm as polyeders and/or cubes (see Fig. 1*a*).

Crystal form *B* was prepared using polyethylene glycol 4000 (Merck) from 8 to 15% as precipitant, 50 m*M* cacodylate buffer pH 6.2–7.0 and 100 m*M* calcium acetate. Protein concentration of the droplet (2 µl reservoir + 2 µl protein in buffer as for crystal form *A*) was varied from 7 to 11 mg cm^{-3}. Single crystals suitable for high-resolution X-ray diffraction experiments appeared after approximately two weeks at room temperature as thick plates with the longest dimension up to 0.8 mm and thickness up to 0.3 mm (see Fig. 1*b*).

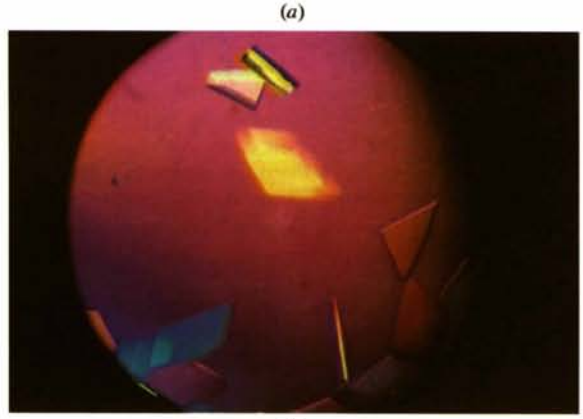
4. X-ray analysis

4.1. *Crystal form A*

X-ray diffraction data were collected at 100 K after flash-freezing the crystals in a cold stream of liquid nitrogen. Before freezing the crystals were transferred to a cryoprotectant consisting of 30% glycerol dissolved in the crystallization buffer. Data collection was performed at beamline X11 of the EMBL Outstation (DESY/Hamburg), 23 frames of 1.2° oscillation, with monochromatic synchrotron radiation (λ = 0.92), using a MAR Research image-plate detector. Data were processed with *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). The data set contained 6531 unique reflections (98.8% completeness to 4.0 Å resolution), had an R_{sym} of 0.162 (11 516 measurements) and indexed in the cubic space group $P4_232$ with cell dimension a = 162.5 Å.



(*a*)



(*b*)

Fig. 1. (*a*) Crystal form *A*, cubic $P4_232$, a = 162.5 Å. The largest crystal has edge dimensions of about 0.4 mm. (*b*) Crystal form *B*, orthorhombic $P2_12_12_1$, a = 64.2, b = 108.8, c = 119.4 Å. The enlargement of photograph as for crystal form *A*.

4.2. *Crystals form B*

Diffraction data were collected at 4° at the station as for form *A*, 89 frames of 1.0° oscillation. The final data set contained 34 872 unique reflections (80% completeness to 2.2 Å resolution) and an R_{sym} of 0.083 (266 396 measurements). Data were indexed in the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions of a = 63.2, b = 108.3, c = 117.4 Å.

Experimental data from gel filtration suggested that the active enzyme form in the solution is a tetramer (see §2); but, according to the criteria of Matthews (1968), both crystal forms contain three monomers in the asymmetric unit: V_m = 1.99 and 2.33 Å^3 Da^{-1} for cubic and orthorhombic crystals, respectively. Therefore, density measurements of the crystals were performed using a Ficoll gradient (Westbrook, 1985). Crystal form *A* dissolved in the Ficoll solution. For form *B* a density of 1.21 g cm^{-3} was determined, suggesting that the enzyme is a trimer in the crystalline state, since theoretical density values for the trimeric and tetrameric forms of crystal form *B* are 1.19 and 1.25 g cm^{-3}, respectively. An additional experiment was carried out by placing a crystal of form *B* in a 50% Ficoll solution with density 1.22 g cm^{-3} in the middle of a long tube. The crystal floated up, which clearly excludes the tetrameric form.

A self-rotation function for the orthorhombic crystal form, calculated with the program *POLARRFN* (Collaborative Computational Project, Number 4, 1994), showed the highest non-origin peak at κ = 120, ω = 50.1 and φ = 0.0° with 25.6% height of the origin peak (Fig. 2). κ = 120° indicates a threefold symmetry, and confirms the trimeric structure of PNP from *Cellulomonas sp.*

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