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## STRUCTURE OF MACROMOLECULAR COMPOUNDS

# Purification, Crystallization, and Preliminary X-ray Diffraction Study of Purine Nucleoside Phosphorylase from *E. coli*

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**Abstract**—Crystals of *E. coli* purine nucleoside phosphorylase were grown in microgravity by the capillary counter-diffusion method through a gel layer. The X-ray diffraction data set suitable for the determination of the three-dimensional structure at atomic resolution was collected from one crystal at the Spring-8 synchrotron facility to 0.99 Å resolution. The crystals belong to sp. gr. $P2_1$ and have the following unit-cell parameters: $a = 74.1$ Å, $b = 110.2$ Å, $c = 88.2$ Å, $\alpha = \gamma = 90°$, $\beta = 111.08°$. The crystal contains six subunits of the enzyme comprising a hexamer per asymmetric unit. The hexamer is the biological active form of *E. coli*. purine nucleoside phosphorylase.

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## INTRODUCTION

Purine nucleoside phosphorylases (PNPs) (EC 2.4.2.1) are key enzymes involved in purine metabolism. They catalyze the reversible phosphorolysis of purine nucleosides, resulting in the cleavage of the glycosidic bond between the ribose and the base (C1'–N) to give the free base and α-D-ribofuranosyl 1-phosphate (ribose 1-phosphate) [1–3]. PNPs catalyze the transglycosylation, *i.e.*, the transfer of a carbohydrate moiety from one base to another. This is a key reaction in the salvage pathway for the synthesis of nucleosides [4].

Due to the differences in the quaternary structure and specificity, as well as because of the low level of amino-acid-sequence homology, PNPs from mammals and microorganisms belong to two different classes. The amino-acid-sequence homology between representatives of two classes—PNPs from mammals and microorganisms—is no higher than 16%. Mammalian purine nucleoside phosphorylases consist of three identical subunits with a molecular weight of 90 kDa. Only 6-oxopurine nucleosides are substrates of these enzymes. Purine nucleoside phosphorylases from microorganisms, including *E. coli* PNP, exist as hexamers with a molecular weight of about 110–150 kDa and are characterized by a wider range of specificity. Apart from 6-oxopurine nucleosides, 6-aminopurine nucleosides and nucleosides with other substituents at the carbon 6 of the purine base are substrates of these enzymes. Among microbial PNPs, the enzyme from *E. coli* is the least selective with respect to the carbohydrate component of the nucleoside. This enzyme can cleave purine derivatives not only of ribose but also of deoxyribose and arabinose [5].

The difference in the specificity between microbial and mammalian PNPs was employed in the development of a strategy for cancer treatment by gene therapy based on gene transfection of PNP to the tumor tissue [6–8]. This method resides on the fact that some analogues of purine nucleosides are prodrugs. They are resistant against human PNP but are cleaved by *E. coli* PNP to form purine bases highly toxic to cancer cells. In experiments on the gene transfection of PNP to the tumor tissue after the gene expression, cancer cell death was observed because of the activation of the administered prodrug by purine nucleoside phosphorylase.

Due to the PNP-catalyzed transglycosylation, *E. coli* PNP is widely employed in the biotechnology industry for the combined chemical–enzymatic synthesis of analogues of natural purine nucleosides, many of which are used as effective anticancer and antiviral drugs [9, 10].

The chemical–enzymatic approach to the synthesis of medically important analogues of natural nucle-

**Fig. 1.** Crystals of *E. coli* purine nucleoside phosphorylase grown by the vapor-diffusion technique using sulfate ammonium as the precipitant. The crystal length is 0.7 mm.

osides rose in popularity since it can be used to perform key transformations with high efficiency, as well as with high regio- and stereoselectivity. The ability of *E. coli* PNP to catalyze the transglycosylation underlies the synthesis of a series of drugs used in the therapy of hard-to-cure viral and autoimmune diseases and tumor damage of the human hematopoietic system.

In view of the application of *E. coli* PNP in biotechnology and medicine, the construction of mutant forms of the enzyme with desired specificity is of considerable interest. The determination of the atomic coordinates of the model of the enzyme at high resolution will make it possible to increase the accuracy of the results obtained by computer simulation methods for the rational design of new inhibitors and mutant forms of the enzyme.

In the present work, we obtained crystals of *E. coli* PNP in microgravity on the International Space Station. These crystals are suitable for determining the three-dimensional structure of the enzyme at 0.99 Å resolution.

## MATERIALS AND METHODS

The enzyme *E. coli* PNP for the crystal growth in microgravity was produced using the producer strain *E. coli* BL21(DE3)/pERPUPHOI [11]. The producer strain of PNP was cultured in the YT medium supplemented with ampicillin (100 μg/mL) at 37°C until the absorbance $A_{595} = 0.8$ was achieved. The gene expression was induced by isopropyl β-D-thiogalactopyranoside at a concentration of 0.4 mM. The biomass was grown at 37°C for 4 h. The cells were separated by centrifugation (5000 rpm, 20 min, 4°C). The wet weight of the cells from 1 L of the culture was 4 g.

The biomass containing the target protein was resuspended in a lysis buffer and disintegrated by ultrasound for 10 min. The homogenate was centrifuged for 40 min at 12 000 rpm. The supernatant was fractionated on a Sepharose QXL column using a NaCl gradient from 0 to 0.5 M; the column size was 1.6 × 12 cm. The recombinant protein was salted out and purified on Sepharose QHP (HiTrap 5 mL) using a NaCl gradient from 0.25 to 0.4 M. The eluate was concentrated by ultrafiltration using an YM30 membrane, and minor amounts of protein and nonprotein impurities were separated by gel filtration using Sephadex S-200. After the gel filtration, the protein was concentrated to 32 mg/mL and stored at –80°C.

### *Crystal Growth by Vapor-Diffusion Technique*

We chose the conditions reported in [12] as the initial crystallization conditions for *E. coli* PNP. However, in these conditions we obtained poor-quality needlelike crystals unsuitable for X-ray diffraction. Therefore, we performed the screening of crystallization conditions by varying the concentration of ammonium sulfate in the pH range of 4.0–6.0. The best crystals were obtained at a protein concentration of 22 mg/mL (0.02 M Tris-HCl, pH 7.4, 0.1 M NaCl) using a 2 M ammonium sulfate solution containing 0.05 M sodium citrate, pH 5.0, as the reservoir solution (Fig. 1).

In addition to ammonium sulfate, polyethylene glycol (PEG) with molecular weights from 2000 to 15000 in the pH range of 4.0–8.5 was used as the precipitant for the crystallization by the vapor-diffusion technique. The best crystals were obtained at a protein concentration of 24 mg/mL using 5% PEG 2000 in 0.05 M sodium citrate, pH 5.0, as the precipitant.

### *Crystal Growth by the Capillary Counter-Diffusion Method*

The crystallization conditions for *E. coli* PNP found by the vapor-diffusion technique were adapted and optimized to apply the capillary counter-diffusion technique. The experiments were performed as described in [13, 14]. A protein solution (7 μL) was placed in a glass capillary 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. In experiments employing the capillary counter-diffusion method, we varied the protein and precipitant concentrations and the length of the silicone tube containing agarose gel, which was attached to the capillary. Single crystals were obtained using ammonium sulfate or PEG 2000 as the precipitant (Fig. 2).

A solution of the protein PNP with a concentration of 21.6 mg/mL contained 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.04% $NaN_3$. The reservoir solution was composed of 0.05 M Na citrate, pH 5.0, 0.02 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.04% $NaN_3$, and 15% PEG 2000 (or 25% ammonium sulfate). The experi-

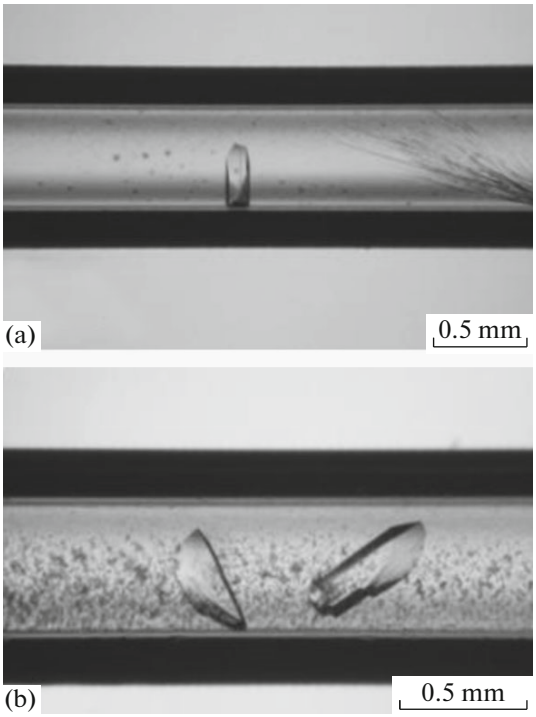


**Fig. 2.** Crystals of *E.coli* purine nucleoside phosphorylase grown by the capillary counter-diffusion method on earth using (a) sulfate ammonium and (b) PEG 2000 as the precipitant.

ments in microgravity were performed in the same conditions.

### *X-ray Diffraction Data Collection and Processing*

The X-ray data set was collected to 0.99 Å resolution at the Spring-8 synchrotron facility (Japan) from one crystal grown in microgravity using 15% PEG 2000 as the precipitant (Fig. 3). Before exposure to X-rays, the crystal was frozen in a cryoprotectant solution containing 15% glycerol. The X-ray data were obtained by the rotation method at a wavelength of 0.8 Å. The rotation angle was 360°, the oscillation angle was 0.5°, and the crystal-to-detector distance was 100 mm. The X-ray diffraction intensities were processed to 0.99 Å resolution using the imosflm program package [15]. The X-ray data collection statistics are given in the table.

### *Structure Solution*

The X-ray diffraction data were employed to solve the three-dimensional structure of the enzyme by the molecular-replacement method using the PHASER program [16]. The atomic coordinates of the unligated enzyme molecule determined at lower resolution (PDB_ID: 1ECP) [17] served as the starting model. The solution with Rf = 0.291 gave the packing containing one PNP hexamer per asymmetric unit of sp. gr. $P2_1$. The structure refinement is currently underway.

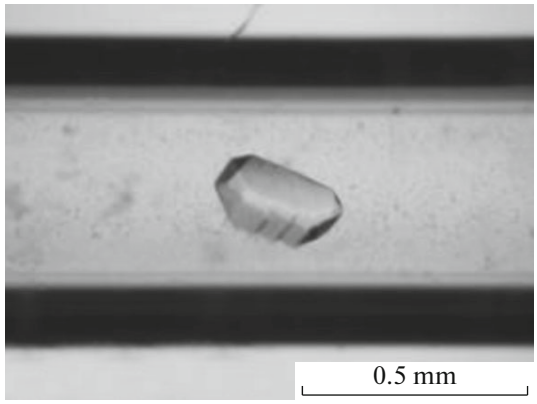


**Fig. 3.** Crystal of *E. coli* purine nucleoside phosphorylase, which was grown in microgravity by the capillary counter-diffusion method and was used for X-ray data collection at the synchrotron radiation source (15% PEG 2000 as the precipitant).

## RESULTS AND DISCUSSION

The crystals suitable for determining the three-dimensional structure of *E. coli* PNP were grown in microgravity on the International Space Station by the capillary counter-diffusion method through a layer of agarose gel using 15% PEG 2000 or ammonium sulfate as the precipitant. It is known that the slow diffusion of the precipitant into a protein solution through a gel layer and the crystal growth in microgravity facilitate the improvement of the X-ray diffraction quality of the crystals [18]. The crystals were grown employing the Japan Aerospace Exploration Agency (JAXA) facilities [19] according to a procedure described in [13, 14]. Several crystallization units were packed in special boxes and delivered to the International Space

Crystallographic data and X-ray data-collection statistics

| | |
|---|---|
| Sp. gr. | $P2_1$ |
| *a*, *b*, *c*, Å | 74.1, 110.2, 88.2 |
| α = γ, β, deg | 90, 111.08 |
| *T*, K | 100 |
| λ, Å | 0.8 |
| Resolution, Å | 30.00–0.99 (1.04–0.99)* |
| Number of unique reflections | 718472 (104431) |
| Redundancy | 3.71 (3.60) |
| Completeness, % | 98.41 (98.05) |
| $I/\sigma(I)$ | 4.9 (2.11) |
| Rmrgd-F, % | 9.8 (67) |

* The data for the last high-resolution shell are given in parentheses.

Station, where the crystal growth occurred in a thermostat at 20°C.

The crystals grown in the presence of 15% PEG 2000 as the precipitant (Fig. 3) diffracted to 0.99 Å resolution in experiments on a synchrotron radiation source, whereas crystals grown using ammonium sulfate as the precipitant diffracted to 1.43 Å. The control crystals grown on earth and studied on the same synchrotron gave lower resolution (2.13 Å).

The Matthews coefficient [20] for the crystals is 2.18 $Å^3$/Da, which indicates that the solvent content is 43.54% of the unit-cell volume. There are six subunits of the enzyme per asymmetric unit (sp. gr. $P2_1$). These subunits form a hexamer, which is the biologically active form of *E. coli* PNP. The structure of the enzyme is currently refined at 0.99 Å resolution.

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