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STRUCTURE OF MACROMOLECULAR COMPOUNDS

Crystallization and Preliminary X-ray Diffraction Study of Recombinant Adenine Phosphoribosyltransferase from the Thermophilic Bacterium *Thermus thermophilus* Strain HB27

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Abstract—Adenine phosphoribosyltransferase (APRT) belongs to the type I phosphoribosyltransferase family and catalyzes the formation of adenosine monophosphate *via* transfer of the 5-phosphoribosyl group from phosphoribosyl pyrophosphate to the nitrogen atom N9 of the adenine base. Proteins of this family are involved in a salvage pathway of nucleotide synthesis, thus providing purine base utilization and maintaining the optimal level of purine bases in the body. Adenine phosphoribosyltransferase from the extremely thermophilic *Thermus thermophilus* strain HB27 was produced using a highly efficient *E. coli* producer strain and was then purified by affinity and gel-filtration chromatography. This enzyme was successfully employed as a catalyst for the cascade biosynthesis of biologically important nucleotides. The screening of crystallization conditions for recombinant APRT from *T. thermophilus* HB27 was performed in order to determine the enzyme structure by X-ray diffraction. The crystallization conditions, which were found by the vapor-diffusion technique, were then optimized to apply the counter-diffusion technique. The crystals of the enzyme were grown by the capillary counter-diffusion method. The crystals belong to sp. gr. $P12_11$ and have the following unit-cell parameters: $a = 69.86$ Å, $b = 82.16$ Å, $c = 91.39$ Å, $\alpha = \gamma = 90°$, $\beta = 102.58°$. The X-ray diffraction data set suitable for the determination of the APRT structure at 2.6 Å resolution was collected from the crystals at the SPring-8 synchrotron facility (Japan).

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INTRODUCTION

Adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) is involved in nucleotide metabolism, belongs to the type I phosphoribosyltransferase family, and catalyzes the magnesium-dependent transfer of ribose 5-phopshate from 5-phosphoribosyl 1-pyrophosphate to the nitrogen atom N9 of the purine base to form adenosine 5'-monophosphate and pyrophosphate [1–4]. Enzymes of this family play a key role in the salvage pathway of nucleotide synthesis from free bases and control the level of free bases in the cell via activation and inhibition mechanisms [5–9]. Free purine bases are utilized by both adenine-specific phosphoribosyltransferase and enzymes specific for 6-oxypurine bases. Enzymes of the phosphoribosyltransferase family are involved also in the synthesis of pyridine coenzymes (NAD and NADP) and the amino acids histidine and tryptophan [1].

The reutilization of purines via the salvage metabolic pathway plays an important role during vigorous growth, for example, during embryogenesis. The same process is activated during tumor proliferation [10]. Adenine phosphoribosyltransferase deficiency in humans and most other organisms, which can synthesize adenosine monophosphate de novo through the main pathway for purine biosynthesis, is not lethal but leads to the development of a number of kidney diseases. As opposed to mammals, most of simple parasitic organisms lost the ability to synthesize purines de novo and contain additional enzymes, which utilize excess purines of the host for the nucleotide synthesis [11]. Since enzymes with phosphoribosyltransferase activity are essential for the survival of parasites, these enzymes are convenient therapeutic targets for drug design against diseases caused by parasites.

It was shown [12] that APRT, phosphoribosylpyrophosphate synthetase, and ribokinase from the ther-

mophilic *T. thermophilus* strain HB27 can be used for the biosynthesis of biologically important nucleotides through a multi-enzymatic cascade conversion of D-pentoses into purine nucleotides. Nucleotides are important metabolites of DNA and RNA biosynthesis, as well as cosubstrates and cofactors of numerous biochemical conversions. Due to the important role of these compounds in living cells, the synthesis of natural representatives of this class and their various analogs is of great interest. It is know than many analogs of natural nucleotides modified at the heterobase or the carbohydrate moiety are therapeutic agents against viral infections and malignancies [13–16].

Although the cascade synthesis method allows the one-pot preparation of a nucleotide from D-pentose and free heterobase, the limited specificity of the enzymes that act at different steps of the cascade synthesis renders this method impractical. The computational modeling of mutant forms of the enzymes with altered specificity requires the use of atomic coordinates of the native form of the enzyme. The crystallization and preliminary structural study of two enzymes, namely, ribokinase and phosphoribosylpyrophosphate synthetase, from the extremely thermophilic bacterium *T. thermophilus* strain HB27 were described in [14, 15]. These enzymes are promising for the use in the cascade method. In the present study, we found crystallization conditions, obtained crystals of recombinant *Tth*APRT, and collected the X-ray diffraction data set from these crystals at 2.6 Å resolution suitable for the determination of the three-dimensional structure of the enzyme and subsequent structural study.

MATERIALS AND METHODS

Cloning of the Gene

The TT_RS06315 gene (TTC1250) from *T. thermophilus* HB27 encoding *Tth*APRT was amplified by the polymerase chain reaction using genomic DNA of the *T. thermophilus* strain HB27 as the template and synthetic primers and was then cloned into the pET-23d+ expression vector at the NcoI and XhoI restriction endonuclease recognition sites [12].

Cultivation of the Producer Strain

The *E. coli* BL21(DE3) strain was transformed using the constructed pER-APRT-Tth expression vector. The cultivation was performed at 37°C in a LB medium containing 100 μg/mL ampicillin. Once the absorbance of the cultures reached $A_{595} = 0.8$, IPTG was added to the cultures to the final concentration of 0.4 mM, and the cultivation was continued for 4 h at 37°C. After the completion of cultivation, the cell biomass was separated by centrifugation.

Protein Isolation and Purification

The cell biomass was resuspended in 50 mM Tris-HCl buffer, pH 8.7, supplemented with 1 mM phenylmethylsulfonyl fluoride in a ratio of 1 : 10 (w/v) and then disrupted using a Labsonic P ultrasonicator. The cell debris was removed by centrifugation. Sodium chloride was added to the clarified cell lysate to the concentration of 300 mM, and the lysate was heat-treated for 10 min at 65°C. After the removal of the protein precipitate, the lysate was desalted using a PD-10 column (GE Healthcare, USA) equilibrated with 20 mM Tris-HCl buffer, pH 9.0, supplemented with 1.0 mM EDTA. The eluate was loaded onto a XK 16/20 column packed with Q Sepharose XL resin (GE Healthcare, USA) and equilibrated with the same buffer solution. The target protein was eluted with a linear concentration gradient of NaCl from 0 to 400 mM. The fractions containing the target protein were combined and loaded onto a XK 16/20 column packed with Phenyl Sepharose HP (GE Healthcare, USA) and equilibrated with 20 mM Tris-HCl buffer, pH 7.6, supplemented with 1 M $(NH_4)_2SO_4$ and 1.0 mM EDTA. The enzyme *Tth*APRT was eluted with a linear concentration gradient of $(NH_4)_2SO_4$ from 1 to 0 M. The fractions containing the target protein were combined and concentrated by ultrafiltration on a PBGC 10 kDa polysulfone membrane to the final concentration of 5.0 ± 0.5 mg/mL as described earlier. The final purification was performed on a Superdex 200 HiLoad 16/60 column (GE Healthcare, USA) using 20 mM Tris-HCl buffer, pH 8.0, supplemented with 50 mM NaCl, 5% glycerol, and 0.04% NaN_3. The fractions containing the target protein were combined and concentrated by ultrafiltration to the final concentration of 12 ± 1 mg/mL. The protein concentration was determined by the Bradford assay using BSA (bovine serum albumin) as the standard [17]. The purity of the protein was determined by denaturing polyacrylamide-gel electrophoresis [18].

Crystal Growth

The screening of crystallization conditions was performed by the hanging-drop vapor-diffusion technique using a protein solution in 0.02 M Tris-HCl buffer, pH 8.0, supplemented with 50 mM NaCl, 5% glycerol, 0.04% NaN_3, and 5 mM AMP. The protein concentration was 12.5 mg/mL. Solutions of ammonium sulfate and different-molecular-weight polyethylene glycols (PEGs) at different concentrations were used as precipitants. Experiments were performed in the pH range of 4.6–8.6. The crystal formation was observed in PEG 3350 and PEG 8000 solutions. The crystals suitable for X-ray diffraction were grown in a capillary with a diameter of 0.5 mm using the reservoir solution composed of 25% w/v PEG 3350, 0.1 M HEPES, pH 7.0, 0.5 M NaCl, and 0.04% NaN_3.

X-ray Diffraction Data Collection

Before the collection of the X-ray diffraction data set, the crystals were withdrawn from the capillary, placed in the reservoir solution, and then, using a cryoloop, transferred into a cryoprotectant solution, which contained the same components as the reservoir solution with the addition of 20% glycerol. The X-ray diffraction data set was collected from a crystal, which was flash-frozen in a liquid nitrogen flow, at 100 K at the beamline BL41XU of the SPring-8 synchrotron radiation facility (Japan) equipped with a PILATUS detector. 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 600 mm. The X-ray data set was processed using the iMosflm program [19]. The X-ray diffraction data collection statistics are given in the table.

Crystallographic data and the X-ray data collection statistics for the crystal of *Tth*HB27 APRT

Sp. gr.	*P*1211
a, *b*, *c*, Å; α = γ, β, deg	69.86, 82.16, 91.39; 90, 102.58
T, K	100
λ, Å	0.8
Resolution, Å	30.00–2.60 (2.74–2.60)*
Number of unique reflections	30211 (4489)
Redundancy	6.26 (6.54)
Completeness, %	96.96 (98.74)
$I/\sigma(I)$	3.23 (2.41)
Rmrgd *F*, %	11.4 (26.0)

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

RESULTS AND DISCUSSION

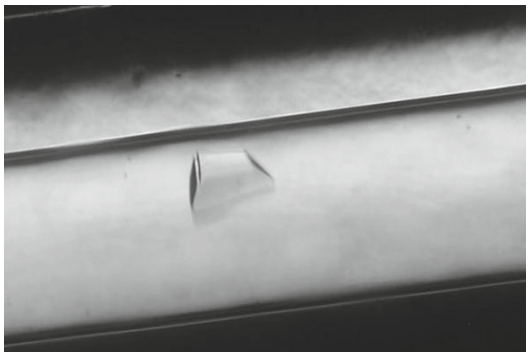
Enzymes from thermophylic microorganisms have a number of advantages as catalysts for biotechnological processes because they are highly stable and can be employed to perform reactions at high temperatures [12].

It was shown [12] that in the presence of magnesium ions at a concentration of at least 0.25 mM, recombinant APRT from the extremely thermophilic *T. thermophilus* strain HB27 has the highest activity at 73°C. A study of the substrate specificity of APRT demonstrated that not only adenine but also a number of its derivatives, including 2-chloro- and 2-fluoroadenine, 2-methoxyadenine, N1-methoxyadenine, and 2,6-diaminpurine, are substrates of this enzyme [12]. Due to the high temperature optimum for activity and rather broad specificity, APRT was successfully employed to synthesize nucleoside 5'-monphosphates from D-ribose and the appropriate bases. 2-Fluoro- and 2-chloroadenosine 5'-monophosphates were synthesized in high yields by the sequential use of three enzymes from the extremely thermophilic *T. thermophilus* strain HB27—phosphoribosylpyrophosphate synthetase, ribokinase, and adenine phosphoribosyltransferase. These experiments demonstrated that recombinant APRT is a promising enzyme for the multi-enzymatic synthesis of nucleotides. The search for new mutant forms based on the three-dimensional structure will make it possible to extend the specificity of the enzyme and, consequently, to increase the number of its possible substrates.

Adenine phosphoribosyltransferase was produced using a prokaryotic expression system with the goal of growing crystals and performing the X-ray diffraction study. The procedure developed for the isolation of the enzyme includes six successive steps. The enzyme with a purity of 96.9% and a specific activity of 14 μmol/min per μg of protein was used in crystallization experiments.

The screening of crystallization conditions was carried out by the vapor-diffusion technique in the pH range from 4.6 to 8.5 using solutions of ammonium sulfate and different-molecular-weight PEGs at different concentrations as precipitants. Crystals of the enzyme were obtained at pH 6.0 in the presence of PEG 8000 or PEG 3350 as the precipitant, but single crystals were grown only in the presence of PEG 3350, whereas the use of PEG 8000 resulted in the formation of small crystalline druses. We succeeded in growing larger crystals in the presence of PEG 3350 by the capillary counter-diffusion technique as described in [14]. The crystals used for the collection of the X-ray diffraction data set were obtained in a capillary 0.5 mm in diameter. They reached the size of 0.1 mm within two weeks. The concentration of PEG 3350 in the reservoir solution was 20% (figure).

Before the X-ray diffraction data collection at the SPring-8 synchrotron facility (Japan), the crystals were withdrawn from the capillary, placed in the reservoir solution, and then frozen in a cryoprotectant solution, which contained the same components as



Crystal of *Tth*HB27 adenine phosphoribosyltransferase grown in a capillary 0.5 mm in diameter by the counter-diffusion technique through a gel layer.

the reservoir solution with the addition of 20% glycerol. The X-ray diffraction data collection statistics are given in the table. The X-ray data set is suitable for the solution of the three-dimensional structure of the enzyme at 2.6 Å resolution.

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