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STRUCTURE OF MACROMOLECULAR COMPOUNDS

Crystallization and Preliminary X-Ray Diffraction Analysis of Recombinant Phosphoribosylpyrophosphate Synthetase from the Thermophilic *Thermus Thermophilus* Strain HB27

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Abstract—Phosphoribosylpyrophosphate synthetases (PRPP synthetases) are among the key enzymes essential for vital functions of organisms and are involved in the biosynthesis of purine and pyrimidine nucleotides, coenzymes, and the amino acids histidine and tryptophan. These enzymes are used in biotechnology for the combined chemoenzymatic synthesis of natural nucleotide analogs. Recombinant phosphoribosylpyrophosphate synthetase I from the thermophilic strain HB27 of the bacterium *Thermus thermophilus* (*T. th* HB27) has high thermal stability and shows maximum activity at 75°C, due to which this enzyme holds promise for biotechnological applications. In order to grow crystals and study them by X-ray crystallography, an enzyme sample, which was produced using a highly efficient producer strain, was purified by affinity and gel-filtration chromatography. The screening of crystallization conditions was performed by the vapor-diffusion technique. The crystals of the enzyme suitable for X-ray diffraction were grown by the counter-diffusion method through a gel layer. These crystals were used to collect the X-ray diffraction data set at the SPring-8 synchrotron radiation facility (Japan) to 3-Å resolution. The crystals belong to sp. gr. $P2_1$ and have the following unit-cell parameters: $a = 107.7$ Å, $b = 112.6$ Å, $c = 110.2$ Å, $\alpha = \gamma = 90°$, $\beta = 116.6°$. The X-ray diffraction data set is suitable for determining the three-dimensional structure of the enzyme at 3.0-Å resolution.

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INTRODUCTION

Enzymes belonging to the posphoribosylpyrophosphate synthetase family (PRPP synthetases, EC 2.7.6.1) catalyze the synthesis of 5-phosphoribosyl 1-pyrophosphate (5-PRPP) from adenosine triphosphate and ribose 5-phosphate [1]. The product of the catalyzed reaction (5-PRPP) is an intermediate in a number of important metabolic pathways of synthesis of purine and pyrimidine nucleotides, coenzymes, and the amino acids histidine and tryptophan and is among metabolites permanently required by the cell [2–6]. In some bacteria, more than a dozen enzymes utilize 5-PRPP as the substrate. Therefore, PRPP synthetases are among the key enzymes ensuring the viability of the organism [7].

Enzymes involved in nucleotide metabolism are of considerable interest for biotechnology because they are widely used in the combined chemoenzymatic synthesis of natural nucleotide analogs, many of which are antiviral and antitumor agents [8]. It was shown [9] that enzymes of nucleotide metabolism, including PRPP synthetases, hold promise for the use in the so-called cascade strategy for the biosynthesis of biologically important nucleotides. This strategy is based on the multienzyme cascade transformation of D-pentoses into purine nucleotides. The cascade synthesis involves the one-pot ribokinase-catalyzed transformation of D-pentoses into 5-phosphopentoses followed by the PRPP-synthetase-catalyzed transformation of the latter into 5-phospho-α-D-pentofuranosyl 1-pyrophosphates. The attachment of the 5-phosphoribosyl group to the heterocyclic purine base in the presence of adenine phosphoribosyltransferase (APR transferase) gives the final nucleotides. The enzymes with PRPP-synthetase activity are required for the second step of the cascade synthesis.

Enzymes with high stability and high temperature optimum are preferred for biotechnological applica-

tions. Enzymes from thermophilic organisms meet these requirements. The substrate specificity of the enzymes is also of substantial importance. The lower degree of selectivity for the purine base and the carbohydrate substrate enables the extension of the range of the synthesized compounds. Therefore, enzymes having broader specificity are preferred for this purpose.

Recently, genes encoding two proteins with PRPP-synthetase activity have been cloned from the bacterium *Thermus thermophilus* (*T. th*) HB27 belonging to a group of extreme thermophiles. The recombinant enzymes were isolated and characterized [10]. Both enzymes have the maximum activity at high temperature (75 and 85°C) and, consequently, they are suitable for biotechnological applications, although they exhibit strict specificity toward the pyrophosphate donor and acceptor. In view of the prospects of biotechnological applications of this enzyme, knowledge of its three-dimensional structure is an essential prerequisite for the rational search for new mutant forms of the enzyme with desired properties.

In the present work, recombinant thermally stable *T. th* PRPP synthetase I from the thermophilic strain HB27 was isolated and purified with the aim of subsequent X-ray diffraction analysis. This enzyme has the maximum activity at 75°C. The screening of crystallization conditions was performed, and crystals of the enzyme were grown by the counter-diffusion method. These crystals were used to collect the X-ray diffraction data set suitable for determining the three-dimensional structure of the enzyme at 3.0-Å resolution.

MATERIALS AND METHODS

Cloning of the Gene

The producer strain of recombinant *T. th* PRPP synthetase I was constructed using an expression system based on the plasmid vector pET23d+ and the *E. coli* Rosetta (DE3) host strain [10].

Isolation and Purification of the Protein

The producer strain *E. coli* Rosetta (DE3)/pER-PRPP1TTHhis was cultured at 37°C in LB medium supplemented with 100 μg/mL ampicillin until the absorbance of the culture was A595 = 0.8, induced by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to 0.4-mM concentration, and grown for 4 h. The cells were separated by centrifugation at 5000 rpm for 20 min at 4°C. Then the cell biomass was resuspended in 150 mL of a buffer solution (50 mM Tris, pH 8.7, 1 mM phenylmercuric sulfonyl fluoride) and disrupted using an ultrasonic disintegrator. The cellular debris was separated by centrifugation at 12 000 rpm for 30 min at +4°C. The clarified cell lysates were loaded onto metal-affinity chromatography columns packed with the Ni^{2+}-IDA sorbent, which were pre-equilibrated with 50 mM Tris buffer, pH 8.7. Ballast proteins were eluted with a buffer solution composed of 50 mM imidazole and 50 mM Tris-HCl, pH 8.7. The target protein was eluted with a buffer solution (200 mM imidazole, 50 mM Tris-HCl, pH 8.7). After the metal-affinity chromatography, ethylenediaminetetraacetic acid was added to the fractions containing the target protein, and the solutions were concentrated using an Amicon ultrafiltration cell.

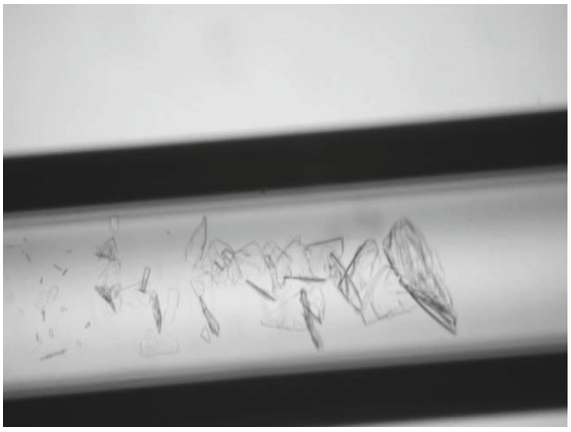
The subsequent purification was performed by means of gel-filtration chromatography on a column packed with Superdex 200 in 20 mM Tris-HCl buffer, pH 8.5, supplemented with 1 mM ATP, 1 mM $MgCl_2$, 5% glycerol, and 0.04% NaN_3. After the gel filtration, the fractions containing the protein were combined and concentrated to the final concentration of 12 mg/mL. The protein was stored in the frozen state at −80°C.

Crystal Growth

The screening of crystallization conditions was carried out by the hanging-drop vapor-diffusion technique using a 1% protein solution in 20 mM Tris buffer, pH 8.5, containing 1 mM ATP, 1 mM $MgCl_2$, 5% glycerol, and 0.04% sodium azide. Ammonium sulfate solutions in the concentration range from 5 to 15% and solutions of different-molecular-weight PEG in the concentration range from 5 to 20% were used as precipitants. Experiments were performed in the pH range 4.9–7.1. The crystal formation was observed in solutions composed of 1 mM ATP, 1 mM $MgCl_2$, and 5 to 10% ammonium sulfate. These crystallization conditions were optimized to apply to the counter-diffusion method [11]. The crystals suitable for X-ray diffraction were grown by the counter-diffusion method through a gel layer in a glass capillary with a diameter of 0.5 mm as described in [12]. The protein concentration was 12 mg/mL. The reservoir solution was composed of 7.5% ammonium sulfate, 0.45 M KCl, 0.1 M sodium citrate, pH 4.9, 5 mM $MgCl_2$, 5 mM ATP, and 0.04% sodium azide. The crystals appeared in the capillary within 7–10 days (figure).

X-ray Diffraction Data Collection and Processing

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) using a PILATUS detector. The X-ray data were obtained by the rotation method at a wavelength of 0.8 Å. The rotation angle



Capillary containing crystals of *T. Thermophilus* HB27 phosphoribosylpyrophosphate synthetase.

was 360°, the oscillation angle was 0.5°, and the crystal-to-detector distance was 600 mm. The X-ray diffraction data collection statistics are given in the table.

RESULTS AND DISCUSSION

A new strategy for the biosynthesis of biologically important 5'-phosphorylated nucleosides was described in [9]. This strategy is based on the multienzyme cascade transformation of D-pentoses into purine nucleotides exposed to the successive action of several enzymes of nucleotide metabolism. The second step of the cascade synthesis—the formation of 5-phospho-α-D-pentofuranosyl 1-pyrophosphate from 5-phosphopentose—is catalyzed by enzymes with phosphoribosylpyrophosphate-synthetase activity.

Phosphorylated nucleoside (nucleotides) are essential metabolites for DNA and RNA biosynthesis, as well as cosubstrates and cofactors in many biochemical transformations [1–4]. Since these compounds play an important role in the living cell, there is a great interest in the synthesis of not only natural representatives of this class but also their diverse analogs, the use of which will make it possible to influence metabolic pathways in a desired way [13–18]. Many nucleotides modified at the heterocyclic base and the carbohydrate moiety are important therapeutic agents against viral infections and malignant tumors [8, 19].

Crystallographic data and the X-ray data collection statistics for *T. th* HB27 phosphoribosylpyrophosphate synthetase

Sp. gr.	$P2_1$
a, *b*, *c*, Å; α = γ, β, deg	107.7, 112.6, 110.2; 90, 116.6
T, K	100
λ, Å	0.8
Resolution, Å	30.00–3.00 (3.15–3.00)*
Number of unique reflections	52760 (9451)
Redundancy	2.8 (1.4)
Completeness, %	90.1 (79.3)
I/σ (*I*)	3.0 (2.1)
Rmrgd F, %	11.7 (69.4)

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

The second step of the cascade synthesis of phosphorylated nucleotides is catalyzed by enzymes with phosphoribosylpyrophosphate-synthetase activity. Phosphoribosylpyrophosphate synthetases from thermophilic microorganisms have some advantages as catalysts in the cascade synthesis of nucleotides because the reactions performed at higher temperatures led to an increase in the solubility of the reaction products [9]. Phosphoribosylpyrophosphate synthetases from the extreme thermophilic strain of the bacterium *T. th* HB7 hold promise as catalysts for the second step of the cascade synthesis [10]. In the genome of the bacterium *T. th* HB7, the proteins with phosphoribosylpyrophosphate-synthetase activity are encoded by two genes. Both genes were cloned and expressed, and the corresponding *T. th* PRPP synthetases I and II were isolated and characterized [10].

Both proteins, despite a high degree of sequence homology (89%), differ in some properties, including the kinetic parameters and the optimum activity temperature [10]. The data on the three-dimensional structure are of interest as the basis for the directed modification of enzymes, in particular, in order to extend their selectivity.

Recombinant *T. th* PRPP synthetase I was produced using an expression system comprising the plasmid vector pET23d+ and the *E. coli* Rosetta (DE3) host strain and was then used for the crystallization and subsequent X-ray crystallography. After the cell disruption by ultrasound, the recombinant enzyme, which was produced in a soluble form, was successively purified by affinity chromatography and gel filtration and then concentrated to 12 mg/mL in Tris-HCl buffer, pH 8.0, supplemented with $MgCl_2$ and potassium phosphate. Since PRPP synthetase is a metal-dependent enzyme, magnesium ions added to a solution increase the stability of the enzyme. In addition, magnesium ions, like phosphate ions, are activators of the enzyme. The reservoir solutions, which were used for the screening of crystallization conditions, also contained $MgCl_2$ and ATP. Ammonium sulfate solutions and solutions of different-molecular-weight PEG at different concentrations were utilized as precipitants. Crystals were obtained in ammonium sulfate solutions in the salt-concentration range from 4.5 to 8%. The addition of 0.1 mM ATP and 0.1 mM magnesium chloride to the reservoir solution resulted in an increase in the crystal size. The crystallization conditions were optimized to apply to the counter-diffusion method. The concentration of ammonium sulfate in the reservoir solution was increased to 7.5% versus 5% in the reservoir solution used for the crystalli-

zation in the drop. The crystals suitable for X-ray diffraction were grown by the counter-diffusion method in a glass capillary 0.5 mm in diameter [11, 12]. The crystals appeared in the capillary within 7–10 days (figure).

Before the X-ray diffraction data collection at the SPring-8 synchrotron radiation facility (Japan), the crystals were withdrawn from the capillary, transferred into the reservoir solution, and then frozen in a cryoprotectant solution, which contained the same components as 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 determining the three-dimensional structure of the enzyme at 3.0-Å resolution.

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REFERENCES

1. H. G. Khorana, J. F. Fernandes, and A. Kornberg, J. Biol. Chem. **230**, 941 (1958).
2. R. L. Switzer, J. Biol. Chem. **244**, 2854 (1969).
3. M. A. Becker, K. O. Raivio, B. Bakay, et al., J. Clin. Invest. **65**, 109 (1980).
4. B. Hove-Jensen and P. Nygaard, Eur. J. Biochem. **126**, 327 (1982).
5. B. Hove-Jensen, Mol. Microbiol. **3**, 1487 (1983).
6. B. Hove-Jensen, J. Bacteriol. **170**, 1148 (1988).
7. T. A. Eriksen, A. Kadziola, A. K. Bentsen, et al., Nat. Struct. Biol. **7**, 303 (2000).
8. A. I. Mikhailopulo and A. I. Miroshnikov, Acta Naturae. **10**, 36 (2010).
9. R. S. Esipov, Yu. A. Abramchik, I. V. Fateev, et al., Acta Naturae ? (2016).
10. R. S. Esipov, Yu. A. Abramchik, I. V. Fateev, et al., Bioorg. Khim. **42** (4), 1 (2016).
11. H. Tanaka, K. Inaka, S. Sugiyama, et al., J. Synchrotron Radiat. **11**, 45 (2004).
12. I. P. Kuranova, E. A. Smirnova, Yu. A. Abramchik, et al., Crystallogr. Rep. **56** (5), 884 (2011).
13. L. P. Jordheim, D. Durantel, F. Zoulim, et al., Nature Rev. Drug Discov. **12**, 447 (2013).
14. E. De Clercq, Rev. Med. Virol. **19**, 287 (2009).
15. B. Diop-Frimpong, T. P. Prakash, K. G. Rajeev, et al., Nucl. Acids Res. **33** (16), 5297 (2005).
16. S. R. Jackson, Cancer Res. **67**, 1121 (2007).
17. M. Egli and S. M. Gryaznov, Cell Mol. Life Sci. **57**, 1440 (2000).
18. C. G. Peng and M. J. Damha, Nucl. Acids Res. **35**, 4977 (2007).

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