Crystal structure of recombinant phosphoribosylpyrophosphate synthetase 2 from *Thermus thermophilus* HB27 complexed with ADP and sulfate ions

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Phosphoribosylpyrophosphate synthetase (PRPPS) from the thermophilic bacterial strain *Thermus thermophilus* HB27 catalyzes the synthesis of phosphoribosylpyrophosphate from ribose 5-phosphate and ATP, and belongs to the class I PRPPSs. The three-dimensional structure of the recombinant enzyme was solved at 2.2 Å resolution using crystals grown in microgravity from protein solution containing ATP, magnesium and sulfate ions. An ADP molecule was located in the active site of each subunit of the hexameric enzyme molecule and sulfate ions were located in both the active and allosteric sites. It was found that the catalytic loop that restricts the active-site area and is usually missing from the electron-density map of class I PRPPSs adopts different conformations in three independent subunits in *T. thermophilus* PRPPS. A closed conformation of the active site was found in one of subunits where the highly ordered catalytic β -hairpin delivers the Lys and Arg residues that are essential for activity directly to the ADP molecule, which occupies the ATP-binding site. A comparison of the conformations of the catalytic loop in the three independent subunits reveals a possible mode of transition from the open to the closed state of the active site during the course of the catalyzed reaction.

1. Introduction

Phosphoribosylpyrophosphate synthetases (PRPPSs; EC 2.7.6.1), which catalyse the reaction of ribose 5-phosphate (R5P) with ATP to yield 5-phosphoribosyl- α -1-pyrophosphate (PRPP) and AMP, are widespread in various eukaryotic and prokaryotic organisms (Kornberg *et al.*, 1955). They link the pentose phosphate pathway to the pyrimidine and purine nucleotide *de novo* and salvage pathways in the biosynthesis of histidine, tryptophan and pyridine nucleotide coenzymes (Jensen, 1983; Khorana *et al.*, 1958). PRPP is a metabolite that is required at all times in the cell, and thus the function of PRPP synthetase is central to life (Eriksen *et al.*, 2000). All PRPPSs are metal-dependent enzymes that require Mg^{2+} and phosphate ions for activity (Switzer, 1969; Willemoës & Hove-Jensen, 1997). Depending on their mode of activity regulation, their specificity for the pyrophosphate donor and their ability to be activated by magnesium and phosphate ions, PRPP synthetases are divided into three classes (Hove-Jensen & McGuire, 2004; Krath & Hove-Jensen, 1999, 2001; Kadziola *et*

al., 2005). The mammalian and the majority of bacterial enzymes belong to class I. The PRPP synthetases in class I adopt a hexameric quaternary structure and their activity is regulated not only in the active site but also in the allosteric site. Phosphate in the allosteric site activates the enzyme, whereas ADP or GDP inhibit it (Switzer & Sogin, 1973). Crystal structures are known for PRPPs that belong to class I from *Bacillus subtilis*, human and *Escherichia coli* (Eriksen *et al.*, 2000, 2002; Timofeev *et al.*, 2016; Li *et al.*, 2007; Chen *et al.*, 2015), and for enzymes from *Methanocaldococcus janaschii* and *Thermoplasma volcanium* that belong to class III (Kadziola *et al.*, 2005; Cherney *et al.*, 2011). The enzymes of class III adopt a tetrameric or dimeric quaternary structure and do not contain the allosteric regulatory site.

Despite the low level of sequence homology between PRPP synthetases from different classes (about 20%), they show a similar fold of the polypeptide chain in the subunit (Kadziola *et al.*, 2005). Each subunit consists of N-terminal and C-terminal domains related by a twofold pseudosymmetry axis. Both domains reveal the type 1 phosphoribosyl-transferase fold that is found in all PRPP synthetases (Eriksen *et al.*, 2000; Timofeev *et al.*, 2016).

The hexameric molecule in the class I enzymes consists of subunits related by a threefold axis with a perpendicular twofold axis and with point-group symmetry 32. The six subunits are arranged with their N-terminal domains closest to the threefold axis; the C-terminal domains are oriented towards the outside (Eriksen *et al.*, 2000).

In a number of biochemical and structural studies, the functionally essential regions of the molecules were characterized and a catalytic mechanism common to the whole family of PRPP synthetases was suggested (Kadziola *et al.*, 2005; Cherney *et al.*, 2011; Hove-Jensen *et al.*, 1986; Miller *et al.*, 1975).

Recently, two genes that encode proteins with PRPP synthetase activity in the thermophilic strain *Thermus thermophilus* HB27 have been cloned and expressed, and the PRPP synthetases (*Tth1PRPPS* and *Tth2PRPPS*) produced by these genes were isolated, purified and characterized (Esipov, Abramchik, Fateev, Muravyova *et al.*, 2016; Esipov, Abramchik, Fateev, Konstantinova *et al.*, 2016). It was shown that *Tth1PRPPS* and *Tth2PRPPS* have a high degree of sequence homology (their identity is 48.8% and their similarity is 89%) but differ in several properties. *Tth1PRPPS* shows maximum activity at 75°C, whereas *Tth2PRPPS* shows maximum activity at 85°C. Enzymes from thermophilic organisms with maximum activity at high temperatures are of special interest for applications in biotechnology.

Here, we present the crystal structure of *Tth2PRPPS* synthetase complexed with ADP and sulfate ions. The structure was determined using crystals grown in microgravity from protein solution containing ATP, Mg²⁺ and sulfate ions. An ADP molecule and one sulfate ion were located in the active site of the enzyme and a second sulfate ion was located in the allosteric regulatory site. The positions of the ligands and their nearest environment are described. It is found that the three independent subunits in the asymmetric unit differ in the

Table 1

Macromolecule-production information.

Source organism	<i>T. thermophilus</i> HB27
DNA source	<i>T. thermophilus</i> HB27
Forward primer	5'-GGTGGTCCATGGCGGACCGCCCCCTCTGA-TCTTC-3'
Reverse primer	5'-GGTGGTCTCGAGTGTGAAGAGGTGGACACCGAC-3'
Cloning vector	pET-23d(+)
Expression vector	pET-23d(+)
Expression host	<i>E. coli</i> C3029H/pG-Tf2 (New England Biolabs/Takara Bio Inc.)
Complete amino-acid sequence of the construct produced	PLLIFSGQSNRPLAQIAEALGLPLGKSTTLRFA-NDNLFVRYEESLREGDVFIVQSFVPPVQDHLMELLMMVDAAGASAAVTAVIPYFSYARSDKK-DAPRISITARLIADLLQTAGADRVLTMTLHSPQVHGFIPVDHLSAEPVIANFYFATRVDELNAVVPADAGDLKRASALARRLGLPLAFIDKERVSDTEVRVRLVGEVEGKTALIVDDEISTAGSLVEAVEALMQAGAKEVYAAATHGVYVGPALDRI-AKSPVKEVAATDTCPPKEGPKRLTLTVAPLFAEAIWRIHRGESVSSSLFT

conformation of the flexible catalytic loop restricting the active-site area. This loop, which is usually missing in the electron-density map of class I PRPP synthetases, is clearly visible in one of the independent subunits of *Tth2PRPPS*, and the Arg and Lys residues that are essential for catalysis are found close to the ADP molecule which occupies the ATP-binding site. It is suggested that the different conformations of the catalytic loop in the independent subunits may be similar to the movement of the catalytic loop during the course of reaction.

2. Materials and methods

2.1. Cloning, expression and purification of 5-phospho-D-ribosyl α -1-diphosphate (PRPP) synthetase from *T. thermophilus* HB27

The TT_RS06430 gene encoding *Tth2PRPPS* from *T. thermophilus* HB27 genomic DNA was cloned in the pET-23d(+) plasmid and expressed in *E. coli* C3029/pG-Tf2 cells, and the desired enzyme was purified by immobilized metal-affinity chromatography and size-exclusion chromatography to give a product with ~96.1% purity and with a specific activity of 11 $\mu\text{mol min}^{-1}$ per milligram of protein. The full cloning, expression, purification and enzymatic activity assay protocol is given in the Supporting Information. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization of recombinant *Tth2PRPPS*

The protein was purified for crystallization according to Esipov, Abramchik, Fateev, Muravyova *et al.* (2016) and Esipov, Abramchik, Fateev, Konstantinova *et al.* (2016). The crystals were grown in a microgravity environment at 293 K using the crystallization facilities of the Japan Aerospace Exploration Agency (JAXA) and the support service provided by JAXA (Tanaka *et al.*, 2004; Takahashi *et al.*, 2013) in the frame of the JAXA PCG experiment. Crystal growth was carried out by the counter-diffusion technique in a

Table 2

Crystallization.

Method	Liquid diffusion
Crystallization device	Capillary
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	12
Buffer composition of protein solution	0.02 M Tris pH 8.5 containing 1 mM ATP, 1 mM MgCl ₂ , 5% Gln, 0.04% NaN ₃
Composition of reservoir solution	0.8 M ammonium sulfate, 0.45 M KCl, 0.1 M sodium citrate pH 4.9, 5 mM MgCl ₂ , 5 mM ATP, 0.04% NaN ₃
Volume of capillary (μl)	8

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline BL41XU, SPring-8
Wavelength (Å)	0.8
Temperature (K)	100
Detector	Dectris PILATUS3 6M
Crystal-to-detector distance (mm)	100
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	0.1
Space group	<i>P</i> ₄ ₁ ₂ ₁ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	115.97, 115.97, 207.99
α , β , γ (°)	90, 90, 90
Resolution range (Å)	77.4–2.2 (2.32–2.20)
No. of unique reflections	72677 (10442)
Completeness (%)	99.97 (99.96)
Multiplicity	11.43 (6.55)
$\langle I/\sigma(I) \rangle$	4.1075 (2.01)
$R_{\text{r.i.m.}}^{\dagger}$	0.095 (0.36)

$\dagger$ Estimated $R_{\text{r.i.m.}} = R_{\text{merge}}[N/(N-1)]^{1/2}$, where N is the data multiplicity.

capillary through a gel layer as described in Kuranova *et al.* (2011).

A thick-walled glass capillary of 0.5 mm inner diameter and 60 mm in length was loaded with 8 μl protein solution in 0.02 M Tris pH 8.5 containing 1 mM ATP, 1 mM MgCl₂, 5% Gln and 0.04% NaN₃. The protein concentration was 12 mg ml⁻¹. The top end of the capillary was sealed with plasticine. The other end of the capillary was inserted into a gel tube of 5 mm in length. The capillary with the attached gel tube was firmly placed into a plastic sack with 0.5 ml precipitant solution consisting of 0.8 M ammonium sulfate, 0.45 M KCl, 0.1 M sodium citrate pH 4.9, 5 mM MgCl₂, 5 mM ATP, 0.04% NaN₃. The sack was sealed hermetically. Crystal growth took place in microgravity during three weeks. Crystallization information is summarized in Table 2.

2.3. Structure determination and refinement

A diffraction data set was collected at the BL41XU station of the SPring-8 synchrotron, Japan at 100 K. A Dectris PILATUS3 6M device was used as a detector. The diffraction data were obtained by rotation using a single crystal. The wavelength was 0.8 Å, the crystal-to-detector distance was 100 mm, the oscillation angle was 0.5° and the rotation angle was 180°. The experimental intensities were processed using *iMosflm* (Battye *et al.*, 2011). The data-collection statistics are presented in Table 3.

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	64.39–2.20 (2.257–2.200)
Completeness (%)	100.0
σ Cutoff	0.0
No. of reflections, working set	69040 (5020)
No. of reflections, test set	3548 (250)
Final R_{cryst}	0.227 (0.265)
Final R_{free}	0.254 (0.300)
No. of non-H atoms	
Protein	6993
Ion	30
Ligand	81
Water	190
Total	7294
R.m.s. deviations	
Bonds (Å)	0.009
Angles (°)	1.453
Average <i>B</i> factors (Å ²)	
Protein	47.5
Ion	38.6
Ligand	53.6
Water	31.1
Ramachandran plot	
Most favoured (%)	94
Allowed (%)	5

The structure of *Tth2PRPPS* was determined at 2.20 Å resolution by the molecular-replacement method using *Phaser* (McCoy *et al.*, 2007), with the atomic coordinates of *E. coli* PRPPS (PDB entry 1dkr; Eriksen *et al.*, 2000) as a starting model. *REFMAC* was used for structure refinement (Murshudov *et al.*, 2011). Manual correction of the model was performed using *Coot* (Emsley *et al.*, 2010) and electron-density maps were calculated with $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ coefficients. Water molecules were located in the electron-density maps and difference Fourier synthesis revealed electron density in the active site which was identified as ADP and a sulfate ion. The ligands were refined with an occupancy of 100%. The coordinates of the structure have been deposited in the PDB (PDB entry 5t3o). The refinement statistics are presented in Table 4.

3. Results and discussion

The recently isolated *Tth2PRPPS* from the extremely thermophilic strain *T. thermophilus* HB27 belongs to the class I PRPPs and reveals maximum activity at 85°C (Esipov, Abramchik, Fateev, Muravyova *et al.*, 2016). It is a prospective candidate for use in the cascade of thermophilic enzymes for the synthesis of modified nucleotides (Esipov, Abramchik, Fateev, Konstantinova *et al.*, 2016). The crystal structure of the enzyme was determined at 2.2 Å resolution using crystals grown in microgravity by the counter-diffusion technique from solutions containing ATP, magnesium and sulfate ions. The crystals, which belonged to space group *P*₄₁₂₁₂, contained three independent subunits in the asymmetric unit. Clear electron density in the active site in each independent subunit was interpreted as an ADP molecule (Supplementary Fig. S1). Two other peaks of electron density in the active and allosteric sites were interpreted as sulfate ions. The biological assembly

is built by the application of a twofold noncrystallographic axis to each subunit (Supplementary Fig. S2).

The overall structure of *Tth2*PRPPS is very similar to the structures of other known PRPP synthetases from class I.

The sequence alignment and secondary structure of subunit *B* of *Tth2*PRPPS in comparison with those of the enzyme from *B. subtilis* (*Bs*PRPPS) are presented in Fig. 1(a). The sequence identity between *Tth2*PRPPS and *Bs*PRPPS is 46.8%.

The subunit of *Tth2*PRPPS comprises two domains related by a noncrystallographic twofold axis. The sequence identity between the domains is 18.55%. Both domains reveal the type 1 phosphoribosyltransferase fold that is found in all PRPP synthetases (Smith, 1995; Hove-Jensen *et al.*, 2005; Khorana *et al.*, 1958; Switzer & Sogin, 1973). The N-terminal and C-terminal domains contain a central five-stranded parallel β -sheet surrounded by four helices from the same domain (Fig. 1b). In the N-terminal domain there is one additional 3_{10} -helix and an α -helix consisting of C-terminal amino-acid residues. The central core in both domains is flanked by a short antiparallel β -sheet designated the flag region (Eriksen *et al.*, 2000). This is β -hairpin $\beta 3$ – $\beta 4$ in the N-terminal domain and $\beta 9$ – $\beta 10$ in the C-terminal domain. The domains are connected by a short linker (residues 147–149). The hexamer is organized

as a propeller with N-terminal domains at the centre and C-terminal domains on the outside (Supplementary Fig. S2).

Based on the results of biochemical and structural studies, as well as sequence alignment with family I phosphoribosyltransferases, several segments of the polypeptide chain that are essential for the function of PRPP synthetases have been identified (Fig. 1a). These segments, which are mostly arranged in the flexible parts of the polypeptide chain, have been designated the PP_i loop, the R5P loop and the flexible loop (Eriksen *et al.*, 2000; Hove-Jensen *et al.*, 1986; Smith, 1995). Residues from the flexible loop participate in binding the substrate ATP in the active site and the inhibitor ADP in the regulatory site of the neighbouring subunit. The flag region of the C-terminal domain, containing the Lys194 and Arg196 residues that are essential for activity, was designated the catalytic flexible loop. The corresponding regions in the *Tth2*PRPPS structure were located by comparison with *Bs*PRPPS (Fig. 1a). As seen in Fig. 1(a), the main difference in the secondary structures of the enzymes is observed in the region of the catalytic loop (strands $\beta 9$ – $\beta 10$). Strands $\beta 9$ – $\beta 10$ in subunit *B* of *Tth2*PRPPS form a highly ordered β -hairpin, whereas in *Bs*PRPPS residues 199–207 have no corresponding electron density.

Figure 1
(a) Alignment of the amino-acid sequences of *Tth2*PRPPS and *Bs*PRPPS. Secondary-structure elements are shown as helices and arrows above the alignment. Functionally important segments of the enzyme are denoted by lines above the alignment. (b) The polypeptide fold in the *Tth2*PRPPS subunit. Helices are coloured red, β -strands yellow and irregular loops green.

3.1. The differences in the structure of independent subunits

Superposition of the three independent subunits of *Tth2PRPPS* on C^α atoms reveals a significant deviation of the amino-acid residues in the catalytic loop. The shift of the C^α atoms of Thr200 located at the turn of the catalytic loop reaches 8 Å between subunits *B* and *A* and 20 Å between subunits *B* and *C*. Consequently, a large difference is observed in the conformation of the catalytic loop.

The catalytic loop in subunit *B* forms one of the walls of the active site and appears as an ordered hairpin consisting of strands $\beta 9$ and $\beta 10$ (Fig. 2). In subunit *A* the hydrogen bonds between the $\beta 9$ and $\beta 10$ strands are broken; the $\beta 10$ strand is partly disordered and removed from the $\beta 9$ strand. However, the $\beta 9$ strand retains its position and its interactions with the bound ligands do not change. The catalytic loop in subunit *C* is completely disordered and removed from the ligands. Owing to this, the accessibility of the active site in subunit *C* is increased significantly.

3.2. Sulfate in the allosteric site

The allosteric sites in subunits *B* and *F* are shown in Supplementary Fig. S3. The allosteric site is situated at the junction of three subunits. Two of the subunits belong to the same dimer, and the third is from an adjacent dimer. It has been shown that sulfate in the allosteric site occupies the position of the β -phosphate of the inhibiting ADP molecule (Eriksen *et al.*, 2000, 2002).

The C-terminal amino-acid residues Ser304*B*, Val305*B*, Ser306*B*, Ser48 and Arg50 from the N-terminal flag region of subunit *A* and Arg105 from the flexible loop of the adjacent subunit *F* participate in the binding of sulfate ion in the allosteric site of subunit *B*. As discussed below, two amino-acid residues from the flexible loop (Arg97 and Lys100) of each subunit participate in the binding of substrate (ATP) in the active site. Thus, the amino-acid residues of the flexible loop are simultaneously involved in binding the substrate ATP in the active site in one subunit and binding the inhibitor ADP in the neighbouring subunit. This complicated network of interactions clearly demonstrates the importance of the quaternary structure in the enzymatic function of PRPP synthetases.

3.3. ADP and sulfate ion in the active site

As in other PRPP synthetases, the active site of *Tth2PRPPS* is located in the cleft between the N-terminal and C-terminal domains. In the N-terminal domain it is restricted by the amino-acid residues of a flexible loop (Phe92–Ser108) and the flag region of the adjacent subunit (Val30–Ile44). The active site in the C-terminal domain is restricted by residues from the PP_i loop (Asp170–Gly175) and the R5P loop (Asp220–Thr226).

An ADP molecule was found in the active site of each subunit, despite the fact that only ATP was present in the

Figure 2

The catalytic loops in the independent subunits *A*, *B* and *C* of *Tth2PRPPS*. The catalytic loops in subunits *A* (green), *B* (red) and *C* (blue) are shown in cartoon representation on the van der Waals surfaces of subunits *A* (a), *B* (b) and *C* (c). The ADP molecule in the active site is shown as red sticks.

The arrangement of the ADP molecule in subunit *B* is

As in other PRPP synthetases, the sulfate in the R5P region occupies the position of the 5'-phosphate group of the second substrate ribose 5-phosphate. The interactions of the sulfate with the main-chain N atoms of Ser224, Thr225, Gly227 and Ser228 provide the specific conformation of the R5P loop. One of the sulfate O atoms forms water-mediated hydrogen bonds to Arg97.

The active sites of TvPRPPS (PDB entries 3nag and 3mbi; Cherney *et al.*, 2011) in the open and closed conformations and in the *Tth*2PRPPS-ADP complex superimposed on the C α

atoms of three invariant amino-acid residues are presented in Fig. 4. The conformation of the catalytic loop in the closed forms of both enzymes coincides perfectly. The position of the catalytic loop in the open form of *Tv*PRPPS increases the accessibility to the active site remarkably.

These results are in good agreement with the suggestion that the catalytic β -hairpin acts as a lid that blocks solvent access to the active site and thus shields the intermediate complex during reaction (Cherney *et al.*, 2011). The various conformations of the catalytic loop observed in the subunits of *Tth*2PRPPS are possibly realised during the transition between the closed and open conformations of the active site.

The arrangement of the amino-acid residues that are essential for activity around the bound ligands in the *Tth*2PRPPS–ADP complex coincides well with the reaction mechanism suggested for PRPP synthetases (Smith, 1995; Khorana *et al.*, 1958; Switzer & Sogin, 1973).

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