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Expression, purification, crystallization and preliminary X-ray diffraction analysis of a galactose 1-phosphate uridylyltransferase from the hyperthermophilic archaeon *Pyrobaculum aerophilum*

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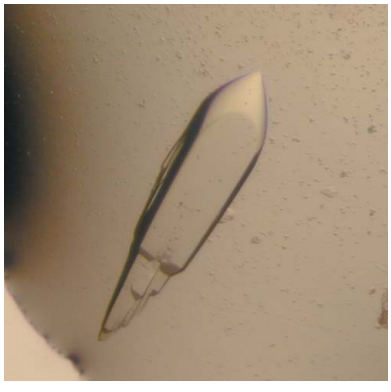

A galactose 1-phosphate uridylyltransferase from the hyperthermophilic archaeon *Pyrobaculum aerophilum* was crystallized using the sitting-drop vapour-diffusion method with polyethylene glycol 8000 as the precipitant. The crystals belonged to the tetragonal space group $P4_1$, with unit-cell parameters $a = b = 73.3$, $c = 126.1$ Å, and diffracted to 2.73 Å resolution on beamline BL5A at the Photon Factory. The overall R_{merge} was 7.3% and the data completeness was 99.8%.

1. Introduction

Galactose 1-phosphate uridylyltransferase (GalT; EC 2.7.7.12) catalyzes nucleotide exchange between UDP-glucose (UDP-Glu) and galactose 1-phosphate, which is a pivotal step in the Leloir pathway of galactose metabolism (Holden *et al.*, 2003). In this pathway, galactose is first phosphorylated to galactose 1-phosphate by galactokinase. GalT then catalyzes the transfer of the uridylyl group from UDP-Glu to galactose 1-phosphate to produce UDP-galactose (UDP-Gal) and glucose 1-phosphate, which enters the glycolytic pathway. Finally, UDP-galactose 4-epimerase (GalE; EC 5.1.3.2) regenerates UDP-Glu from UDP-Gal, thereby maintaining the production of glucose 1-phosphate. The aforementioned three enzymes comprising the Leloir pathway are widely distributed in bacteria and eukarya, and structural and functional analyses of several examples have been reported (Holden *et al.*, 2003). Galactokinases from the hyperthermophilic archaea *Pyrococcus furiosus* and *Pyrococcus horikoshii* (Verhees *et al.*, 2002; Inagaki *et al.*, 2006; Hartley *et al.*, 2004) have also been characterized and the molecular basis of substrate recognition by the *P. furiosus* enzyme is now known. However, information about the other two enzymes involved in the Leloir pathway in archaea remains limited.

We recently identified a gene (Pcal_0885) encoding what we predicted would be a GalE homologue within the genomic sequence of the hyperthermophilic archaeon *Pyrobaculum calidifontis*. By expressing the gene in *Escherichia coli* and characterizing the enzyme produced (Sakuraba *et al.*, 2011), we confirmed that the enzyme is an extremely thermostable GalE. We also solved the crystal structure of this enzyme at a resolution of 1.8 Å and evaluated the structural features responsible for its high thermostability compared with GalEs from other sources (Sakuraba *et al.*, 2011). This was the first example of a GalE that is found in both the archaeal domain and a hyperthermophile.

Within the *P. calidifontis* genome, we also identified the genes encoding the homologues of the other two enzymes involved in the Leloir pathway (Pcal_0776 and Pcal_0777, encoding galactokinase and GalT, respectively). Moreover, a database search showed the presence of homologues of all three enzymes among phylogenetically distant archaeal species, including *Pyrobaculum* and *Pyrococcus* species, which suggests that this pathway is functional in hyperthermophilic archaea. Thus, structural and functional analysis of the

constituents of the Leloir pathway may shed light on the features and diversity of the pathway in hyperthermophilic archaea. Among these enzymes, the *Pyrobaculum aerophilum* GalT homologue shares 44–71% amino-acid sequence identity with other archaeal GalT homologues. In this paper, we describe the crystallization and preliminary X-ray analysis of *P. aerophilum* GalT, as well as the expression of the gene in *E. coli*, as a first step in the structural analysis of GalTs from hyperthermophilic archaea.

2. Methods and results

2.1. Construction of the expression system and purification of the recombinant protein

To construct the expression plasmid for the putative *P. aerophilum* GalT homologue, a 1.0 kbp gene fragment composed of the gene (PAE1184) encoding the enzyme plus *Nde*I and *Bam*HI restriction sites was amplified by PCR using the primers 5′-ACATATGG-AGATTAGGAAAGACCCC-3′, which contains a unique *Nde*I restriction site overlapping the 5′ initial codon, and 5′-TTG-GATCCTTAATCCGCCGAATGC-3′, which contains a unique *Bam*HI restriction site proximal to the 3′-end of the termination codon. The genomic DNA was prepared using a Genomic DNA Isolation Kit for Bacteria (Nexttec GmbH Biotechnologie, Leverkusen, Germany). The amplified 1.0 kbp fragment was digested with *Nde*I and *Bam*HI and then ligated into the expression vector pET11a (Novagen, Madison, Wisconsin, USA) previously linearized using *Nde*I and *Bam*HI, yielding pET1184. *E. coli* strain BL21 (DE3) Codon Plus RIL (DE3) (Stratagene, La Jolla, California, USA) was then transformed with the vector and the transformants were cultivated at 310 K in 1 l SB medium (1.2% tryptone peptone, 2.4% yeast extract, 1.25% K_2HPO_4, 0.38% KH_2PO_4, 0.5% glycerol) containing 50 µg ml^{-1} ampicillin until the optical density at 600 nm reached 0.6. Expression was then induced by adding 0.1 m*M* isopropyl *β*-D-1-thiogalactopyranoside to the medium and cultivation was continued for an additional 21 h at 293 K. The cells were then harvested by centrifugation, suspended in 10 m*M* potassium phosphate buffer pH 7.0 supplemented with lysozyme (1 mg ml^{-1}) and DNase (0.1 mg ml^{-1}) and disrupted by ultrasonication. GalT activity was assayed at 323 K essentially as described by Wong & Frey (1974). Protein levels were determined using the method of Bradford (1976) with bovine serum albumin serving as the standard.

To isolate *P. aerophilum* GalT, the crude extract was heated to 353 K for 10 min, after which the denatured protein was removed by centrifugation (15 000*g* for 10 min). The resultant supernatant was loaded onto a DEAE-Toyopearl 650M column (Tosoh, Japan) equilibrated with 10 m*M* potassium phosphate buffer pH 7.0 and the column was washed with the same buffer. The enzyme was eluted without absorption. The active fractions in the flowthrough were pooled and loaded onto a HiPrep 16/10 SP XL column (GE Healthcare, Buckinghamshire, England) equilibrated with 10 m*M* potassium phosphate buffer pH 7.0. After washing the column with the same buffer, the enzyme was eluted with 200 ml of a linear gradient of 0–200 m*M* NaCl in the same buffer. The active fraction was then pooled and used as the purified enzyme preparation. The entire procedure was carried out at room temperature (∼298 K).

We found that the transformant cells exhibited a high level of GalT activity, and the enzyme was readily purified from the crude cell extract in three simple steps: heat treatment, DEAE-Toyopearl 650M column chromatography and HiPrep 16/10 SP XL column chromatography. About 16 mg purified enzyme was obtained from 1 l *E. coli* culture.

2.2. Molecular-mass determination

The molecular mass of the recombinant enzyme was determined using a Superose 6 10/300 GL column (GE Healthcare) with 10 m*M* potassium phosphate buffer pH 7.0 containing 200 m*M* NaCl as the elution buffer. Gel-filtration calibration kits (GE Healthcare) were used for the molecular-mass standards. The subunit molecular mass was determined by SDS–PAGE using eight marker proteins (6–175 kDa; New England Biolabs, Ipswich, Massachusetts, USA). The native and subunit molecular masses of the enzyme were found to be about 59 and 36 kDa, respectively, suggesting that the enzyme is a homodimer in solution.

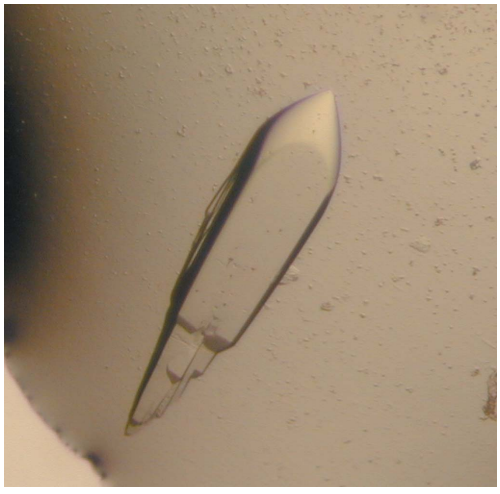

Figure 1
Photograph of a *P. aerophilum* GalT crystal. The dimensions of the crystal are 0.6 × 0.1 × 0.1 mm.

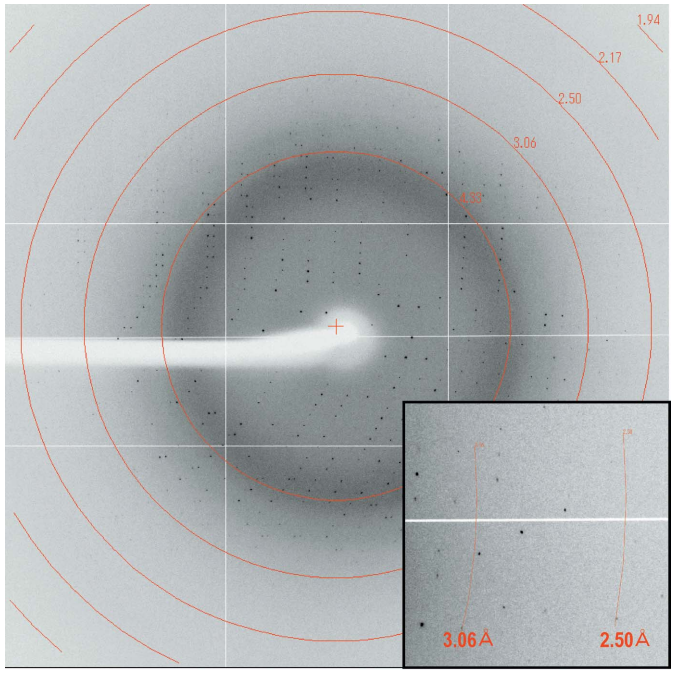

Figure 2
X-ray diffraction of a *P. aerophilum* GalT crystal. The high-resolution area is enlarged (insert).

Table 1
Data-collection and processing statistics for *P. aerophilum* GalT.

Values in parentheses are for the highest resolution shell.

Source	BL5A, Photon Factory
Wavelength (Å)	1.00
Rotation range per frame (°)	1
Total rotation range (°)	180
Exposure per frame (s)	1
Crystal-to-detector distance (mm)	350
Temperature (K)	100
Space group	$P4_1$
Unit-cell parameters	$a = b = 73.3$, $c = 126.1$
Resolution range (Å)	50–2.73 (2.78–2.73)
No. of measured reflections	132516 (6559)
No. of unique reflections	17691 (863)
Multiplicity	7.5 (7.6)
Completeness (%)	99.8 (99.9)
$R_{\rm merge}$†	0.073 (0.287)
$\langle I/\sigma(I)\rangle$	15.7 (8.9)
Mosaicity (°)	0.48

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

2.3. Crystallization

The purified enzyme was concentrated to 10 mg ml^{-1} for crystallization trials. Initial screening was carried out using Crystal Screen, Crystal Screen 2 (Hampton Research, Aliso Viejo, California, USA), the Crystallization Kit for Protein Complexes (Sigma–Aldrich, St Louis, Missouri, USA) and Wizard I, II and III (Emerald BioSystems, Bainbridge Island, Washington, USA). Crystallization was accomplished at 293 K using the sitting-drop vapour-diffusion method, in which 1 µl drops of protein solution were mixed with an equal volume of reservoir solution and equilibrated against 0.1 ml reservoir solution using CompactClover Crystallization Plates (Emerald BioSystems). Initially, small crystals were grown from reagent 31 [12.0% polyethylene glycol (PEG) 8000, 100 m*M* 2-(*N*-morpholino)ethanesulfonic acid buffer (MES) pH 6.5, 100 m*M* magnesium acetate] from the Crystallization Kit for Protein Complexes. This precipitant solution was taken as a starting point and was optimized by variation of the PEG 8000 concentration; diffraction-quality crystals (maximum dimensions of ∼0.6 × 0.1 × 0.1 mm; Fig. 1) were obtained within one week using a reservoir solution composed of 10.0–11.0% PEG 8000, 100 m*M* MES pH 6.5, 100 m*M* magnesium acetate.

2.4. Data collection and preliminary X-ray analysis

The *P. aerophilum* GalT crystals were flash-cooled in liquid nitrogen at 100 K after first stirring them gently in Paratone-N (Hampton Research). Diffraction data were collected to 2.73 Å resolution using monochromated radiation of wavelength 1.0 Å and an ADSC Quantum CCD detector system on beamline BL5A at the Photon Factory (Tsukuba, Japan; Fig. 2). The data were processed using *HKL*-2000 (Otwinowski & Minor, 1997).

The crystals belonged to the tetragonal space group $P4_1$. A summary of the data statistics is presented in Table 1. Assuming two protein molecules in the asymmetric unit, the crystal volume per enzyme mass ($V_{\rm M}$) and the solvent content were calculated to be 2.4 Å^3 Da^{-1} and 47.7%, respectively, which are within the frequently observed ranges for protein crystals (Matthews, 1968).

When we compared the amino-acid sequence of *P. aerophilum* GalT with the sequences of GalT homologues for which crystal structures have previously been determined, we found that *P. aerophilum* GalT exhibits the highest identity (29%) to a GalT-like protein from *Arabidopsis thaliana* (At5g18200; PDB entry 1z84; McCoy *et al.*, 2006). Based on this structure, we used the molecular-replacement method for phase calculation, but were unable to obtain useful data. We next soaked the crystals in reservoir solutions containing several mercury (mercury chloride, mercury acetate, ethylmercuric phosphate, sodium ethylmercurithiosalicylate and 1,4-diacetoxymercuri-2,3-dimethoxybutane), gold (gold chloride, gold potassium bromide and gold potassium cyanide), platinum (platinum potassium thiocyanate) or samarium (samarium chloride hexahydrate) compounds in an effort to solve the phase using the heavy-atom isomorphous replacement method; however, we have not yet obtained an adequate heavy-atom derivative. We are currently attempting to prepare selenomethionine-substituted *P. aerophilum* GalT.

To date, no GalT from an archaeal species or from a hyperthermophile has been structurally characterized. In the present study, the first diffraction-quality crystals were obtained for a GalT from a hyperthermophilic archaeon. We expect that elucidation of the three-dimensional structure of this enzyme will increase our understanding of the structure–function relationships in hyperthermophilic GalTs.

Data collection was performed on BL5A at the Photon Factory. We thank Drs Matsugaki, Igarashi and Wakatsuki for their kind assistance with the data collection. This work was supported in part by a Grant-in-Aid for Scientific Research (C) to HS from the Japan Society for the Promotion of Science.

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