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addenda and errata

Expression, purification, crystallization and preliminary X-ray diffraction analysis of galactokinase from *Pyrococcus horikoshii*. Erratum

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An error is corrected in the paper by Inagaki *et al.* [(2006), *Acta Cryst.* F**62**, 169–171].

This erratum refers to the paper by Inagaki *et al.* [(2006), *Acta Cryst.* F**62**, 169–171]. In §2.3 the amount of silicone and paraffin oil mixture used in the crystallization of the apo form of the enzyme was given incorrectly. The correct amount is 15 µl.

References

Inagaki, E., Sakamoto, K., Obayashi, N., Terada, T., Shirouzu, M., Bessho, Y., Kuroishi, C., Kuramitsu, S., Shinkai, A. & Yokoyama, S. (2006). *Acta Cryst.* F**62**, 169–171.

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Expression, purification, crystallization and preliminary X-ray diffraction analysis of galactokinase from *Pyrococcus horikoshii*

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Galactokinase (EC 2.7.1.6) catalyzes the ATP-dependent phosphorylation of α-D-galactose to α-D-galactose-1-phosphate, in an additional metabolic branch of glycolysis. The apo-form crystal structure of the enzyme has not yet been elucidated. Crystals of galactokinase from *Pyrococcus horikoshii* were prepared in both the apo form and as a ternary complex with α-D-galactose and an ATP analogue. Diffraction data sets were collected to 1.24 Å resolution for the apo form and to 1.7 Å for the ternary complex form using synchrotron radiation. The apo-form crystals belong to space group *C*2, with unit-cell parameters $a = 108.08$, $b = 38.91$, $c = 81.57$ Å, $\beta = 109.8^\circ$. The ternary complex form was isomorphous with the apo form, except for the length of the *a* axis. The galactokinase activity of the enzyme was confirmed and the kinetic parameters at 323 K were determined.

1. Introduction

The biological conversion of galactose to glucose takes place *via* the Leloir pathway, which is an additional metabolic branch of glycolysis (Frey, 1996). Galactokinase (EC 2.7.1.6) catalyzes the ATP-dependent phosphorylation of α-D-galactose to α-D-galactose-1-phosphate in the pathway (Trucco *et al.*, 1948; Wilkinson, 1949). In humans, deficiencies of this enzyme can result in type II galactosaemia (MIM 230200), the main symptom of which is cataract development (Holton *et al.*, 2001). Recently, *Escherichia coli* galactokinase has been utilized *via* a directed-evolution approach to create natural and unnatural sugar 1-phosphates in a process of glycorandomization, with the aim of enhancement of drug-discovery efforts (Hoffmeister *et al.*, 2003; Yang *et al.*, 2005).

In recent years, the crystal structures of the galactokinases from *Lactococcus lactis* complexed with galactose (Thoden & Holden, 2003), *Pyrococcus furiosus* with ADP and galactose (Hartley *et al.*, 2004), human with adenosine 5′-(β,γ-imino)-triphosphate (AMP-PNP) and galactose (Thoden, Timson *et al.*, 2005) and *Saccharomyces cerevisiae* with AMP-PNP and galactose (Thoden, Sellick *et al.*, 2005) have been solved to 2.1, 2.9, 2.4 and 2.5 Å resolution, respectively. The structures were all in ligand-bound forms; therefore, determination of both apo and ligand-bound structures may shed light on the catalytic mechanism of galactokinases.

Here, we report the crystallization and preliminary crystallographic studies of *P. horikoshii* galactokinase (*Ph*GalK), which is encoded by the *PH0369* gene of *P. horikoshii* OT3 (GenBank accession No. NP_142343) and shares 78% amino-acid sequence homology with *P. furiosus* galactokinase, in both the apo form and as a ternary complex with AMP-PNP and D-galactose. In addition, we have confirmed the galactokinase activity of *Ph*GalK and determined the kinetic parameters at 323 K.

2. Materials and methods

2.1. Expression and purification

The *Ph*GalK gene was amplified by the polymerase chain reaction (PCR) using *P. horikoshii* OT3 genomic DNA as the template. The amplified fragment was cloned under the control of the T7 promoter, using the *Nde*I and *Bam*HI sites of the *E. coli* expression vector

Table 1
Crystal parameters and data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Apo form	Ternary complex
Unit-cell parameters		
a (Å)	108.08	106.33
b (Å)	38.91	39.07
c (Å)	81.57	81.41
β (°)	109.8	109.5
Space group	*C*2	*C*2
Z	4	4
Solvent content (%)	39.2	39.3
Data collection		
Temperature (K)	100	100
X-ray source	BL26B2	BL26B1
Detector	R-AXIS V	R-AXIS V
Wavelength (Å)	0.95	1.0
Resolution (Å)	30–1.24 (1.26–1.24)	30–1.70 (1.74–1.70)
Unique reflections	86789	34628
Redundancy	2.8 (2.5)	3.0 (3.1)
Completeness (%)	97.4 (94.2)	98.9 (99.3)
$R_{\rm merge}$ (%)	5.4 (46.4)	8.6 (36.4)
$\langle I/\sigma(I)\rangle$	21.6 (2.0)	13.0 (3.2)

pET11a (Novagen), by the super-rare-cutter system (Kanagawa *et al.*, in preparation). The expression vector was introduced into the *E. coli* BL21 codon plus (DE3)-RIL strain (Stratagene), which was cultured at 310 K for 20 h in 4.5 l LB broth supplemented with 50 µg ml^{-1} ampicillin. The cells (19.8 g) were collected by centrifugation, washed with 40 ml of buffer [20 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl, 5 m*M* 2-mercaptoethanol (2-ME)] and then resuspended in 20 ml of the same buffer. The cells were disrupted by sonication on ice and the cell lysate was then incubated at 363 K for 13 min. The sample was centrifuged at 15 000*g* for 30 min and the supernatant was then dialyzed against 20 m*M* Tris–HCl pH 8.5 containing 10 m*M* NaCl and 5 m*M* 2-ME. The sample was applied onto a 10 ml HiTrap Q (Amersham Biosciences) column pre-equilibrated with 20 m*M* Tris–HCl pH 8.5 containing 10 m*M* NaCl and 5 m*M* 2-ME. The column was washed with four bed volumes of the same buffer and the bound protein was then eluted with 20 bed volumes of a linear gradient of 0.01–1 *M* NaCl in 20 m*M* Tris–HCl pH 8.5 containing 5 m*M* 2-ME. The fractions containing *Ph*GalK were pooled. After the concentration of $(NH_4)_2SO_4$ had been adjusted to 1.2 *M*, the pooled solution was applied onto a 10 ml HiTrap Phenyl FF (Amersham Biosciences) column pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 1.2 *M* $(NH_4)_2SO_4$ and 5 m*M* 2-ME. After washing with four bed volumes of the same buffer, the bound protein was eluted with five bed volumes of a linear gradient of 1.2–0 *M* $(NH_4)_2SO_4$. The fractions containing *Ph*GalK were collected and concentrated with an Amicon Ultra-15 concentrator (10 kDa molecular-weight cutoff, Millipore). The concentrated enzyme solution was applied onto a HiLoad 16/60 Superdex 75 (Amersham Biosciences) column pre-equilibrated with 20 m*M* Tris–HCl pH 8.0 containing 150 m*M* NaCl and 2 m*M* dithiothreitol (DTT). The fractions containing *Ph*GalK were collected and concentrated with an Amicon Ultra-15 concentrator (10 kDa molecular-weight cutoff) to 23 mg ml^{-1}. The purified protein was homogeneous as determined by SDS–PAGE.

2.2. Enzyme assay

Galactokinase activity was measured by coupling the production of ADP to the reactions catalyzed by pyruvate kinase and lactate dehydrogenase. To determine the kinetic parameters, the decrease in absorbance at 340 nm which results from the oxidation of NADH was followed at 310 and 323 K in a 500 µl mixture containing 50 m*M* HEPES–NaOH pH 7.5, 1.6–1.8 µg ml^{-1} *Ph*GalK, 1 m*M* freshly prepared NADH (Sigma), 5 m*M* $MgCl_2$, 1 m*M* phosphoenolpyruvate, 0.98 U pyruvate kinase (Sigma), 1.4 U lactate dehydrogenase (Sigma), various concentrations of ATP (0.001–1.5 m*M*) and various concentrations of D-galactose (0.001–5 m*M*). Reactions were initiated by the addition of ATP. Kinetic parameters were calculated with a rigorous nonlinear least-squares method (*Enzyme Kinetics Pro*, ChemSW Software), using the mean initial velocity of triplicate measurements for each substrate concentration.

2.3. Crystallization of apo form

The initial crystallization conditions were examined by the microbatch method (Chayen *et al.*, 1990) using the TERA crystallization robot and a screening kit designed for high-throughput protein crystallization (Sugahara & Miyano, 2002). Each droplet, prepared by mixing 0.5 µl protein solution (23 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl and 2 m*M* DTT) with 0.5 µl of the screen solution, was covered with 150 µl of a silicone and paraffin oil mixture and incubated at 291 K. Plate-shaped crystals appeared from a screen solution containing PEG 4000, $MgCl_2$ and MES pH 6.3 after one week. The best crystals grew to dimensions of 0.3 × 0.2 × 0.05 mm from solution containing 27.5%(*w*/*v*) PEG 4000, 50 m*M* $MgCl_2$ and 0.1 *M* MES pH 6.3. A mixture of Paratone-N (Hampton Research), paraffin oil and glycerol was used for cryoprotection (Sugahara & Kunishima, in preparation).

2.4. Crystallization of ternary complex

Crystallization of the complex of *Ph*GalK with α-D-galactose and AMP-PNP was attempted by a seeding method in combination with the sitting-drop vapour-diffusion method as follows. 1.0 µl drops of protein solution (16 mg ml^{-1} in 1 *M* D-galactose, 5 m*M* AMP-PNP, 14 m*M* Tris–HCl pH 8.0, 0.1 *M* NaCl and 1.4 m*M* DTT) with 1.0 µl reservoir solution were equilibrated against 500 µl reservoir solution at 295 K in 24-well plates. After 5 d, a small apo-form crystal was seeded in each well. Plate-shaped crystals were produced after a few days and the best crystals grew to dimensions of 0.15 × 0.1 × 0.03 mm in one week using a precipitant solution containing 2.5%(*w*/*v*) PEG 4000, 50 m*M* $MgCl_2$ and 0.1 *M* MES pH 6.3. The cryoprotectant solution contained 30%(*v*/*v*) PEG 400, 1 *M* D-galactose, 5 m*M* AMP-PNP, 5%(*w*/*v*) PEG 4000, 50 m*M* $MgCl_2$ and 0.1 *M* MES pH 6.3.

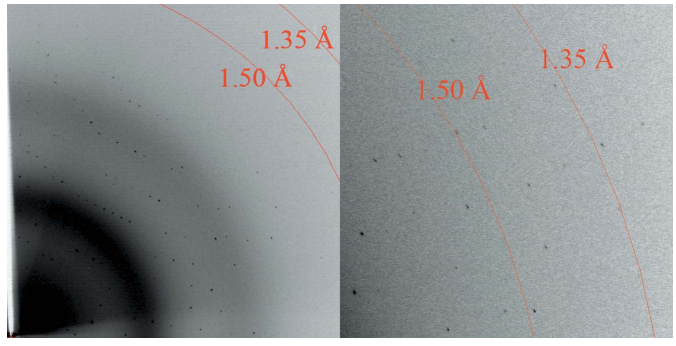

Figure 1
Diffraction pattern of a *Ph*GalK crystal collected at the RIKEN Structural Genomics Beamline I at SPring8 at 100 K. The exposure time was 20 s, with an oscillation range of 0.5° and a crystal-to-detector distance of 200 mm. The 1.35 and 1.50 Å resolution rings are also marked.

Table 2
Kinetic parameters of *Ph*GalK at 323 K.

The parameters for D-galactose and ATP were determined in the presence of 1 m*M* ATP and 5 m*M* D-galactose, respectively.

k_{cat} (s^{-1})	$K_{m,gal}$ (μM)	$K_{m,ATP}$ (μM)	$k_{cat}/K_{m,gal}$ (M^{-1} s^{-1})	$k_{cat}/K_{m,ATP}$ ($M-$ s^{-1})
0.91	403	132	2.26×10^3	5.44×10^3

2.5. Data collection

For the data collection, the crystals were mounted in nylon-fibre loops and flash-cooled in a dry nitrogen stream at 100 K. The crystals of both forms diffracted to well over 2.0 Å resolution (Fig. 1). Diffraction data sets were collected at 100 K using RIKEN Structural Genomics Beamline I or II (BL26B1 or BL26B2) at SPring-8 (Hyogo, Japan). Diffraction images were processed using the *HKL*2000 program package (Otwinowski & Minor, 1997). The crystal parameters and the data-processing statistics are summarized in Table 1.

3. Results and discussion

The purified *Ph*GalK exhibited significant galactokinase activity at 323 K (Table 2), but very little activity at 310 K (data not shown). Complete diffraction data sets for each form were collected using a single crystal and were processed to resolutions of 1.24 and 1.7 Å for the apo and ternary complex forms, respectively. Both forms were isomorphous except for the length of the *a* axis, which is 1.8 Å shorter in the ternary complex form than the apo form (Table 1). Assuming the presence of one *Ph*GalK molecule in the asymmetric unit of each form, the calculated Matthews coefficient (Matthews, 1968) is 2.0 $Å^3$ Da^{-1}, which corresponds to a solvent content of 39% (Table 1). The preliminary structure of the apo form was successfully obtained by the molecular-replacement method with *CNS* (Brünger *et al.*, 1998) using the coordinates of the *P. furiosus* galactokinase structure (PDB code 1s4e) as a search model. Further refinement is in progress and the current *R* factor is 20.9% (R_{free} = 22.4%). A refined structure of the apo form was used as the starting model for the ternary complex form. A molecular-replacement search was not necessary, since there were few differences in the unit-cell parameters between the two forms. In the early stage of refinement, the electron-density map clearly showed the presence of α-D-galactose and an ATP analogue at the ligand-binding sites. The details of the structure determination and refinement will be reported elsewhere.

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