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Expression, purification, crystallization and preliminary X-ray diffraction analysis of a ribokinase from the thermohalophile *Halothermothrix orenii*

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A ribokinase gene (*rbk*) from the anaerobic halothermophilic bacterium *Halothermothrix orenii* was cloned and overexpressed in *Escherichia coli*. The recombinant protein (Ho-Rbk) was purified using immobilized metal-ion affinity chromatography and crystals were obtained using the sitting-drop method. Diffraction data were collected to a resolution of 3.1 Å using synchrotron radiation. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 45.6, b = 61.1, c = 220.2, and contained two molecules per asymmetric unit. A molecular-replacement solution has been found and attempts are currently under way to build a model of the ribokinase. Efforts to improve crystal quality so that higher resolution data can be obtained are also being considered.

1. Introduction

Halothermothrix orenii is a heterotrophic, halophilic and thermophilic bacterium which grows optimally at 333 K with 5–10% NaCl at pH 6.5–7.0 (Cayol *et al.*, 1994). The bacterium was isolated from the sediment of a Tunisian hypersaline lake, a habitat which is subject to seasonal changes in temperature and salinity. Sequencing of the genome of *H. orenii* identified a large battery of intracellular and extracellular glycosyl hydrolases and kinases (Mijts & Patel, 2001; Mavromatis *et al.*, 2009). In an attempt to understand the adaptation of proteins from *H. orenii* to simultaneous exposure to high temperature and salts, its response to the fluctuating environmental conditions and the potential application of its enzymes in biotechnology, we initiated studies of the biochemistry and three-dimensional structures of amylase A (amyA), amylase B (amyB), sucrose phosphate synthase (SPS), fructokinase (FRK), β-glucosidase (BglA) (Li *et al.*, 2002; Tan *et al.*, 2003, 2008; Huynh *et al.*, 2005; Sivakumar *et al.*, 2006; Chua *et al.*, 2008, 2010; Kori *et al.*, 2011), β-glucosidase (Kori *et al.*, unpublished work) and α-L-arabinofuranosidase (Kori & Patel, unpublished work) from *H. orenii*. As part of these ongoing investigations, we have cloned and expressed a ribokinase, a member of the PfkB-family carbohydrate kinases, from *H. orenii*. Other members of the PfkB family include fructokinase, 1-phosphofructokinase, 6-phosphofructokinase, hexokinase, ketohexokinase, adenosine kinase, ribokinase and 2-dehydro-3-deoxygluconokinase (Sigrell *et al.*, 1997).

Ribokinase is involved in ribose phosphorylation and catalyses the following reaction in the presence of magnesium: ATP + D-ribose → ADP + D-ribose 5-phosphate. The substrate ribose, a monosaccharide, is the most abundant sugar in the environment and is metabolized by almost every living organism. D-Ribose is used in the synthesis of nucleotides and amino acids (histidine and tryptophan) and is also an important source of energy. To date, sugar phosphorylation is not completely understood and therefore the mechanism of sugar phosphorylation by ribokinase is a worthwhile target to explore. The core domain of ribokinase is an α/β-fold which harbours the catalytic site. A protruding four-stranded β-sheet with distinct topology forms a lid covering the active site where the enzymatic reaction takes place (Chua *et al.*, 2010). Previously determined structures of *Escherichia*

coli ribokinase show that D-ribose is enclosed in the substrate-binding site under the protruding β-sheet, and an induced-fit mechanism which enables the recognition and binding of the donor trinucleotide ATP as a co-substrate has been proposed (Sigrell *et al.*, 1998, 1999).

2. Materials and methods

2.1. Expression and purification of recombinant ribokinase

A 918 bp ribokinase gene (GenBank accession No. YP_002508722) was PCR-amplified from the genomic DNA of *H. orenii* using Mango*Taq* DNA polymerase (Bioline, Australia) with an upstream primer 5′-TTC**CATATG**ATGGGAAAAAGATAATTATG-3′ (*Nde*I restriction-enzyme site shown in bold) and a downstream primer 5′-TA**CTCGAG**TCA*ATGATGGTGATGGTGATG*CTTTTATCCGG-ACTCTTTC-3′ (*Xho*I restriction-enzyme site shown in bold and the oligonucleotide encoding the hexahistidine tag shown in italics). The PCR conditions used for DNA amplification have been described previously (Kori *et al.*, 2011). The amplified product was purified using a QIAquick gel-extraction kit (Qiagen, Germany) and digested with *Nde*I and *Xho*I. The fragment was ligated with the plasmid vector pET22b(+) (Novagen) and transformed into *E. coli* DH5α (Bioline, Australia) using procedures described by Sambrook *et al.* (1989). After overnight incubation at 310 K, white colonies that developed on MacConkey ampicillin (100 µg ml^{-1}) agar plates were selected and the presence of inserts was confirmed by colony PCR using T7 promoter (5′-TAATACGACTCACTATAGGG-3′) and T7 terminator (5′-GCTAGTTATTGCTCAGCGG-3′) primers and *Nde*I and *Xho*I restriction-enzyme digestion analysis. Plasmids that were confirmed as positive were used to transform *E. coli* BL21 (DE3) competent cells (Novagen) according to the manufacturer's instructions and were screened for expression of ribokinase using previously described methods (Kori *et al.*, 2011). A colony that overexpressed the recombinant ribokinase (designated Ho-Rbk) was used for further studies.

For overexpression of Ho-Rbk, the clone was inoculated into Luria Broth (LB) supplemented with 100 µg ml^{-1} ampicillin and grown to an OD_{650} of 0.6 at 310 K, at which stage IPTG was added to a final concentration of 3 m*M* and the culture was incubated for a further 6 h. The cells were harvested by centrifugation at 8000*g* for 10 min and resuspended in 5 ml 100 m*M* HEPES pH 7.0, 2 mg ml^{-1} lysozyme, 2 U ml^{-1} DNase I per gram of cells. The suspension was incubated for 45 min at 310 K and sonicated on ice. The cell lysate was clarified by centrifugation at 18 000*g* for 30 min at 278 K and the host proteins in the supernatant were denatured by incubation at 323 K for 45 min. The resulting precipitate was removed by centrifugation (18 000*g* for 30 min at 278 K) and the supernatant was filtered through a low-protein-binding PVDF filter (Millipore, USA) to remove any remaining particulate material. The filtrate was loaded onto an $NiSO_4$-charged Fractogel resin column (Merck) and incubated for 1 h at 278 K with gentle rotation on a tube rotator. The column was then washed with an equilibration buffer consisting of 500 m*M* NaCl, 20 m*M* Tris pH 8.0, washed with equilibration buffer containing a step gradient of 8, 15, 50, 75 and 100 m*M* imidazole and Ho-Rbk was eluted with 350 m*M* imidazole. The eluate was applied onto a U-Tube concentrator (30 kDa cutoff; Novagen), centrifuged at 10 000*g* for 8 min at 298 K and desalted with 100 m*M* NaCl, 20 m*M* HEPES pH 7.2. The final concentration of the recombinant protein was 9 mg ml^{-1} as determined by the Bradford assay (Bradford, 1976).

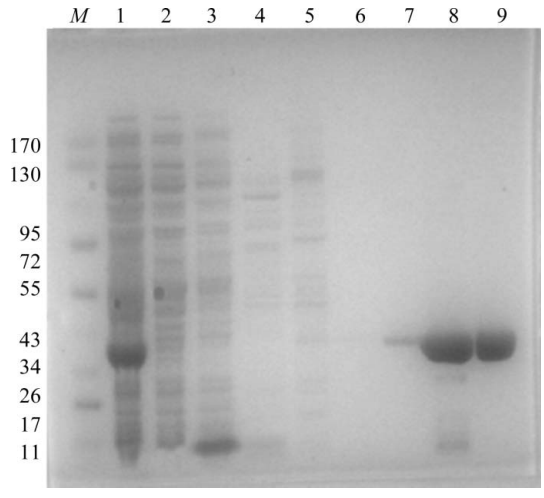

Figure 1
Ni^{2+}-affinity chromatography purification of Ho-RbK. Fractions were collected and separated on a 4–12% Bis-Tris NuPAGE gel and the gel was stained with SimplyBlue Safe Stain (Invitrogen, USA). Lane *M*, molecular-weight markers (labelled in kDa); lane 1, cell lysate; lane 2, column flowthrough; lanes 3–8, imidazole wash (8, 15, 50, 75, 100 and 350 m*M*); lane 9, concentrated and desalted Ho-Rbk from 350 m*M* imidazole eluted fraction.

2.2. Crystallization

Screening of crystallization conditions was carried out at 289 K in 96-well MRC sitting-drop crystallization plates (Molecular Dimensions Ltd, Suffolk, England) using our large in-house factorial collection (1056 pre-formulated conditions). 1 µl recombinant Ho-Rbk (9 mg ml^{-1}) was mixed with 1 µl reservoir solution and equilibrated against 100 µl reservoir solution.

2.3. Data collection and analysis

Crystals that developed over up to 10 d were mounted in nylon loops and cryoprotected with a 1:1(*v*:*v*) mixture of Paratone-N (Hampton Research, USA) and mineral oil (Oxoid, Australia) (Hope, 1988; Kori *et al.*, 2011). X-ray diffraction experiments were carried out at the in-house diffractometer (Rigaku MicroMax-007 HF with VariMax optics and an R-AXIS IV^{++} detector) as well as on Australian Synchrotron beamline MX2. Data sets were indexed with *MOSFLM* (Leslie, 1992) and further processed with *SCALA* and *TRUNCATE*, which are part of the *CCP*4 program suite (Winn *et al.*, 2011). Self-rotation functions were calculated with *GLRF* (Tong &

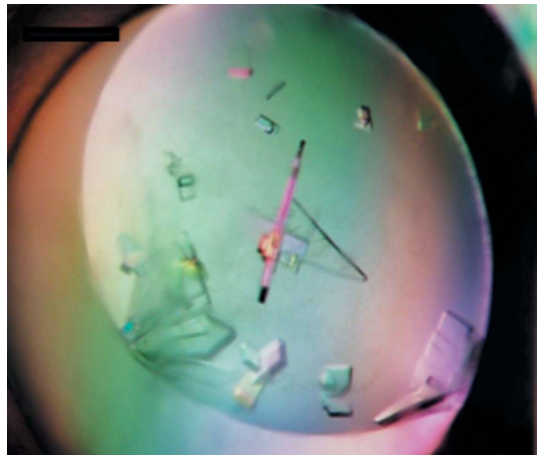

Figure 2
Rectangular-shaped crystals of *H. orenii* ribokinase with typical dimensions of 0.5 × 0.1 mm. The black bar indicates 0.5 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Australian Synchrotron beamline MX2
Detector	ADSC Quantum
Wavelength (Å)	0.95375
Temperature (K)	100
Crystal-to-detector distance (mm)	250
Rotation per image (°)	1
Total rotation (°)	180
Exposure time (s)	2
No. of crystals	1
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 45.6, b = 61.1, c = 220.2
Resolution range (Å)	59–3.1 (3.27–3.10)
Wilson B factor (Å^2)	57.2
Mosaicity (°)	0.52
Total No. of measurements	57751 (7084)
No. of unique reflections	11229 (1440)
$\langle I/\sigma(I)\rangle$	5.3 (3.1)
Multiplicity	5.1 (4.9)
Completeness (%)	94.8 (85.1)
$R_{\rm merge}$	0.108 (0.238)

Rossmann, 1990) and molecular-replacement calculations were carried out using *Phaser*/*PHENIX* (Adams *et al.*, 2010).

3. Results and discussion

The *rbk* gene of *H. orenii* was successfully cloned and overexpressed in *E. coli* BL21 (DE3) cells and the protein was purified using an $NiSO_4$-charged Fractogel resin column (Merck). Fig. 1 shows the protein profile of fractions collected during the purification process with imidazole.

Out of 1056 tested factorial conditions, crystals were obtained in 181 different conditions. Only crystals obtained from one condition [20%(*w*/*v*) PEG 4000, 0.02 *M* Tris pH 9.0] after about 6 d showed X-ray diffraction. The rectangular plate-like crystals had dimensions of 0.5 × 0.1 mm (Fig. 2) and diffracted to 3.1 Å resolution (Fig. 3). The unit-cell parameters of the orthorhombic crystals were a = 45.6, b = 61.0, c = 220.2 Å. Data-collection statistics are given in Table 1.

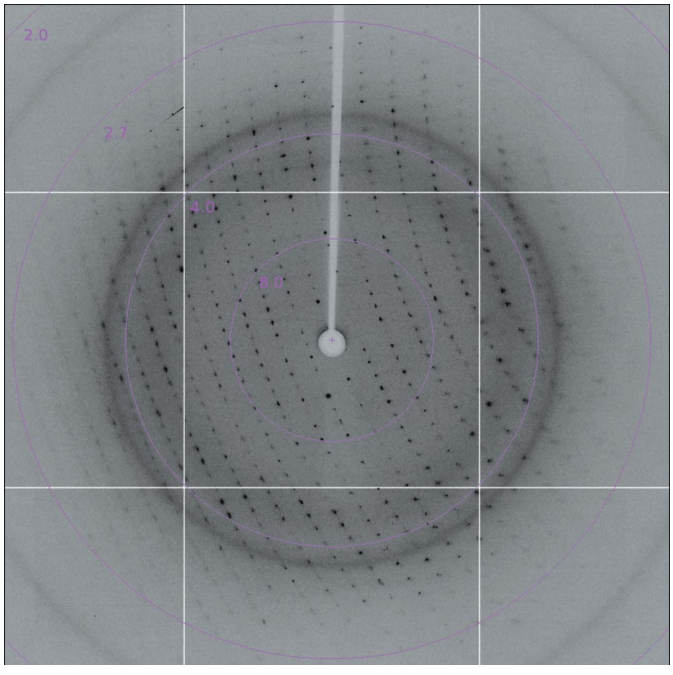

Figure 3
X-ray diffraction pattern from the best diffracting crystal of Ho-Rbk acquired at the Australian Synchrotron beamline MX2. The image shows a complete 1° oscillation frame. The resolutions of the magenta rings are given in Å.

Table 2
Calculation of Matthews coefficients for different numbers of molecules per asymmetric unit indicates the presence of a noncrystallographic dimer.

No. of molecules in asymmetric unit	Matthews coefficient (Å^3 Da^{-1})	Solvent content (%)
1	4.3	71.2
2	2.1	42.4
3	1.4	13.6

Serial extinctions were clearly visible in all three directions, resulting in the final assignment of $P2_12_12_1$ as the space group. The Matthews coefficient (Matthews, 1968) indicated that there were two molecules in the asymmetric unit (see Table 2). In the self-rotation function calculated for ω = 180° no noncrystallographic peaks were observed. Therefore, the asymmetric unit may contain two molecules that are not related by a rotational symmetry axis. Alternatively, the asymmetric unit may consist of a $P2$-symmetric dimer that is oriented parallel to one of the crystallographic twofold axes ([100], [010], [001]).

Using *PSIPRED* (Bryson *et al.*, 2005), mannose-binding protein from *Rattus rattus* (PDB entry 1rdk; Ng *et al.*, 1996) was identified as the protein of known structure with highest homology to Ho-Rbk. Molecular-replacement calculations with various models based on 1rdk were conducted. The best solution was obtained with a poly-Ala model of 1rdk that had the protruding four-stranded β-sheet truncated. This solution suggested the presence of two Ho-Rbk monomers in the asymmetric unit which are not related by a rotational symmetry axis.

Current efforts to improve crystallization conditions in order to obtain better quality crystals of the apo and ligand-bound enzyme are under way. We are also attempting to build a model of Ho-Rbk using the available molecular-replacement solution.

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