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Production and crystallization of α -phosphoglucomutase from *Lactococcus lactis*

α -Phosphoglucomutase (α -PGM) is an enzyme that is essential for the growth of *Lactococcus lactis*. The enzyme links bacterial anabolism with sugar utilization through glycolysis by catalyzing the reversible interconversion of glucose 6-phosphate and α -glucose 1-phosphate. The gene encoding α -PGM was cloned and overexpressed in *L. lactis*. The purified protein was functionally active and was crystallized with ammonium sulfate as a precipitant using vapour-diffusion and seeding techniques. Optimized crystals diffracted to 1.5 Å resolution at a synchrotron source.

1. Introduction

Phosphoglucomutases (EC 5.4.2.2) are widely found in all domains of life (Whitehouse *et al.*, 1998). They catalyze the interconversion of α -glucose 1-phosphate to glucose 6-phosphate. The majority of enzymes with α -phosphoglucomutase activity are classified into the α -D-phosphohexomutase superfamily (IPR005841). These enzymes typically contain over 450 residues and comprise a conserved serine residue for phosphoryl transfer to the substrate (Shackelford *et al.*, 2004). In bacteria, phosphoglucomutases are involved in sugar utilization *via* glycolysis and in the synthesis of UDP-glucose, a sugar donor in cell-wall synthesis (Ugalde *et al.*, 2000; Hardy *et al.*, 2001).

Lactococcus lactis is a member of the lactic acid bacteria (LAB) that is widely used in starter cultures for the manufacture of fermented dairy products such as cheese and buttermilk. Interestingly, *L. lactis* lacks α -phosphoglucomutase genes related to the α -D-phosphohexomutase superfamily. Instead, it possesses a smaller enzyme of 252 residues with α -phosphoglucomutase activity which is related to eukaryotic phosphomannomutases (IPR005002; Neves *et al.*, 2006). This enzyme, α -phosphoglucomutase (α -PGM), from *L. lactis* does not show activity towards α -mannose 1-phosphate (Neves *et al.*, 2006), whereas eukaryotic phosphomannomutases can use both α -glucose 1-phosphate and α -mannose 1-phosphate as substrates.

Here, we report the production, crystallization and X-ray diffraction data of *L. lactis* α -PGM. The atomic structure should provide insight into the strict substrate specificity of the enzyme and how it compares with eukaryotic phosphomannomutases. A better understanding of the catalytic mechanism of α -PGM may provide useful tools for re-routing the metabolism of *L. lactis* towards the production of high-value polysaccharides.

2. Materials and methods

2.1. Cloning, expression and purification

The nucleotide sequence *pgmH* encoding α -PGM was amplified by PCR from *L. lactis* subsp. *cremoris* strain NZ9000 using the forward primer 5'-CATGCCATGGGCCATCATCATCATCATGACGACGACGACAAGATGAAAAAATATTAAGT-3' and the reverse primer 5'-GCTCTAGATTAAGCTTCTCCATCGCAATAATTGCTTTTAGAATAGCTGCAG-3'. The gene was cloned into the pNZ8048 vector, introducing a His₆ tag at the N-terminus followed by an enterokinase cleavage site. The cloned DNA sequence was confirmed to be correct using the AGOWA GmbH DNA-sequencing service.

Homologous expression was performed in *L. lactis* strain NZ9000 grown in 5 l fermentors with M17 medium (Difco) containing 0.5% glucose and 5 mg l⁻¹ chloroamphenicol at 303 K under anaerobic

Figure 1
SDS-PAGE of purified α -PGM (30 kDa; construct with His tag) stained with Coomassie Blue. Lane *M*, molecular-weight marker (labelled in kDa) from Bio-Rad (Precision Plus Protein Standards); lane *P*, protein sample (10 μ g).

conditions and at a controlled pH of 6.5. At an OD₆₀₀ of 0.5, protein overexpression was induced by adding nisin to a final concentration of 1 μ g l⁻¹ and subsequent growth for a further 2 h. The biomass was harvested by centrifugation at 4000g, resuspended in 50 mM phosphate buffer pH 8.0, 300 mM NaCl, 20 mM imidazole and lysed using a French press. The cell debris was removed by centrifugation at 30 000g for 1 h. The soluble extract was loaded onto a 5 ml Ni-NTA Superflow cartridge (Qiagen). The column was washed with 50 mM phosphate buffer pH 8.0, 600 mM NaCl, 20 mM imidazole until the UV absorbance at 280 nm reached baseline. A gradient elution was performed using 50 mM phosphate buffer pH 8.0, 300 mM NaCl, 500 mM imidazole as the elution buffer. The purity of the protein was assessed by SDS-PAGE (Fig. 1) and its activity was confirmed by a functional assay for α -phosphoglucomutase activity as described by Neves *et al.* (2006) (data not shown). The sample buffer was exchanged to 10 mM HEPES pH 7.4 and the protein was concentrated to about 10 mg ml⁻¹ using Vivaspin concentrators with 10 kDa molecular-weight cutoff. The protein sample was divided into 100 μ l aliquots, flash-cooled in liquid nitrogen and stored at 193 K.

2.2. Crystallization

Screening of the crystallization conditions was performed with a Cartesian Mini-Bee nanorobot at 293 K using The Classics and Classics II Suites from Qiagen. The crystallization sitting drops were prepared by mixing 100 nl protein sample and 100 nl reservoir solution. Multiple needle-shaped crystals were obtained in a

Figure 2
Crystals of α -PGM. (a) Crystals obtained in 2 M ammonium sulfate, 0.1 M HEPES pH 7.5, 2% PEG 400. (b) Crystals grown without PEG 400. (c, d) Crystals obtained by streak-seeding into crystallization solution containing 2% PEG 400 from needles (c) or multiple plates (d).

Table 1

Data collection and processing.

Values in parentheses are for the outer resolution shell.

X-ray source	Beamline ID14-2, ESRF
Wavelength (Å)	0.9334
Temperature (K)	100
Detector	ADSC Q4
Rotation range per image (°)	0.1
Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 67.2$, $c = 210.4$
Resolution range (Å)	33.00–1.50 (1.58–1.50)
Total No. of reflections	744514 (105892)
No. of unique reflections	89350 (12888)
Completeness (%)	100.0 (100.0)
Average multiplicity	8.3 (8.2)
Average $I/\sigma(I)$	16.8 (3.1)
R_{meas}	0.089 (0.754)
Overall B factor from Wilson plot (Å ²)	15.9

crystallization condition consisting of 2 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5, 2% PEG 400 (Fig. 2a). Removal of PEG 400 from this condition led to the formation of multiple plate-like crystals (Fig. 2b). Crystal optimization included streak-seeding trials from both needle-shaped and plate-like multiple crystals into new crystallization solutions comprising 2 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5. A cat whisker was used to touch the crystal and seeds were immediately transferred in one quick movement across the new drop. Multiple crystals appeared with similar shapes as the crystals used for seeding (not shown). Furthermore, streak-seeding of needles grown in the presence of PEG 400 into the same crystallization condition yielded single tiny crystals that were not suitable for diffraction experiments (Fig. 2c).

The crystallization procedure was optimized so that single hexagonal plate crystals were obtained by cross-seeding of multiple plate-like crystals grown in the absence of PEG 400 and were transferred after 24 h equilibration into crystallization solutions consisting of 1.9–2.1 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5, 2% PEG 400 (Fig. 2d). Hanging drops were prepared by mixing protein solution and reservoir solution in 2 µl:1 µl, 1 µl:1 µl and 1 µl:2 µl ratios, and were equilibrated against 500 µl reservoir solution at 293 K.

2.3. Data collection and processing

Crystals were transferred from the drop using a cryoloop into a new drop consisting of the crystallization solution supplemented with 20% glycerol and were flash-cooled in liquid nitrogen.

X-ray diffraction data were collected on beamline ID14-2 at the European Synchrotron Radiation Facility (ESRF, Grenoble) at 100 K. 1400 images with an oscillation angle of 0.1° and an exposure time of 1.5 s per frame were recorded on an ADSC Q4 CCD detector at a wavelength of 0.9334 Å. Data were integrated using *MOSFLM* (Battye *et al.*, 2011) and were scaled with *SCALA* (Evans, 2006). The Matthews coefficient was calculated using the *CCP4* package (Winn *et al.*, 2011).

3. Results and discussion

Homologous expression of α -PGM in *L. lactis* was successfully achieved. Purification of α -PGM by affinity chromatography was sufficient to produce pure and functional protein (Fig. 1) with a yield of ~1.5 mg per litre of culture. The problem of crystal multiplicity

observed during the initial screens was overcome by seeding techniques. Single hexagonal crystals were grown by streak-seeding from multiple crystals grown in ammonium sulfate in the absence of PEG 400 into the same crystallization solution supplemented with 2% PEG 400. Crystals grew to 150 µm within one week of streak-seeding (Fig. 2d). These new single crystals obtained from seeding were subsequently used as an initial source of seeds. The crystals diffracted to 1.5 Å resolution and belonged to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 67.17$, $c = 210.39$ Å. The calculated Matthews coefficient (Matthews, 1968; Kantardjieff & Rupp, 2003) was 2.3 Å³ Da⁻¹ with a solvent content of around 46%, indicating the presence of two molecules in the asymmetric unit. The relevant parameters for data collection and processing are summarized in Table 1.

A *BLAST* search revealed that the highest identity of the α -PGM sequence was to phosphomannomutases (around 22–24%). The best three hits for which three-dimensional structure were available were used as search models for molecular replacement [human phosphomannomutase 2 (PDB entry 2amy; Center for Eukaryotic Structural Genomics, unpublished work), human phosphomannomutase 1 (PDB entry 2fue; Silvaggi *et al.*, 2006) and *Leishmania* phosphomannomutase (PDB entry 2i54; Kedzierski *et al.*, 2006)]. No solution was found in various molecular-replacement trials with *MOLREP* (Vagin & Teplyakov, 2010) and *Phaser* (McCoy *et al.*, 2007). The phase problem therefore needs to be addressed by experimental phasing.

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