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Crystallization and preliminary X-ray diffraction analysis of the putative aldose 1-epimerase YeaD from *Escherichia coli*

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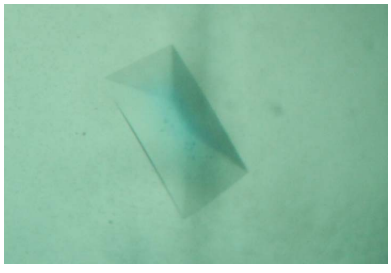

Escherichia coli YeaD (ecYeaD) is suggested to be a member of the galactose mutarotase-like superfamily. Galactose mutarotase is an enzyme that converts α-galactose to β-galactose. The known structures of these galactose mutarotase-like proteins are similar to those of galactose mutarotases, with the catalytic residues being conserved, but there are some differences between them in the substrate-binding pocket. In order to reveal the specificity of ecYeaD, a three-dimensional structure is essential. Full-length ecYeaD with an additional 6×His tag at the C-terminus was crystallized by the hanging-drop vapour-diffusion method using polyethylene glycol 4000 as a precipitant at 283 K. An X-ray diffraction data set was collected to a resolution of 1.9 Å from a single flash-cooled crystal that belonged to space group $P2_12_12_1$.

1. Introduction

Galactose and glucose are produced by the hydrolysis of lactose by β-galactosidase. Glucose can directly enter the glycolysis pathway, while galactose, an equal energy source, is transformed into glucose 1-phosphate by the Leloir pathway (Holden *et al.*, 2003). Glucose 1-phosphate is further converted to glucose 6-phosphate, an intermediate compound in glycolysis, by the action of phosphoglucomutase. The Leloir pathway was first delineated by L. F. Leloir and associates in 1948–1951; it is the only pathway for the biological conversion of galactose to glucose (Wilkinson, 1949) and is comprised of three steps involving the action of four enzymes. In the first step of this pathway, galactose mutarotase produces α-D-galactose from β-D-galactose by stereochemical inversion at carbon 1 of galactose (Frey, 1996). Galactose mutarotase is important in galactose metabolism, because in the second step of the Leloir pathway galactokinase requires α-D-galactose as its substrate, whereas β-galactosidase produces β-D-galactose from lactose. The reaction mechanism of galactose mutarotase has been revealed to involve the transfer of a proton from the C-1 hydroxyl group of the substrate to the C-5 ring O atom of the substrate with a transient ring opening, followed by a rotation about the C-1–C-2 bond and subsequent reversal of the process, leading to product formation (Thoden *et al.*, 2003).

Galactose mutarotase has been observed in bacteria (Wallenfels & Herrmann, 1965), plants (Bailey *et al.*, 1966), fungi (Bentley & Bhate, 1960) and mammals (Keston, 1954), and crystal structures have been reported for galactose mutarotases from human (hsGalM), *Lactococcus lactis* (lacGalM) and *Caenorhabditis elegans* (ceGalM). All are topologically similar in molecular architecture, with some difference in the loop regions between the β-strands (Thoden *et al.*, 2004). The sequence identity and homology between hsGalM and lacGalM are 32% and 49% for 337 comparable residues, respectively.

The crystal structure of YeaD from *Salmonella typhimurium* (stYeaD) has been reported (Chittori *et al.*, 2007). The crystallographic models of stYeaD and galactose mutarotases are topologically similar in structure and active-site architecture, but have minor differences in the substrate-binding pocket, suggesting that stYeaD might be a galactose mutarotase-like protein. Sequence analysis showed that YeaD from *Escherichia coli* (ecYeaD) shares 80% amino-acid sequence identity with stYeaD. The presence of YeaD in

E. coli in addition to galactose mutarotases is enigmatic. Although the catalytic residues and a few of the substrate-binding residues of galactose mutarotase-like proteins are conserved in other well characterized mutarotases, there are some differences in the substrate-binding pocket which may be important for the specificity of the enzyme. In order to elucidate the specificity of ecYeaD on ligand anchoring, a three-dimensional structure is essential, as protein function is tightly correlated to structure. In this paper, we report the crystallization and preliminary crystallographic analysis of ecYeaD.

2. Materials and methods

2.1. Cloning, expression and purification

The primers used were 5′-GGAATTC**CATATG**ATGATTAAGAAAATTTTTGCCCTTC-3′ (forward) and 5′-CCG**CTCGAG**ACGTTTCGCAACGCGAATGGATTGC-3′ (reverse). They were designed with *Nde*I and *Xho*I restriction sites, which are depicted in bold. The gene (gene ID 946572) encoding full-length ecYeaD (residues 1–294) was amplified by polymerase chain reaction (PCR) from *E. coli* genomic DNA. The PCR-amplified fragment was digested with *Nde*I and *Xho*I and cloned into the pET-22b(+) vector (Novagen) previously digested with the same restriction enzymes. The sequence of the recombinant plasmid was confirmed by nucleotide sequencing. The final plasmid that encodes the ecYeaD polypeptide with an addition C-terminal LEHHHHHH tag was transformed into Rosetta (DE3) cells (Novagen). The cells containing the target plasmid were grown in Luria–Bertani (LB) medium supplemented with 50 mg ml^{-1} ampicillin at 310 K until the culture reached an OD_{600} of 0.6 and were then induced with 1 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) for a further 18 h at 289 K. The cells were harvested by centrifugation at 8000*g* for 6 min, suspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl) and lysed by sonication on ice. The cell lysates were centrifuged at 12 000*g* for 30 min. The supernatant containing soluble protein was loaded onto a nickel-chelating column (GE Healthcare) pre-equilibrated with buffer *A*. Nonspecifically bound proteins were washed from the column using 50 ml buffer *A* containing 20 m*M* imidazole. The recombinant protein was eluted with 40 ml buffer *A* containing 300 m*M* imidazole. To remove the remaining impurities, the fraction containing the target protein was loaded onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with buffer *A*. The pooled fractions which contained the target protein were desalted by ultrafiltration (Millipore, 10 kDa cutoff) with buffer *B* (10 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl) and concentrated to 24 mg ml^{-1} as determined using the BCA Protein Assay Kit (Pierce). According to SDS–PAGE (Fig. 1), the purity of the protein was greater than 95%. Finally, the purified protein was stored at 193 K.

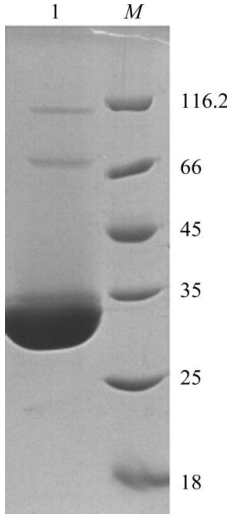

Figure 1
SDS–PAGE of YeaD. About 10 µg protein was loaded. Lane 1, purified YeaD; lane *M*, molecular-weight markers (kDa).

2.2. Crystallization

Crystallization experiments on ecYeaD with the addition of 5 mg ml^{-1} DTT were carried out by the hanging-drop vapour-diffusion method using the Crystal Screen and Crystal Screen 2 reagent kits from Hampton Research and the Structure Screen 1 and 2 reagent kits from Molecular Dimensions at 277 K. The crystallization droplets contained 1 µl 24 mg ml^{-1} protein solution and 1 µl crystallization cocktail and were equilibrated against 100 µl reservoir solution. A well diffracting single crystal was observed in condition No. 22 of Crystal Screen comprising 30%(*w*/*v*) polyethylene glycol 4000 (PEG 4000), 200 m*M* sodium acetate and 100 m*M* Tris–HCl pH 8.5 after 3 d and grew to a size suitable for data collection in 10 d (Fig. 2).

2.3. X-ray diffraction data collection and preliminary analysis

Initial X-ray diffraction experiments were performed using in-house Cu *Kα* X-rays generated by an RA-Micro007 rotating-anode X-ray source (Rigaku) and diffraction images (Fig. 3) were collected using a MAR345dtb imaging-plate detector (MAR Research). Crystals were quickly passed through a cryoprotectant solution [30%(*w*/*v*) PEG 4000, 200 m*M* sodium acetate, 100 m*M* Tris–HCl pH 8.5 and 20%(*v*/*v*) glycerol] and flash-cooled to 100 K in a stream of cold nitrogen gas produced by an Oxford Cryosystems cooling device (Oxford Cryosystems Ltd). The crystal-to-detector distance was 150 mm. The oscillation angle was 1° and the exposure time was 5 min per frame. A complete diffraction data set consisting of 180 images was collected at 100 K. Diffraction data were processed and scaled using the *HKL*-2000 suite (Otwinowski & Minor, 1997) and programs from *CCP*4 (Collaborative Computational Project, Number 4, 1994). Data-collection and processing statistics are listed in Table 1.

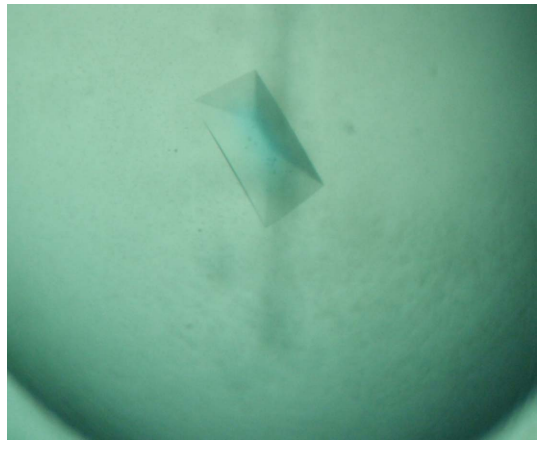

Figure 2
Photomicrograph of an ecYeaD crystal. The dimensions of this single crystal are about 0.3 × 0.1 × 0.1 mm.

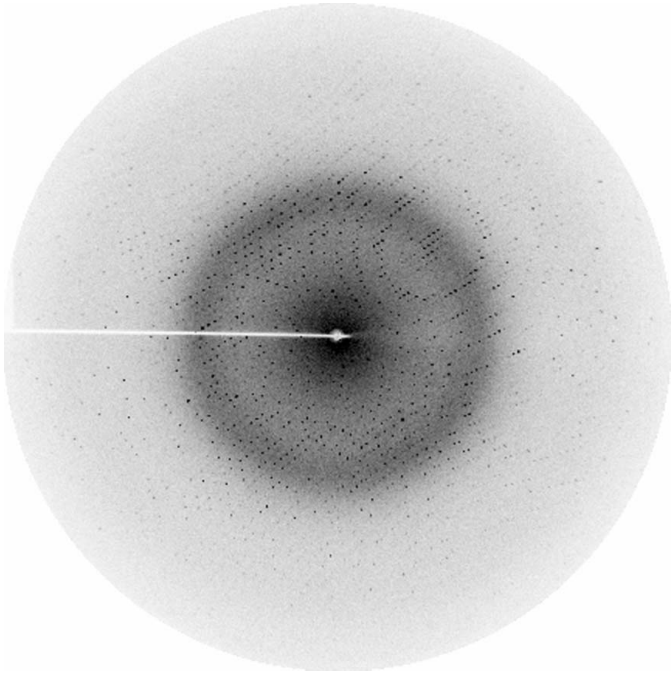

Figure 3
Image of the diffraction pattern of YeaD.

3. Results and discussion

ecYeaD was cloned and overexpressed in a soluble form in *E. coli* and was purified by Ni–NTA affinity chromatography and size-exclusion chromatograpy as described in §2.1. The yield of purified protein was approximately 15 mg per litre of bacterial culture. The molecular weight of ecYeaD was 34 kDa as estimated by SDS–PAGE analysis, which is in good agreement with the calculated molecular weight of ecYeaD and the additional C-terminal six-His tag. The apparent molecular weight of ecYeaD was 34 kDa as estimated by size-exclusion chromatography on Superdex 200 (data not shown), indicating that the purified protein is a monomer in solution.

The crystal of ecYeaD belonged to space group $P2_12_12_1$. The calculated Matthews coefficient and solvent content suggested the presence of two molecules per asymmetric unit in the crystal (Matthews, 1968). stYeaD was also found to contain a dimer in the asymmetric unit but was a monomer in solution; however, a dimer was observed by examination using gel-permeation chromatography when the protein solution was pre-incubated with the crystallization cocktail. It was suggested that the crystallographic dimer of stYeaD may be a consequence of oligomerization induced during crystallization and the same is likely to apply to ecYeaD.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 64.72, b = 97.32, c = 106.99
Resolution (Å)	50.00–1.90 (1.93–1.90)
Unique reflections	54268 (2680)
Redundancy	7.0 (6.7)
Completeness (%)	100 (100)
Average $I/\sigma(I)$	36.45 (7.65)
$R_{\rm merge}$† (%)	5.3 (34.4)
No. of molecules in unit cell (Z)	2
$V_{\rm M}$ (Å^3 Da^{-1})	2.41
Solvent content (%)	48.93

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the mean value of $I_i(hkl)$ for all i measurements.

The *MOLREP* program (Vagin & Teplyakov, 1997) from the *CCP*4 suite was used to perform molecular replacement using stYeaD (PDB code 2hta; Chittori *et al.*, 2007) as the search model. Two molecules were found in the asymmetric unit, with an R factor of 0.416, which confirmed that there was a dimer in the asymmetric unit. Further refinement of the structure is under way.

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