Acta Crystallographica Section F
**Structural Biology and Crystallization Communications**
ISSN 1744-3091

# Expression, purification and preliminary X-ray crystallographic analysis of UDP-galactopyranose mutase from *Deinococcus radiodurans*

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Received 8 May 2009
Accepted 14 July 2009

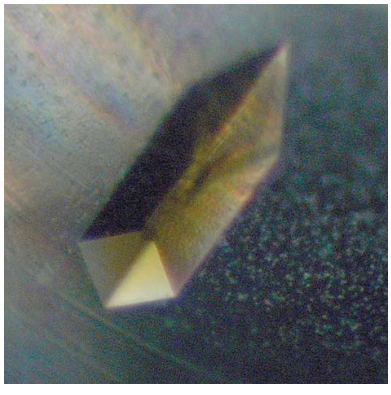
UDP-galactopyranose mutase (UGM) catalyzes the interconversion of UDP-galactopyranose and UDP-galactofuranose. A UGM–substrate complex from *Deinococcccus radiodurans* has been expressed, purified and crystallized. Crystals were obtained by the microbatch-under-oil method at room temperature. The crystals diffracted to 2.36 Å resolution at the Canadian Light Source The space group was found to be $P2_12_12_1$, with unit-cell parameters $a = 134.0$, $b = 176.6$, $c = 221.6$ Å. The initial structure solution was determined by molecular replacement using UGM from *Mycobacterium tuberculosis* (PDB code 1v0j) as a template model.

## 1. Introduction

The cell wall of various pathogenic microbes is a validated antimicrobial target. A number of antimicrobial drugs that are currently in use inhibit cell-wall biosynthesis. The intricate nature of the microbial cell wall and its components are not found in humans and hence cell-wall biosynthesis is an attractive target for the development of novel antimicrobial drugs (Green, 2002). D-Galactofuranose (Gal*f*), the five-membered ring form of the common sugar D-galactose, is one of the vital components found in the cell wall of pathogenic bacteria, including *Escherichia coli*, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* (Pedersen & Turco, 2003). Gal*f* is also found in the cell wall and cell surfaces of certain fungi and protozoa (*Aspergillus nidulans*, *Trypanosoma cruzi* and *Leishmania* species). In 1993, the World Health Organization declared tuberculosis (TB) to be a global health emergency; TB alone kills nearly three million people worldwide per year (World Health Organization, 2002). *M. tuberculosis*, the causative organism of TB, has a unique cell wall with an outer layer of mycolic acid connected to the peptidoglycan through an arabinogalactan layer (Besra *et al.*, 1995). Gal*f* is an essential component of the arabinogalactan complex. UDP-galactofuranose (UDP-Gal*f*) serves as the precursor of Gal*f* residues in the arabinogalactan layer. UDP-galactopyranose mutase (UGM), a flavoenzyme (with bound FAD), catalyzes the interconversion of UDP-galactopyranose (UDP-Gal*p*) and UDP-Gal*f*. The gene encoding UGM in *M. tuberculosis* is essential for its viability, suggesting that UGM is a potential antimycobacterial drug target (Pan *et al.*, 2001)

Previously, crystal structures of UGM (without substrate) from *E. coli*, *K. pneumonia* and *M. tuberculosis* have been determined by X-ray crystallography (Sanders *et al.*, 2001; Beis *et al.*, 2005). UGM is a homodimer in solution and belongs to the $\alpha/\beta$ class of proteins. The unliganded UGM structures have provided insight into the architecture of the UGM–flavin interactions and helped to predict the binding mode of the substrate. It has been found that UGM is only catalytically active when the flavin is in the reduced form (Sanders *et al.*, 2001). Despite the wealth of structural and biochemical information, the mechanism and the role of FAD are not fully understood.

The crystal structure of UGM in complex with substrates or inhibitors is currently unknown. Previous attempts to crystallize UGM



(from *E. coli*, *K. pneumoniae* and *M. tuberculosis*) with substrates (either by cocrystallization or soaking) were unsuccessful (they either produced no crystals or resulted in apoenzyme). A flexible loop at the entrance of the substrate-binding pocket of UGM exists primarily in an open conformation in the crystal structures and the movement of this loop has been proposed to affect the cocrystallization or soaking process (Chad *et al.*, 2007).

In an effort to determine the structure of a UGM–substrate complex, we chose UGM from *Deinococcus radiodurans* (drUGM). Changing the source of an enzyme has proven to be a highly successful strategy for determination of the structure of proteins (McPherson, 1998). drUGM shows ∼35% sequence identity to other bacterial UGMs and the active-site residues are identical to those of other bacterial UGMs. A UGM–substrate complex will greatly improve our understanding of enzyme–substrate interactions and, in conjunction with the other unliganded structures, will help in the design of inhibitors. In this paper, we report the cloning, expression, purification, crystallization and preliminary X-ray crystallographic studies of drUGM complexed with the substrate UDP-Gal*p*.

## 2. Materials and methods

### 2.1. Cloning and overexpression

The UGM gene (DR_A0367) was obtained by the polymerase chain reaction (PCR) using *D. radiodurans* genomic DNA (strain R1) as a template (ATCC 13939). The primers used for PCR were as follows: forward, 5′-ACT CCT GCC ATG GGG AAT GCC GAT GAC TGA-3′; reverse, 5′-ATG GAT CCT TAC TCC GCG TT-3′. The amplified PCR fragment was purified by gel extraction, digested with *Nco*I and *Bam*HI and cloned into a pEHISTEV vector (Liu & Naismith, 2009). The cloned gene was verified by DNA sequencing (Plant Biotechnology Institute, Saskatoon, Canada). The sequencing results confirmed the full-length gene product and also the addition of the affinity tag (six histidine residues at the N-terminus plus the linker DYDIPTTENLYFQG). The construct was then transformed into *E. coli* Tuner cells (Novagen, USA). Transformed cells were grown in Luria–Bertani (LB) medium with 50 µg ml$^{-1}$ kanamycin at 310 K until the optical density reached ∼0.6; this was followed by induction with 0.4 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 300 K for 4–5 h. The cells were harvested by centrifugation for 20 min at 8000*g* and 277 K and the resulting cell pellets were stored at 193 K.

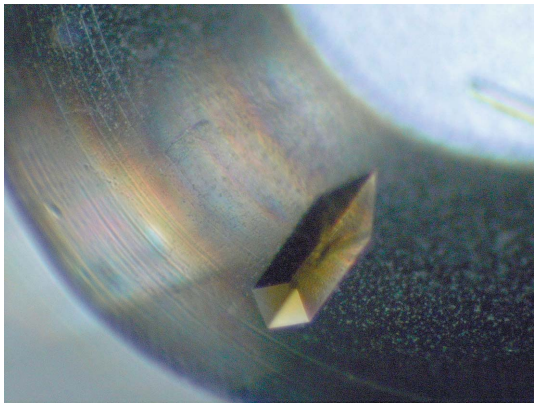


**Figure 1**
Crystal of drUGM–substrate complex. The crystal size is 0.3 × 0.1 × 0.1 mm.

### 2.2. Purification

The frozen cell pellet was resuspended in lysis buffer [100 m*M* potassium phosphate pH 8.0, 1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 0.1%(*v*/*v*) Triton-X, 20 µg ml$^{-1}$ DNase and lysozyme] and stirred at 277 K for 30 min; this was followed by sonication and the suspension was then clarified by centrifugation at 17 000*g* for 30 min. The supernatant was subjected to heat denaturation at 328 K for 10 min followed by centrifugation at 17 000*g* for 30 min. The supernatant was dialyzed against 25 m*M* potassium phosphate pH 8.0 (four changes). The His-tagged protein did not bind to an affinity column and therefore alternate purification methods were used. The dialyzed sample was filtered and applied onto a HQ20 (Applied Biosystems, USA) anion-exchange column pre-equilibrated with 25 m*M* potassium phosphate pH 8.0, which was followed by gradient elution using 25 m*M* potassium phosphate buffer pH 8.0 containing 1 *M* NaCl. Fractions containing drUGM were collected, pooled and dialysed against 50 m*M* potassium phosphate pH 8.0. The sample was concentrated and brought to 30%(*w*/*v*) ammonium sulfate with stirring at 277 K. The resulting solution was filtered and applied onto a pre-equilibrated HP-20 (Applied Biosystems, USA) hydrophobic interaction chromatography column with binding buffer (50 m*M* potassium phosphate pH 8.0 containing 30% ammonium sulfate). Bound proteins were eluted with a decreasing gradient of ammonium sulfate in 50 m*M* potassium phosphate pH 8.0. Fractions containing drUGM were combined and dialysed against 50 m*M* bis-tris propane pH 8.0. The purified drUGM was concentrated to 7.5 mg ml$^{-1}$ (determined by Bradford assay) and the purity of the protein sample was judged from SDS–PAGE analysis. Small aliquots were flash-cooled using liquid nitrogen and stored at 193 K.

### 2.3. Crystallization

Crystallization trials were carried out at room temperature using the microbatch-under-oil method. Initial crystallization trials were carried out using commercial screening kits (from Qiagen). Prior to

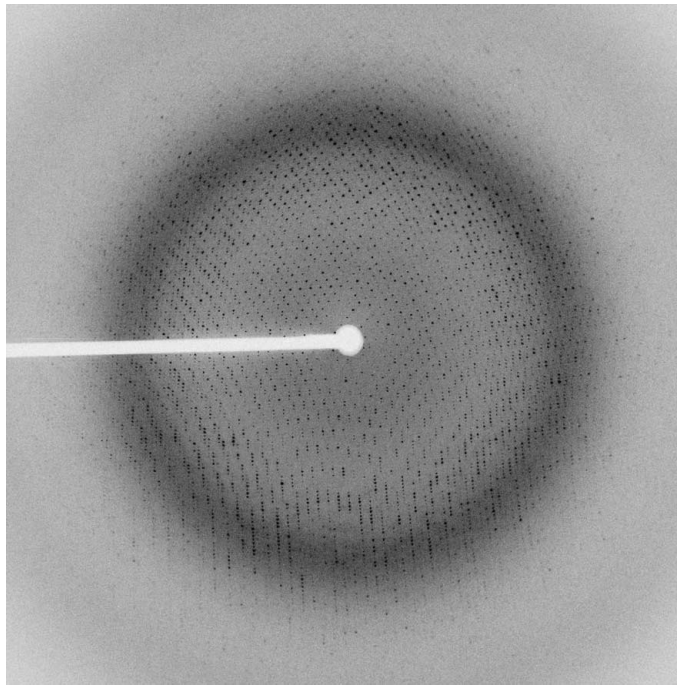


**Figure 2**
Diffraction pattern of the drUGM complex crystal.

**Table 1**
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Temparature (K) | 100 |
| Wavelength (Å) | 0.9797 |
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å, °) | $a$ = 134.0, $b$ = 176.6, $c$ = 221.6, $\alpha = \beta = \gamma = 90$ |
| Resolution limits (Å) | 19.88–2.36 (2.42–2.36) |
| Total No. of reflections | 1509175 (96614) |
| No. of unique reflections | 204925 (13696) |
| Completeness (%) | 95.8 (87.4) |
| $R_{\rm merge}$ (%) | 11.6 (68.9) |
| $I/\sigma(I)$ | 13.07 (3.08) |
| Solvent content (%) | 57.5 |
| No. of molecules in ASU | 10 |
| Matthews coefficient (Å$^3$ Da$^{-1}$) | 2.8 |

crystallization trials, the protein sample was reduced with sodium dithionite (20 m*M*) and incubated with UDP-Gal*p* (10 m*M*) for 30 min. Crystallization drops were made by mixing 2.0 µl protein solution with 2.0 µl precipitant solution and the drops were covered with paraffin oil (Chayen, 1999). Crystals appeared in several conditions; however, good crystals (rod shaped and well defined with sharp edges) were obtained in 0.1 *M* HEPES pH 7.0, 0.2 *M* LiCl and 20%(*w*/*v*) PEG 6000. A grid screen was performed around this condition (varying the pH and the precipitant concentrations) and the best diffracting crystals were obtained using 0.1 *M* HEPES pH 6.5, 0.2 *M* LiCl and 28% PEG 6000. These crystals appeared within a week and grew to dimensions of ~0.1 × 0.1 × 0.3 mm after two weeks (Fig. 1).

### 2.4. Data collection

Prior to data collection, single crystals from the drop were transferred into mother-liquor solution containing 10% xylitol and 20 m*M* UDP-Gal*p*. Crystals were mounted in a cryoloop and flash-cooled with liquid nitrogen. Diffraction data were collected on beamline 08ID-1 at the Canadian Light Source, Saskatoon using a MAR 225 CCD X-ray detector. The crystal-to-detector distance was set to 250 mm with an oscillation range of 0.25° and a total of 720 images (1 s exposure time for each image) were collected that covered a total oscillation range of 180°. The images were integrated and scaled using *XDS*/*XSCALE* (Kabsch, 1993). The diffraction pattern and data-collection statistics are shown in Fig. 2 and Table 1, respectively.

## 3. Results and discussion

The crystals belonged to the orthorhombic space group $P2_12_12_1$. The structure of the drUGM–substrate complex was determined by the molecular-replacement method using *MrBUMP* (Keegan & Winn, 2007), an automated protocol for molecular-replacement solution built within the *CCP*4 package (Collaborative Computational Project, Number 4, 1994). Using the *MOLREP* (Vagin & Teplyakov, 1997) option within *MrBUMP*, an initial solution was found using the crystal structure of UGM from *M. tuberculosis* (PDB code 1v0j). The sequence identity between UGM from *M. tuberculosis* and drUGM is 39%. The initial solution had eight monomers in the aymmetric unit. A search for additional copies was performed by fixing the eight known monomers and a further two monomers were found. The other reported UGMs crystallized with two homodimers, one homodimer or a monomer in the asymmetric unit (Sanders *et al.*, 2001; Beis *et al.*, 2005). Initial restrained refinement carried out using *REFMAC*5 as part of *MrBUMP* (Winn *et al.*, 2001) resulted in an *R* factor and an $R_{\rm free}$ of 0.352 and 0.423, respectively (initial *R* factor of 0.419 and $R_{\rm free}$ of 0.466). The Matthews coefficient of 2.8 Å$^3$ Da$^{-1}$ and the solvent content of 57.5% are consistent with ten monomers (as five homodimers) in the asymmetric unit. Each dimer is related by a twofold rotation; however, no regular relationship exists for the five dimers in the asymmetric unit. The difference map from the initial refinement showed clear density for the cofactor (FAD) and the substrate, with difference peaks exceeding 2.5$\sigma$ for the UDP moiety of the substrate. Further refinement and model building are in progress. The structural details will be described in a separate paper.

We are grateful to the members of the macromolecular beamline 08ID-1, Canadian Light Source, Saskatoon, Canada for their technical assistance during data collection. This work was supported by an NSERC grant to DARS. The research described in this paper was performed at the Canadian Light Source, which is supported by NSERC, NRC, CIHR and the University of Saskatchewan.

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