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Purification, crystallization and preliminary X-ray diffraction studies of a putative UDP-*N*-acetyl-D-mannosamine dehydrogenase from *Pyrococcus horikoshii* OT3

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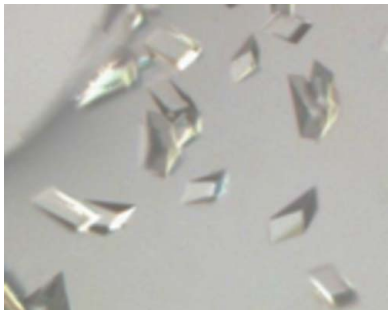
A putative UDP-*N*-acetyl-D-mannosamine dehydrogenase from *Pyrococcus horikoshii* OT3, an essential enzyme for polysaccharide biosynthesis, has been overexpressed in *Escherichia coli* and purified. Crystals were obtained using the oil-microbatch method at 291 K. A native data set extending to 1.8 Å resolution has been collected and processed in space group $P2_1$. Assuming the presence of a dimer in the asymmetric unit, the V_M value is calculated to be 2.3 Å^3 Da^{-1}, which is consistent with the result of a dynamic light-scattering experiment that shows a dimeric state of the protein in solution.

1. Introduction

Uridine-diphospho-*N*-acetyl-D-mannosamine (UDP-D-ManNAc) and uridine-diphospho-*N*-acetyl-D-mannosaminuronic acid (UDP-D-ManNAcA) are ubiquitous and essential metabolites in cellular processes. They act as precursors for proteoglycans and glycoproteins as well as for the cell-wall components of bacteria (peptidoglycans and lipopolysaccharides). *N*-Acetyl-*O*-mannosamine is a major component of the oligosaccharide chains of N- and O-glycans and of glycolipids (Schachter, 2000; Herscovics & Orlean, 1993). O-linked *N*-acetylglucosamine modification of specific residues of intracellular proteins in higher eukaryotes has been shown to directly regulate important processes such as the cell cycle, transcription, translation, cell signalling and the stress response to carbohydrate metabolism (Wells *et al.*, 2003; Zachara *et al.*, 2004).

UDP-D-ManNAcA is synthesized from UDP-*N*-acetyl-D-glucosamine (UDP-D-GlcNAc) by a two-step pathway. In the first step, UDP-*N*-acetylglucosamine 2-epimerase catalyzes the C-2 epimerization of UDP-D-GlcNAc to form UDP-D-ManNAc; in the second step, UDP-D-mannosamine dehydrogenase (UDPManNAcDH) catalyzes the C-6 dehydrogenation of UDP-D-ManNAc to form UDP-D-ManNAcA (Reid & Fewson, 1994).

UDPManNAcDH is essential in the biosynthesis of UDP-*N*-acetylglucosamine and belongs to an enzyme family known as the UDP-glucose/GDP-mannose dehydrogenase family. This family constitutes a small group of enzymes that catalyze the NAD$^+$-dependent twofold oxidation of a sugar nucleotide without the release of an aldehyde intermediate. Two known crystal structures of members of this family are those of UDP-glucose dehydrogenase (Campbell *et al.*, 2000) and of GDP-mannose dehydrogenase (Snook *et al.*, 2003), which show less than 30% sequence identity to UDPManNAcDH. These two enzymes belonging to the UDP-glucose/GDP-mannose dehydrogenase family were found to be dimers with a similar overall fold in their crystal structures. However, the biological role of the dimer formation of these enzymes is not fully understood. Some proteins belonging to this family of enzymes, such as UDPManNAcDH, are also of further interest because of their sugar nucleotide-modifying capabilities, involving a net four-electron oxidation of alcohol and aldehyde (Ge *et al.*, 2004).



As the three-dimensional structure of UDPManNAcDH is unknown, our research on this protein may shed light on the UDPManNAc-biosynthesis pathway. Detailed analysis of the structure–function relationship of this enzyme will help in understanding the chemical principles of the biosynthesis of di-*N*-acetylated mannosamine sugar nucleotide. Here, we report the purification, crystallization and preliminary crystallographic analysis of the putative UDPManNAcDH from *Pyrococcus horikoshii* OT3 (PH1618), with the aim of determining its crystal structure and elucidating the mechanism of the enzymatic reactions. The PH1618 protein was assigned as a putative UDPManNAcDH, as it has a high sequence identity (40%) to the UDPManNAcDH from *Escherichia coli*.

2. Experimental

2.1. Protein expression and purification

The putative UDPManNAcDH from *P. horikoshii* OT3 used in this study has a molecular weight of 46.1 kDa and consists of 418 amino-acid residues (PH1618; gi:3258047). Protein expression and purification were performed by the Structurome Research Group at the RIKEN SPring-8 Center, Harima Institute, Japan. The plasmid encoding UDPManNAcDH was digested with *Nde*I and *Bgl*II and the fragment was inserted into the expression vector pET-11a (Novagen) linearized with *Nde*I and *Bam*HI. *E. coli* BL21 Codon Plus (DE3)-RIL cells were transformed with the recombinant plasmid and grown without IPTG induction at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin for 20 h. The cells were harvested by centrifugation at 4500*g* for 5 min at 277 K and were subsequently suspended in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl and 5 m*M* 2-mercaptoethanol; they were finally disrupted by sonication and heated at 363 K for 10 min. The cell debris and denatured protein were removed by centrifugation (18 000*g* for 30 min). The supernatant solution was used as the crude extract for purification.

The crude extract was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) and applied onto a Super Q Toyopearl 650M (Tosoh) column equilibrated with 20 m*M* Tris–HCl pH 8.0 (buffer *A*). After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing UDPManNAcDH was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) with buffer *A*. The sample was loaded onto a Resource Q column (Amersham Biosciences) equilibrated with buffer *A*. After elution with a linear gradient of 0–0.2 *M* NaCl, the fraction containing UDPManNAcDH was desalted using a HiPrep 26/10 desalting column with 10 m*M* phosphate–NaOH pH 7.0. The sample was then applied onto a Bio-Scale CHT-20-I column (Bio-Rad) equilibrated with 10 m*M* phosphate–NaOH pH 7.0 and eluted with a linear gradient of 10–150 m*M* phosphate–NaOH pH 7.0. The sample was concentrated by ultrafiltration (VivaSpin) and loaded onto a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Biosciences) equilibrated with buffer *A* containing 0.2 *M* NaCl. The homogeneity and identity of the purified sample were estimated by SDS–PAGE (Laemmli, 1970) and N-terminal sequence analysis. Finally, the purified UDPManNAcDH was concentrated by ultrafiltration to 12 mg ml^{-1} in buffer *A* containing 0.2 *M* NaCl.

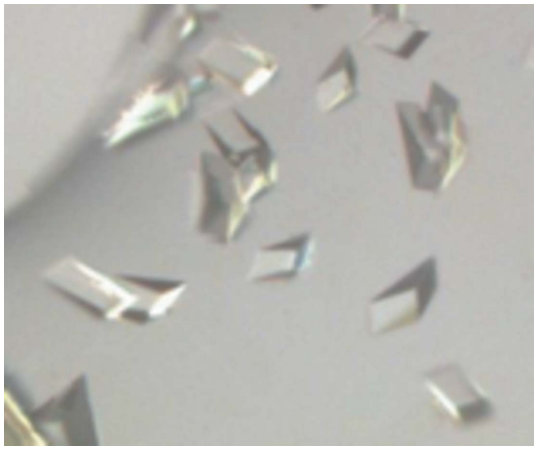


Figure 1
Crystals of the putative UDP-D-ManNAc dehydrogenase. The typical dimensions of the crystals are about 0.1 × 0.1 × 0.1 mm.

Table 1
Crystal data and data-collection statistics of putative UDP-D-ManNAc dehydrogenase.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 80.28, b = 69.24, c = 83.10, β = 114.4
Resolution range (Å)	50.0–1.80 (1.86–1.80)
Total observations	250348
Unique reflections	75480
Redundancy	3.4 (3.3)
Completeness (%)	99.9 (100.0)
Mean $I/\sigma(I)$	14.7 (4.6)
$R_{\rm merge}$† (%)	4.7 (23.3)

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, where $I_j(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of measurement j and the mean intensity of reflections with index hkl, respectively.

The oligomeric state of purified UDPManNAcDH was examined by a dynamic light-scattering experiment using a DynaPro MS/X instrument (Protein Solutions), which was performed at a protein concentration of 12 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.6 with 0.2 *M* NaCl. Several measurements were taken at 291 K and analyzed using the *DYNAMICS* software v.3.30 (Protein Solutions). A bimodal analysis resulted in a molecular weight of 92 kDa, which is consistent with a dimeric state of the protein in solution.

2.2. Crystallization

Crystallization trials were carried out using the oil-microbatch method (Chayen *et al.*, 1990) using Nunc HLA plates at 291 K. Initial screening for crystallization conditions was performed using Hampton Research Crystal Screens I and II (Jancarik & Kim, 1991). Equal volumes of protein solution (1.0 µl) and precipitant solution (1.0 µl) were mixed. The crystallization drop was overlaid with a 6:4 mixture of silicone and paraffin oils, allowing slow evaporation of water in the drop. One condition provided the most well defined crystals. The precipitant solution consisted of 27.5%(*w*/*v*) PEG 4000, 1 *M* lithium chloride and 0.1 *M* MES–NaOH pH 6.1. The initial crystals formed as clusters of multiple crystals. Several rounds of optimization resulted in single crystals having sharp edges and typical dimensions of 0.1 × 0.1 × 0.1 mm (Fig. 1). These crystals appeared about a month after setup.

2.3. Data collection and analysis

The crystals were flash-cooled in a cryoprotectant solution consisting of the precipitant solution diluted with glycerol at 20%(*v*/*v*). X-ray diffraction intensity data were collected at the SPring-8 beamline BL26B1 using a Jupiter 210 detector (Rigaku). A total of 180 frames were collected with 1° oscillation and 15 s expo-

sure time per image. The wavelength of the synchrotron radiation was 1.0 Å and the crystal-to-detector distance was 150 mm. The diffraction data were integrated and scaled to 1.8 Å resolution using *DENZO* and *SCALEPACK* as implemented in the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results

The crystals belong to space group $P2_1$, with unit-cell parameters $a = 80.28$, $b = 69.24$, $c = 83.10$ Å, $\beta = 114.4^\circ$. A dynamic light-scattering experiment gave a result consistent with a dimeric state of the protein in solution (see §2). Assuming the presence of two UDPManNAcDH molecules in the asymmetric unit, the Matthews coefficient V_M (Matthews, 1968) is calculated to be the reasonable value 2.3 $Å^3$ Da^{-1}, corresponding to a solvent content of 48%. A complete data set was collected and the data-collection statistics are summarized in Table 1. Selenomethionine labelling or soaking the crystals in a solution of NaBr are being considered in order to solve the structure of putative UDPManNAcDH by multiple isomorphous replacement/anomalous dispersion phasing. The molecular-replacement method using the coordinates of GDP-mannose dehydrogenase available in the Protein Data Bank (PDB code 1mv8) did not yield a structure solution owing to the low homology (27% identity) between these proteins.

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References

Campbell, R. E., Mosimann, S. C., Van de Rijn, I., Tanner, M. E. & Strynadka, C. J. (2000). *Biochemistry*, **39**, 7012–7023.
Chayen, N. E., Shaw Stewart, P. D., Maeder, D. L. & Blow, D. M. (1990). *J. Appl. Cryst.* **23**, 297–302.
Ge, X., Penney, L. C., van de Rijn, I. & Tanner, M. E. (2004). *Eur. J. Biochem.* **271**, 14–22.
Herscovics, A. & Orlean, P. (1993). *FASEB J.* **7**, 540–550.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Reid, M. F. & Fewson, C. A. (1994). *Crit. Rev. Microbiol.* **20**, 13–56.
Schachter, H. (2000). *Glycoconj. J.* **17**, 465–483.
Snook, C. F., Tipton, P. A. & Beamer, L. J. (2003). *Biochemistry*, **42**, 4658–4668.
Wells, L., Vosseller, K. & Hart, G. W. (2003). *Cell. Mol. Life Sci.* **60**, 222–228.
Zachara, N. E., O'Donnell, N., Cheung, W. D., Mercer, J. J., Marth, J. D. & Hart, G. W. (2004). *J. Biol. Chem.* **279**, 30133–30142.