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Purification, crystallization and preliminary X-ray diffraction studies of UDP-*N*-acetylglucosamine pyrophosphorylase from *Candida albicans*

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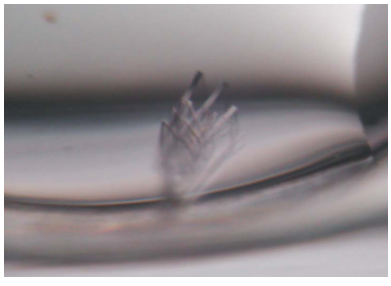

UDP-*N*-acetylglucosamine pyrophosphorylase (UAP) is an essential enzyme in the synthesis of UDP-*N*-acetylglucosamine. UAP from *Candida albicans* was purified and crystallized by the sitting-drop vapour-diffusion method. The crystals of the substrate and product complexes both diffract X-rays to beyond 2.3 Å resolution using synchrotron radiation. The crystals of the substrate complex belong to the triclinic space group $P1$, with unit-cell parameters $a = 47.77$, $b = 62.89$, $c = 90.60$ Å, $\alpha = 90.01$, $\beta = 97.72$, $\gamma = 92.88^\circ$, whereas those of the product complex belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 61.95$, $b = 90.87$, $c = 94.88$ Å.

1. Introduction

Uridine-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) is a ubiquitous and essential metabolite in cellular processes. It acts as a precursor for proteoglycans and glycoproteins as well as the cell-wall components of bacteria (peptidoglycans and lipopolysaccharides) and fungi (chitin). It is also used in the GlcNAc moiety of N-linked glycosylation and the glycosylphosphoinositol anchor of eukaryotic proteins (Herscovics & Orlean, 1993).

UDP-GlcNAc is synthesized from fructose-6-phosphate by several reaction steps (Mengin-Lecreulx & van Heijenoort, 1994; Mio *et al.*, 1998, 1999, 2000; Watzele & Tanner, 1989) and UDP-GlcNAc pyrophosphorylase (UAP; EC 2.7.7.23) is a key enzyme in this biosynthesis. This enzyme condenses *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) and uridine-5′-triphosphate (UTP) by an S_N2-like mechanism to form UDP-GlcNAc. The non-esterified oxygen of the phosphate group of GlcNAc-1-P attacks the α-phosphate group of UTP and its β- and γ-phosphate groups are released as pyrophosphate. This enzyme is found in both prokaryotes and eukaryotes (Kostrewa *et al.*, 2001; Peneff *et al.*, 2001; Sheu & Frey, 1978). The prokaryotic UAP is a bifunctional enzyme called GlmU, which synthesizes GlcNAc-1-P from glucosamine-1-phosphate as well as UDP-GlcNAc from GlcNAc-1-P (Mengin-Lecreulx & van Heijenoort, 1994). The crystal structures of GlmU have been determined from several species and have been revealed to be divided into two domains corresponding to the two functions: the acetylation domain and the pyrophosphorylation domain (Brown *et al.*, 1999; Kostrewa *et al.*, 2001; Olsen & Roderick, 2001; Sulzenbacher *et al.*, 2001). In contrast to prokaryotic UAPs, eukaryotic UAPs only catalyze pyrophosphorylation. Sequence alignment indicates that the homology between the pyrophosphorylation domain of GlmU and eukaryotic UAP is approximately 10%. Thus, prokaryotic UAP can be considered to catalyze the condensation reaction in a different way from the eukaryotic enzyme.

Three crystal structures of mammalian UAP, human AGX1, human AGX2 and mouse AGX2, have been determined (Peneff *et al.*, 2001). All three mammalian enzymes possess insertion loops composed of more than ten amino-acid residues compared with non-mammalian eukaryotic UAP. The insertion loop is found at the dimer interface in the crystal structure. The dimer cannot be considered to be a physiological state as UAP functions as a monomer. In addition, the insertion loop binds to the catalytic site of the other UAP molecule forming the dimer (Peneff *et al.*, 2001). Since the amino-acid residues on the insertion loop are not well conserved and since the loop seems

Table 1
Crystal data and data-collection statistics of CaUAP1.

Values in parentheses are for the highest resolution shell.

	GlcNAc-1-P complex	UDP-GlcNAc complex
Space group	$P1$	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 47.43, b = 61.80, c = 90.44, α = 90.01, β = 97.72, γ = 92.88	a = 61.95, b = 90.87, c = 94.88
No. of measured reflections	87749	173951
No. of independent reflections	43334	23766
Resolution range (Å)	50–2.3 (2.38–2.30)	20–2.3 (2.38–2.30)
$R_{\rm merge}$† (%)	7.0 (12.0)	9.3 (21.3)
Completeness ($I > 1\sigma$) (%)	93.0 (86.2)	96.0 (93.1)
$I/\sigma(I)$	11.19 (6.75)	17.99 (8.47)

† $R_{\rm merge} = \sum |I_i - \langle I_i \rangle| / \sum \langle I_i \rangle$, where I_i is the observed intensity and $\langle I_i \rangle$ is the average intensity over symmetry-equivalent measurements.

to prevent the enzyme from interaction with the ligand, a reasonable catalytic mechanism has not been proposed from the known structures.

The insertion loop of UAP from *Candida albicans* (CaUAP1) is much shorter than those of the structurally determined eukaryotic UAPs and is not expected to occupy the catalytic site of other CaUAP1 molecules in the crystal. This 'loop-free' structure will contribute to the understanding of the catalytic mechanism of eukaryotic UAP. In addition, a specific inhibitor of CaUAP1 may be useful as a novel medicine against candidasis, as *C. albicans* is the pathogen of candidasis (Rogers & Balish, 1980). Here, we report the purification of CaUAP1 and the crystallization of its complexes with substrate (GlcNAc-1-P) and product (UDP-GlcNAc) in order to determine the crystal structure and to elucidate the mechanism of the enzymatic reactions.

2. Cloning, overexpression and purification

CaUAP1 is composed of 486 amino-acid residues and its molecular weight is about 55 kDa. The ORF of CaUAP1 was cloned at the *Sma*I site of pGEX-2T to express CaUAP1 as a glutathione *S*-transferase (GST) fusion protein. The GST-tagged CaUAP1 was overproduced and sonicated using previously described methods (Mio *et al.*, 1998), except that aeration was continued for an additional 16 h at 295 K after the addition of isopropyl 1-thiogalactopyranoside. The supernatant of the sonicated cells was applied onto a Glutathione Sepharose 4 FastFlow column (Amersham Biosciences, Piscataway, NJ, USA) and washed with PBS (phosphate-buffered saline). The GST-tagged protein was eluted with GST elution buffer (50 m*M* Tris–HCl pH 8.0, 0.15 *M* NaCl, 10 m*M* reduced glutathione). The GST tag was removed by thrombin treatment for 20 h at 277 K. The GST-removed sample in a buffer composed of 1.0 *M* ammonium sulfate was applied onto a HiTrap Phenyl Sepharose High Performance column (Amersham Biosciences, Piscataway, NJ, USA). A gradient of 1.4–0 *M* ammonium sulfate in 20 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA and 5.0%(*v*/*v*) glycerol was used to elute the protein at room temperature. Finally, the protein was dissolved in GSTrap buffer composed of 20 m*M* Tris–HCl pH 7.5, 1 m*M* EDTA, 0.15 *M* NaCl, 1 m*M* dithiothreitol (DTT) and 5%(*v*/*v*) glycerol and applied onto a 5 ml GSTrap column (Amersham Biosciences, Piscataway, NJ, USA) and the unbound protein was collected at room temperature. Gel-filtration chromatography analysis suggested that the purified protein forms a monomer in solution (data not shown). Approximately 4.5 mg CaUAP1 was obtained from 1 l culture.

3. Crystallization and data collection

10 mg ml^{-1} UAP with ligand solution (10 m*M* GlcNAc-1-P or 10 m*M* UDP-GlcNAc) was prepared in 50 m*M* Tris–HCl pH 7.5 and 1 m*M* DTT for cocrystallization. 1 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated using the sitting-drop vapour-diffusion method. Crystals of the complexes with GlcNAc-1-P and with UDP-GlcNAc were obtained within one week using precipitant solution composed of 100 m*M* sodium citrate pH 5.5–6.0, 20–30%(*w*/*v*) polyethylene glycol 6000, 80–120 m*M* ammonium sulfate and 5–15%(*v*/*v*) glycerol. Crystals of the apo form of UAP could not be obtained in any screening condition. The crystals of the complexes with substrate (GlcNAc-1-P) and with product (UDP-GlcNAc) grew to approximate dimensions of 0.05 × 0.05 × 0.3 mm and 0.05 × 0.2 × 0.3 mm, as shown in Figs. 1 and 2, respectively.

Diffraction data were collected at BL38B1 and BL45XU, SPring-8, Japan using a Jupiter 210 detector (Rigaku) and at AR-NW12A, Photon Factory, Tsukuba, Japan using a Quantum 210 detector (ADSC). The crystals were flash-cooled in a nitrogen-gas stream at 95 K without using a cryoprotectant solution. The diffraction data were collected using X-rays at a wavelength of 1.0000 Å with exposures of 30 and 20 s and oscillation angles of 1.0 and 2.0° per frame for the complexes with GlcNAc-1-P and with UDP-GlcNAc, respectively. The crystal-to-detector distances were set to 150 and 200 mm for the

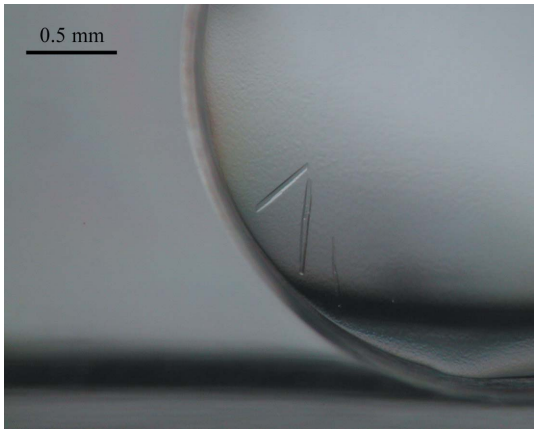

Figure 1
Crystal of CaUAP1 complexed with GlcNAc-1-P.

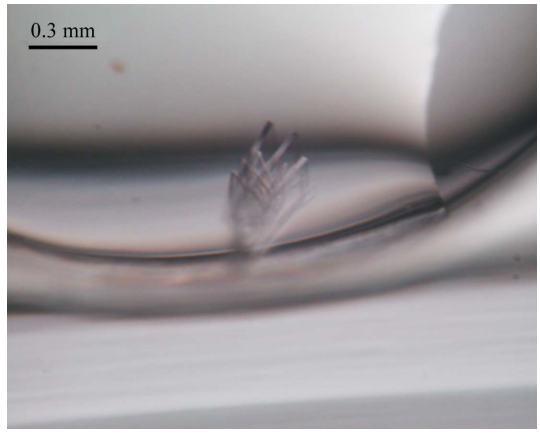

Figure 2
Crystal of CaUAP1 complexed with UDP-GlcNAc.

complexes with GlcNAc-1-P and with UDP-GlcNAc, respectively. Both crystals diffracted to beyond 2.3 Å resolution and the data sets were processed using the *HKL*-2000 package (Otwinowski & Minor, 1997).

The crystals of the complex with GlcNAc-1-P belong to the triclinic space group $P1$, with unit-cell parameters a = 47.43, b = 61.80, c = 90.44 Å, α = 90.01, β = 97.81, γ = 92.88°. The crystals of the complex with UDP-GlcNAc belong to the orthorhombic system, with unit-cell parameters a = 61.95, b = 90.87, c = 94.88 Å, and the space group was determined to be $P2_12_12_1$ based on the systematic absences of reflections ($h = 2n + 1$ at $h00$, $k = 2n + 1$ at $0k0$ and $l = 2n + 1$ at $00l$). The statistics of the intensity data of both crystals are shown in Table 1. Assuming the presence of two CaUAP1 molecules in the asymmetric unit of the substrate complex, the Matthews coefficient V_M and the solvent content are 2.39 Å^3 Da^{-1} and 48.5%, respectively (Matthews, 1968). On the other hand, assuming that one molecule exists in the asymmetric unit of the product complex, the V_M value and the solvent content are calculated as 2.43 Å^3 Da^{-1} and 49.3%, respectively. The structures of the substrate and product complexes were successfully solved by the molecular-replacement method using the atomic coordinates of human AGX2 (PDB code 1jvd; Peneff *et al.*, 2001) as a search model with the program *AMoRe* (Navaza, 1994) from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

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