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Purification, crystallization and preliminary X-ray characterization of the human GTP fucose pyrophosphorylase

Stephen Quirk‡ and Katherine L. Seley-Radtke*

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, 1000 Hilltop Circle, Chemistry 405C, Baltimore, MD 21250, USA

‡ Present address: Kimberly Clark Corp., Roswell, Georgia, USA.

Correspondence e-mail: kseley@umbc.edu

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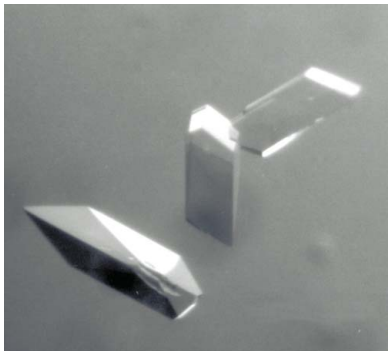

The human nucleotide-sugar metabolizing enzyme GTP fucose pyrophosphorylase (GFPP) has been purified to homogeneity by an affinity chromatographic procedure that utilizes a novel nucleoside analog. This new purification regime results in a protein preparation that produces significantly better crystals than traditional purification methods. The purified 66.6 kDa monomeric protein has been crystallized *via* hanging-drop vapor diffusion at 293 K. Crystals of the native enzyme diffract to 2.8 Å and belong to the orthorhombic space group $P2_12_12_1$. There is a single GFPP monomer in the asymmetric unit, giving a Matthews coefficient of 2.38 Å^3 Da^{-1} and a solvent content of 48.2%. A complete native data set has been collected as a first step in determining the three-dimensional structure of this enzyme.

1. Introduction

Fucose is a deoxyhexose that is found in nearly all plant and animal species and is a critical monosaccharide in several metabolic processes (for a review, see Becker & Lowe, 2003). Usually, fucose is made available during the synthesis of fucosylated glycolipids, oligosaccharides and glycoproteins *via* a sugar nucleotide intermediate, specifically GDP-β-L-fucose. In most organisms, GDP-fucose is formed predominantly by the oxidation–reduction and epimerization of GDP-α-D-mannose (Bulet *et al.*, 1984). In mammals, a second pathway exists for the formation of GDP-β-L-fucose: a salvage pathway that converts the fucose that is liberated from broken-down glycoproteins, glycolipids and fucose-containing oligosaccharides into fucose-1-phosphate and then in turn to GDP-β-L-fucose. The first reaction is catalyzed by a ubiquitous fucosyl kinase (Park *et al.*, 1998). The second reaction is catalyzed by a novel guanosine triphosphate fucose pyrophosphorylase (GFPP; Pastuszak *et al.*, 1998).

GFPP (EC 2.7.7.30) catalyzes the reversible formation of the nucleotide sugar GDP-β-L-fucose from the condensation of GTP and fucose-1-phosphate according to

$$\text{GTP} + \text{fucose-1-P} \longleftrightarrow \text{GDP-L-fucose} + \text{PP}_\text{i}.$$

Like other nucleotide-sugar pyrophosphorylases, the reaction catalyzed by GFPP is magnesium-dependent, but unlike other such enzymes, the enzyme does not catalyze the formation of orthophosphate from the liberated pyrophosphate moiety. GFPP is a monomeric enzyme of 594 amino acids (66.6 kDa), which is also unique among the family of nucleotide-sugar pyrophosphorylases. Not surprisingly, it shares little sequence similarity with other homologous proteins. Several critical catalytic amino acids have been identified through a series of site-directed mutagenesis experiments (Quirk & Seley, 2005*b*) and the active site has been shown to discriminate substrate from non-substrate *via* a complex set of hydrogen-bond interactions (Quirk & Seley, 2005*a*).

Even though the salvage pathway accounts for only 10% of the total cellular GDP-β-L-fucose pool (Yurchenco & Atkinson, 1977), several studies (Pastuszak *et al.*, 1998; Niittymaki *et al.*, 2004) indicate that GFPP and fucose kinase are expressed at high levels in many tissues. Therefore, they clearly play a more significant role in the overall synthesis of GDP-β-L-fucose. GFPP may also be a new target for anti-inflammatory and antimetastatic drugs. In order to further

the understanding of this unusual pyrophosphorylase, structural determination studies have been undertaken.

2. Materials and methods

2.1. Protein expression and purification

The standard GFPP purification scheme (Quirk & Seley, 2005*a*), which utilized a histidine affinity-tag expression system, did not result in diffraction-quality crystals. In fact, the removal of the affinity tag did not improve crystal quality either, so changes to the expression system were attempted. A new expression/purification protocol was devised. The gene encoding the human GFPP enzyme was subcloned into pET12a utilizing terminal *Nde*I and *Bam*HI restriction sites. The enzyme can then be induced and purified without an affinity tag. GFPP was purified to homogeneity by growing 5 l of BL21(DE3) culture (LB supplemented with 50 µg ml^{-1} ampicillin) at 310 K until an OD_{595} of 0.4 was reached. Protein expression was induced by the addition of IPTG to 0.4 m*M* and the culture was grown for an additional 10 h at 303 K. Cells were pelleted by centrifugation at 11 000*g* for 15 min, resuspended in 10 m*M* Tris–HCl pH 7.5, 20 m*M* NaCl, 10 m*M* $MgCl_2$, 0.5 m*M* EGTA and lysed by passage through a French press. The material was cleared *via* centrifugation and the supernatant was decanted. This crude protein extract was applied onto a tricyclic inosine Sepharose 6B affinity column (10 cm × 1.76 cm^2) in the same buffer at a flow rate of 0.1 ml min^{-1}. This column was prepared by reacting tricyclic inosine and epoxy-activated Sepharose 6B in water/NaOH pH 13.0 according to the directions from the manufacturer (Amersham Bioscience). Bound GFPP was eluted from the column by the application of a 0–2 *M* NaSCN gradient. Fractions containing eluted GFPP were pooled, dialyzed into 10 m*M* Tris–HCl pH 7.5 and concentrated to 10 mg ml^{-1} by pressure filtration through an Amicon YM-3 semi-permeable membrane.

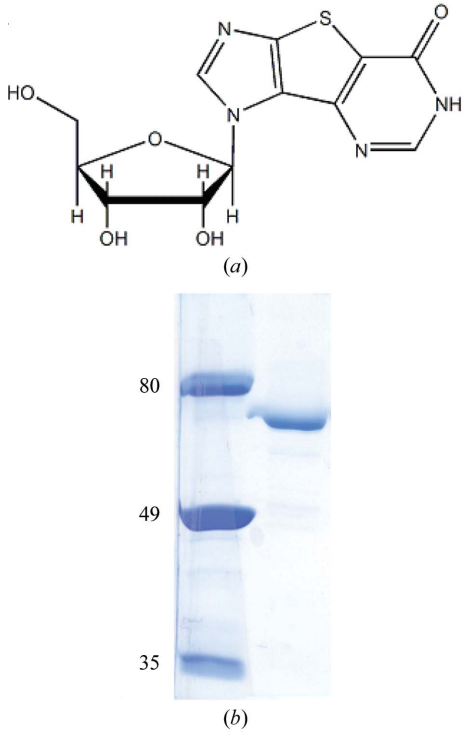

Figure 1
Purification of recombinant GFPP. (*a*) Structure of the tricyclic inosine affinity probe that was coupled to epoxy-activated Sepharose 6B. (*b*) SDS–PAGE analysis through a 12–18% gradient gel. Lane 1, molecular-weight markers with weight (in kDa) indicated; lane 2, purified GFPP. Proteins were visualized by staining the gel with Coomassie brilliant blue.

2.2. Crystallization and data collection

Utilizing the traditional GFPP purification procedure (Quirk & Seley, 2005*a*), it was possible to produce small crystals that diffracted poorly and that grew as small clumps of interconnected microcrystals. Although several conditions in the initial combinatorial crystallization screen (Jancarik & Kim, 1991; McPherson, 1992) produced crystals of various habits, the best crystals did not diffract beyond 3.5 Å resolution. Utilizing the affinity purification protocol, it was possible to produce single crystals that diffracted beyond 3 Å resolution. The highest quality GFPP crystals were formed by the hanging-drop vapor-diffusion method in 24-well Linbro plates over a 1.0 ml reservoir buffer which consisted of 12%(*w*/*v*) PEG 8000, 10%(*w*/*v*) ethylene glycol, 40 m*M* sodium cacodylate pH 6.6, 1 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$. The 8 µl hanging drops were composed of 4 µl protein stock solution mixed with 4 µl reservoir buffer. Diffraction-quality crystals formed over a period of 5–10 d at 293 K. Crystals were equilibrated in cryoprotectant solution comprising 25%(*w*/*v*) PEG 8000, 20%(*w*/*v*) glycerol, 10%(*w*/*v*) ethylene glycol, 40 m*M* cacodylate pH 6.6 for 12 h and were flash-cooled directly in liquid nitrogen prior to diffraction analysis.

A complete native data set was measured on a single GFPP crystal at 100 K by ω scans of 0.25°. Frames were recorded for 200 s each. Diffraction measurements were collected using a Rigaku IV^{++} area detector utilizing Cu $K\alpha$ X-rays from a rotating-anode source. Diffraction data were processed and analyzed using the *HKL* package (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Purification and crystallization

The revised protein purification protocol significantly improved the quality of the GFPP crystals, even though this and the original protein preparations appear to be homogeneous by a variety of biophysical measurements. A likely explanation is that the revised expression protocol slows the protein expression rate (both by lowering the IPTG concentration and the induction temperature for culture growth) to a level where the protein is not trapped into inclusion bodies. The original expression procedure (Quirk & Seley, 2005*a*) produces approximately 45% soluble protein, whereas the revised expression protocol results in approximately 90% soluble protein, albeit at an overall lower level. The use of an affinity-based purification protocol may also reduce sample heterogeneity by selectively binding the subfraction of GFPP which is competent to bind the affinity probe. The use of the tricyclic nucleoside, which was originally synthesized as a novel antiviral compound (Seley *et al.*, 2002), has proven useful as an affinity purification reagent for GFPP and reduces the purification scheme to a single step. The overall yield is 3 mg per litre of induced culture. Fig. 1 illustrates the structure of the tricyclic inosine affinity probe and shows an SDS–PAGE analysis of the pooled GFPP fraction used for crystallization studies.

Diffraction-quality crystals were obtained under several initial screening conditions and improved orthorhombic crystals were formed from reservoir buffers containing PEG 8000, ethylene glycol and cacodylate. Initial crystals diffracted in the range 3.5–4 Å resolution and were typically small (0.1 × 0.05 × 0.02 mm). These crystals were also highly mosaic (greater than 2°). Subsequent reservoir buffer optimization greatly improved the crystal quality, as did

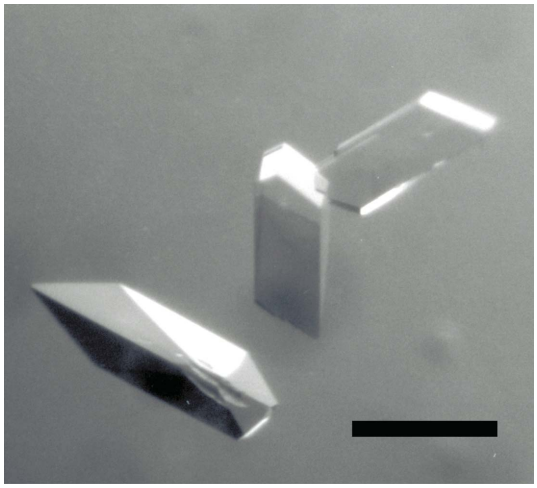

Figure 2
Crystals of human GTP fucose pyrophosphorylase. The bar is 1 mm in length.

dehydration of the crystals in a cryoprotectant buffer containing glycerol. The final crystallization conditions resulted in larger crystals (typically 1.2 × 0.4 × 0.3 mm) with an extended diffraction limit and reduced mosaicity (approximately 0.5°). A typical GFPP crystal is shown in Fig. 2.

3.2. Diffraction data analysis

X-ray diffraction data were collected at a resolution of 2.8 Å. The data set revealed that the crystals belong to the orthorhombic space group $P2_12_12_1$. Assuming a molecular weight of 66 626 Da and the presence of one molecule in the asymmetric unit, the crystal volume per unit weight (V_M) and the solvent content of the crystal were calculated to be 2.38 Å^3 Da^{-1} and 48.2%, respectively. The Matthews coefficient is within the range commonly observed for proteins (Kantardjieff & Rupp, 2003). Table 1 summarizes the data-collection and processing statistics. As human GFPP is only 11% identical to any other homologous protein of known three-dimensional structure (Pastuszak *et al.*, 1998; Quirk & Seley, 2005*b*), the GFPP structure will be solved by the MIR methodology. Since the protein contains 14 methionine residues, SeMet-substituted protein is also being prepared.

Table 1
Crystal and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Crystal dimensions (mm)	1.2 × 0.4 × 0.3
Unit-cell parameters (Å)	a = 75.21, b = 86.13, c = 97.72
Space group	$P2_12_12_1$
Molecules per ASU	1
Resolution range (Å)	20–2.8 (2.9–2.8)
Observed reflections	53206
Unique reflections	15105
Redundancy	3.52
Completeness (%)	96.3 (97.7)
$\langle I/\sigma(I)\rangle$†	18.6
R_{sym}‡ (%)	7.2 (29.3)
B§ (Å^2)	41.2

† Average of the intensity divided by the standard deviation for all reflections. ‡ $R_{sym} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where $I(h)$ is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over the ith measurement. § Overall Debye–Waller temperature factor.

GFPP is unlike any of the other nucleotide-sugar metabolizing pyrophosphorylase. This includes sequence identity, allosteric regulation by a regulatory subunit, tissue distribution and substrate preferences. By unraveling more of the enzyme architecture, structure and mechanism, it will be possible to further the understanding of fucose metabolism in diverse species.

References

Becker, D. J. & Lowe, J. B. (2003). *Glycobiology*, **13**, 41R–51R.
Bulet, P., Hoflack, B., Porchet, M. & Verbert, A. (1984). *Eur. J. Biochem.* **144**, 255–259.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kantardjieff, K. A. & Rupp, B. (2003). *Protein Sci.* **12**, 1865–1871.
McPherson, A. (1992). *J. Cryst. Growth*, **122**, 161–167.
Niittymaki, J., Mattila, P., Roos, C., Huopaniemi, L., Sjoblom, S. & Renkonen, R. (2004). *Eur. J. Biochem.* **271**, 78–86.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Park, S. H., Pastuszak, I., Drake, R. & Elbein, A. D. (1998). *J. Biol. Chem.* **273**, 5685–5691.
Pastuszak, I., Ketchum, C., Hermanson, G., Sjoberg, E. J., Drake, R. & Elbein, A. D. (1998). *J. Biol. Chem.* **273**, 30165–30174.
Quirk, S. & Seley, K. L. (2005*a*). *Biochemistry*, **44**, 10854–10863.
Quirk, S. & Seley, K. L. (2005*b*). *Biochemistry*, **44**, 13172–13178.
Seley, K. L., Zhang, L., Hagos, A. & Quirk, S. (2002). *J. Org. Chem.* **67**, 3365–3373.
Yurchenco, P. D. & Atkinson, P. H. (1977). *Biochemistry*, **16**, 944–953.