Crystallization and preliminary X-ray characterization of the *Vitis vinifera* fucokinase:GDP-fucose pyrophosphorylase

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The *Vitis vinifera* dual-activity fucose and nucleotide-sugar metabolizing enzyme L-fucokinase:GDP-fucose pyrophosphorylase (FKP) has been purified to homogeneity and the 118.8 kDa monomeric protein has been crystallized by vapor diffusion in Zeppenzauer tubes at 277 K. Crystals of the apoenzyme diffracted to 2.6 Å resolution and belonged to the tetragonal space group $P4_12_12$. There is a single FKP monomer in the asymmetric unit, giving a Matthews coefficient of $3.22 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 61.8%. 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). However, in all organisms studied to date a salvage pathway exists for the formation of GDP-β-L-fucose that converts the fucose liberated from degraded glycoconjugates into fucose 1-phosphate and then in turn to GDP-β-L-fucose. In most organisms the first reaction is catalyzed by fucokinase (Park *et al.*, 1998). The second reaction is catalyzed by guanosine diphosphate fucose pyrophosphorylase (Pastuszak *et al.*, 1998). In plants, by way of contrast, these two separate salvage reactions are catalyzed by a single enzyme: fucokinase:GDP-fucose pyrophosphorylase (FKP; Kotake *et al.*, 2008). It is hypothesized that this dual-activity enzyme plays an important role in the formation of fucose-containing glycoconjugates in seed plants by limiting the diffusion of fucose 1-phosphate into the cytoplasm during critical periods in plant development (for a general review, see Ma *et al.*, 2006). Plant dual-activity enzymes such as the *Vitis vinifera* FKP protein have not been extensively studied beyond basic kinetics.

FKP first phosphorylates free fucose in the presence of ATP to form fucose 1-phosphate and then catalyzes the reversible formation of GDP-β-L-fucose from the condensation of GTP and fucose 1-phosphate according to the following scheme:

The *Arabidopsis* FKP enzyme has been characterized (Kotake *et al.*, 2008). Like other nucleotide-sugar pyrophosphorylases and sugar kinases, the reactions catalyzed by FKP are magnesium/manganese-dependent. The separate active sites, as identified by consensus sequence homology and limited site-directed mutagenesis, show distinct pH-dependent behavior and thermal denaturation characteristics. However, the mechanism of how newly synthesized fucose 1-phosphate is shuttled to the pyrophosphorylase active site is unknown (as is the mechanism). The enzyme from *V. vinifera* is a monomeric protein of 1079 amino acids (118.8 kDa) and shares kinetic similarities with the *Arabidopsis* protein. Both enzymes are inherently unstable during purification, which has limited both the

amount of recombinant protein that can be produced as well as the suitability of the enzyme to form diffraction-quality crystals.

Even though the salvage pathway accounts for only 10% of the total cellular GDP- β -L-fucose pool (Yurchenco & Atkinson, 1977), the *Arabidopsis* FKP protein is expressed at a high level and null mutants do not express a growth phenotype (Kotake *et al.*, 2008). In order to further the understanding of this unusual kinase/pyrophosphorylase, structure-determination studies have been undertaken.

2. Materials and methods

2.1. Protein expression and purification

The amino-acid sequence of *V. vinifera* FKP (NCBI reference sequence XP_002264541) was reverse-translated and codon optimized for expression in *Escherichia coli* recombinant systems. The open reading frame was synthesized by DNA 2.0 Inc. and was subcloned into pMAL-p5G (New England Biolabs Inc.) utilizing terminal *Nde*I and *Bam*HI restriction sites. This vector system creates an N-terminal fusion with maltose-binding protein (MBP). The affinity tag was utilized in hope of increasing the expression level and stabilizing FKP in addition to improving the purification yield. The enzyme was purified to homogeneity by growing 5 l *E. coli* BL21 (DE3) culture (LB supplemented with 50 μ g ml⁻¹ ampicillin) at 293 K until an OD₅₉₅ of 0.4 was reached. Protein expression was induced by addition of IPTG to 0.3 mM and the culture was grown for an additional 24 h at 283 K. Cells were pelleted by centrifugation at 11 000g for 15 min, resuspended in 10 mM Tris-HCl pH 7.5, 20 mM NaCl, 0.5 mM EDTA and lysed by passage through a French press. The material was cleared *via* centrifugation and the supernatant was

decanted. The fusion protein was purified on an amylose affinity column following the instructions supplied by New England Biolabs. Fractions containing eluted MBP-FKP were pooled, dialyzed into 10 mM Tris-HCl pH 7.5 and concentrated to 2 mg ml⁻¹ by pressure filtration through an Amicon YM-3 semipermeable membrane. The maltose-binding protein tag was cleaved from FKP using Genease I protease (following the New England Biolabs Inc. procedure). This mixture was applied onto a guanosine Sepharose 6B affinity column (5.0 cm $\times$ 1.76 cm²) in the same buffer at a flow rate of 0.1 ml min⁻¹. This column was prepared by reacting guanosine and epoxy-activated Sepharose 6B in water/NaOH pH 13.0 according to the directions from the manufacturer (Amersham Bioscience). Bound FKP was eluted from the column by the application of a 0–1 M NaSCN gradient. Fractions containing eluted FKP were pooled, dialyzed into 10 mM Tris-HCl pH 7.5 and concentrated to 10 mg ml⁻¹ by pressure filtration through an Amicon YM-3 semipermeable membrane.

2.2. Crystallization and data collection

Few conditions in the initial combinatorial crystallization screen (Jancarik & Kim, 1991; McPherson, 1992) produced crystals; even the best crystals, when they formed at all, did not diffract well enough to collect and reduce a complete data set (either owing to severe radiation sensitivity, crystal size or a high degree of twinning or mosaicity). In an effort to obtain usable crystals, several experiments were undertaken using the rotary cell-culture system from Synthecon Inc. to simulate reduced-gravity conditions. Zeppezauer-type crystallization tubes (Zeppezauer *et al.*, 1986) of 2 mm internal diameter were loaded with protein solution/50% crystallization buffer (20 μ l) at one end and 100% crystallization buffer (40 μ l) at the other end. The tubes were sealed with hematocrit clay and placed into the rotary unit. The assembly was then rotated at 80 rev min⁻¹ at 277 K. The rotation angle of the device can be varied from 0° to 90° to simulate reduced gravity (meaning a nearly randomized gravity vector) or normal gravity, respectively. The device was rotated in 5° intervals between 0° and 90° in order to screen for a rotation angle that facilitated crystal growth.

Utilizing this technique in conjunction with the original screening results, it was possible to produce single crystals that diffracted to beyond 3 Å resolution. The highest quality FKP crystals were formed against 9%(w/v) PEG 6K, 5%(v/v) ethylene glycol, 5%(v/v) glycerol, 25 mM HEPES pH 7.5, 2% *tert*-butanol, 1 mM MgCl₂, 1 mM MnCl₂. Diffraction-quality crystals formed over a period of 20–30 d at 277 K. The crystals were equilibrated in cryoprotectant solution comprising 9%(w/v) PEG 6K, 20%(v/v) glycerol, 5%(v/v) ethylene glycol, 25 mM HEPES pH 7.5 for 12 h and flash-cooled directly in liquid nitrogen prior to diffraction analysis.

A complete native data set was measured on a single FKP crystal at 100 K by ω scans of 0.25°. Frames were recorded for 200 s each. Diffraction measurements were collected using a Rigaku R-Axis IV⁺⁺ area detector utilizing Cu K α 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 purification of *Vitis* FKP was greatly facilitated (and simplified) by using the maltose-binding protein affinity tag. The final relative yield of the protein was increased to 10 mg homogeneous FKP (devoid of affinity tag) per litre of starting culture. As with the *Arabidopsis* protein (Kotake *et al.*, 2008), low-temperature induction

Figure 1

The low-shear simulated reduced-gravity crystallization setup. (a) Diagram showing the capillary-tube array mounted on the rotation wheel. The small arrow indicates the wheel rotation direction. The array can be rotated through $0 < \theta < 90^\circ$ to go from fully simulated reduced gravity to normal gravity. The gravity vector G is shown. (b) Organization of the Zeppezauer crystallization tubes: clay plugs (black), crystallization buffer (gray), protein solution (stripes).

Table 1

Crystal and data-collection statistics.

Values in parentheses are for the highest resolution shell.

Crystal dimensions (mm)	1.3 × 1.3 × 0.4
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 72.4, <i>c</i> = 583.3
Space group	<i>P</i> 4 ₁ 2 ₁ 2
Molecules per asymmetric unit	1
Resolution range (Å)	20–2.6 (2.8–2.6)
Unique reflections	53989 (8118)
Redundancy	6.1 (3.52)
Completeness (%)	98.6 (96.4)
$\langle I/\sigma(I) \rangle^\dagger$	19.9 (3.3)
$R_{\text{merge}}^\ddagger$ (%)	9.5 (29.3)
$B^\S$ (Å ²)	50.2
Mosaicity (°)	0.55
Twinning fraction (%)	1.0

† Average of the intensity divided by the standard deviation for all reflections. $^\ddagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl . § Overall Debye–Waller temperature factor.

was key to isolating properly folded and active enzyme. Previous expression experiments utilizing fully native protein (no expression tag) or an N-terminal or C-terminal hexahistidine tag resulted in a significantly lower yield of protein (0.1 mg l^{−1} for native expression, ~2.0 mg l^{−1} with an N-terminal His tag and no expression with a C-terminal His tag). The MBP-FKP protein could be stored at 253 K for up to three months without loss of enzymatic activity, compared with approximately one month for native FKP. Thus, it appears that MBP has a stabilizing effect on FKP.

The final FKP protein was indeed enzymatically active when assayed as described by Kotake *et al.* (2008). In our hands, the values were $K_m = 0.8$ mM (for ATP), $k_{\text{cat}} = 0.22$ s^{−1} for the fucokinase reaction and $K_m = 0.2$ mM (for GTP), $k_{\text{cat}} = 1.55$ s^{−1} for the forward GDP-fucose pyrophosphorylase reaction. Only enzymatically active enzyme was utilized for crystallization studies.

All initial attempts to crystallize FKP or MBP-FKP were not successful. Screening a wide array of combinatorial screen conditions and temperatures resulted in either precipitated protein or microcrystals that did not diffract. Hanging-drop, sitting-drop and under-oil conditions produced equally poor crystals. Seeding experiments did not improve the crystal quality under any of the conditions. The ‘best’ crystals were obtained from PEG 6K-containing buffers with added metal salts; however, they were small thin hexagonal crystals (typi-

cally 0.05 × 0.1 × 0.1 mm) and diffracted to approximately 5 Å resolution. These crystals were also highly mosaic and twinned. As a last effort to improve crystal quality, a simulated low-shear reduced-gravity approach was conducted using a rotary cell-culture device. A schematic of the device is shown in Fig. 1. Crystals, when observed, were tested for diffraction quality. Useful crystals were only obtained at a rotation angle θ centered around 18° and in a small range of PEG 6K concentrations [8.5–9.5% (w/v)]. The addition of both MgCl₂ and MnCl₂ improved crystal quality in these PEG 6K and rotation-angle ranges. No diffraction-quality crystals were observed in the absence of rotation or when the device was rotated at angles outside of the optimal ~15–20° range. Fig. 2 shows the relationship between PEG 6K concentration, rotation angle θ and crystal diffraction limit. The best crystals were hexagonal in habit and measured approximately 1.3 × 1.3 × 0.4 mm. A typical FKP crystal is shown in Fig. 3.

3.2. Diffraction data analysis

X-ray diffraction data were collected to a resolution of 2.6 Å. The data set revealed that the crystals belonged to the tetragonal space group *P*4₁2₁2. Assuming a molecular weight of 118.8 kDa and one molecule in the asymmetric unit, the crystal volume per unit mass (V_M) and the solvent content of the crystal were calculated to be 3.22 Å³ Da^{−1} and 61.8%, respectively. The Matthews coefficient is within the range commonly observed for proteins (Kantardjieff & Rupp, 2003). Table 1 summarizes the data-collection and processing statistics.

Of interest in the data-collection statistics in Table 1 is the observation that there is a large fall-off in redundancy as a function of resolution (6.1 overall *versus* 3.5 in the highest shell). This may indicate a high degree of anisotropy. This is indeed the case, as shown by the calculation of an anisotropic ΔB statistic. The value for the data set in Table 1 is 34.4 Å², which indicates strong anisotropy. Hence, the structure solution will most likely be improved *via* ellipsoidal truncation and anisotropic scaling. However, there was an improvement in the anisotropic ΔB statistic compared with the ‘best’ data set collected prior to application of the reduced-gravity technique (see §3.1 above). This data set, although also having a high degree of mosaicity and twinning, had an anisotropic ΔB statistic of 52.2 Å², indicating severe anisotropy.

Figure 2

Crystallization-space map. Relative crystal diffraction is plotted as a function of polyethylene glycol concentration and the device rotation angle (θ). The crystals with the highest diffraction limit (2.6 Å; yellow regions) were arbitrarily set to 100%. A relative diffraction value of zero (blue regions) either indicates that no crystals were formed or that any crystals that were encountered did not diffract.

Figure 3

Crystals of *V. vinifera* fucose kinase:GDP-fucose pyrophosphorylase. The scale bar is 2 mm in length.

The enzyme structure will be solved by the MIR methodology as the *Vitis* FKP shares a low overall sequence identity with other nucleotide-sugar metabolizing enzymes. The N-terminal domain shares an average of 14% sequence identity with other pyrophosphatases (see, for example, Pastuszak *et al.*, 1998; Quirk & Seley, 2005) and the C-terminal fucokinase domain is only 12% identical to glucokinase-like enzymes of known three-dimensional structure (Kotake *et al.*, 2008). It is possible to attempt a molecular-replacement solution using both of the domains as search models; however, this route may not be fruitful as these two homology domains only account for 70% of the overall FKP protein (and have very low identity). As the protein contains 22 Met residues, it may be possible to utilize selenomethionine for phasing.

Plant fucose salvage is unlike the pathway in mammals and most bacteria. The conversion of free fucose to the activated GDP-fucose form is catalyzed by a single polypeptide instead of two distinctly separate enzymes. Within a single enzyme, *Vitis* FKP has two distinct active sites that are differentially regulated by substrate binding and are thermodynamically/kinetically different (Quirk *et al.*, manuscript in preparation). The enzyme shares low sequence identity to its enzymatic cousins and is inherently unstable. *Vitis* FKP will be the first enzyme of this type to be structurally characterized, which will further the understanding of the evolution of fucose salvage in diverse species and provide structural information on how fucose 1-phosphate is moved between active sites.

A historical frustration for the structural enzymology community has been the inability of some proteins to form diffraction-quality crystals. Many new methods to improve crystallization success have been developed over the years, including the development of vast combinatorial screens, microbatch robotic methods and crystallization under oil. Another technique that has shown promise is crystallization under reduced-gravity conditions. Since the early 1980s several hundred protein-crystallization experiments have been conducted aboard the Space Shuttle, the Russian Space Station and the International Space Station. Many of these experiments have demonstrated significant improvement in crystals as a result of a reduced-gravity environment (for reviews, see Vergara *et al.*, 2003; Judge *et al.*, 2005). It is increasingly difficult to find opportunities for space-based crystallization trials; it is therefore important to develop alternative systems to simulate reduced gravity that can be used in the average protein-biophysics laboratory. One such device is the high-aspect ratio vessel bioreactor. This device has been used for over a decade to study the effect of reduced gravity on cells (Lynch *et al.*,

2004). As the vessel is rotated about a horizontal axis (normal to the gravitational field), the liquid system reaches a steady-state velocity where the gravitational force is zeroed out by equal and opposing hydrodynamic forces (shear, centrifugal and Coriolis forces). This results in a significantly reduced effective gravity. The bioreactor device has the added feature that the degree of relative gravity can be adjusted by simply changing the rotation angle at a constant rotation speed. The theory of the device is well understood for cell growth (Tsao *et al.*, 1994; Nickerson *et al.*, 2003), but not for crystallization. Still, the laboratory-based rotary-culture system, and its effect of producing low-shear simulated reduced gravity, can be a useful tool to crystallize recalcitrant proteins.

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