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Cloning, expression, purification, crystallization and preliminary X-ray analysis of a fructokinase from *Vibrio cholerae* O395

Fructokinase (FK), one of the crucial enzymes for sugar metabolism in bacterial systems, catalyses the unidirectional phosphorylation reaction from fructose to fructose 6-phosphate, thereby allowing parallel entry of fructose into glycolysis beside glucose. The *cscK* gene from *Vibrio cholerae* O395 coding for the enzyme FK has been cloned, overexpressed in *Escherichia coli* BL21 (DE3) and purified using Ni-NTA affinity chromatography. Crystals of *V. cholerae* FK (*Vc*-FK) and its cocrystal with fructose, adenosine diphosphate (ADP) and Mg^{2+} were grown in the presence of polyethylene glycol 6000 and diffracted to 2.45 and 1.75 Å resolution, respectively. Analysis of the diffraction data showed that both crystal forms have symmetry consistent with space group $P2_12_12$, but with different unit-cell parameters. Assuming the presence of two molecules in the asymmetric unit, the Matthews coefficient for the apo *Vc*-FK crystals was estimated to be $2.4 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to a solvent content of 48%. The corresponding values for the ADP- and sugar-bound *Vc*-FK crystals were $2.1 \text{ Å}^3 \text{ Da}^{-1}$ and 40%, respectively, assuming the presence of one molecule in the asymmetric unit.

1. Introduction

Kinases that catalyse the phosphorylation of carbohydrates are termed sugar kinases and can be divided into at least three distinct non-homologous families: the hexokinase (HK), ribokinase (RK) and galactokinase families (Bork & Sander, 1993). These enzymes have evolved in different metabolic pathways from bacteria to eukaryotic systems. Fructose, a monosaccharide that is widely available in nature, cannot directly participate in metabolism unless it is phosphorylated. Free fructose can be phosphorylated by either HK or fructokinase (FK), depending upon the substrate specificity and affinity of the enzyme. HK phosphorylates both glucose and fructose, with a higher affinity for glucose, whereas FK is specific for fructose. The specificity of FK has evolved independently in both the HK-like and the RK-like superfamilies.

The members of the RK-like superfamily (EC 2.7.1.–) are phosphotransferases that have an alcohol group as an acceptor and can broadly be divided into six families: the adenosine diphosphate-specific phosphofructokinase/glucokinase (ADP_PFK_GK), aldolase 2, carbohydrate kinase, hydroxyethyl-thiazole kinase, PfkB and phosphomethylpyrimidine kinase families. A Pfam database search (Bateman *et al.*, 2000) indicates that FK belongs to the PfkB family of carbohydrate kinases. Phosphorylation of D-fructose to D-fructose 6-phosphate catalysed by FK using ATP is an irreversible step which is central to many regulatory processes in plants and bacteria, and allows direct entry of D-fructose 6-phosphate into glycolysis, starch biosynthesis and the oxidative pentose phosphate pathway. This key step is also required for the mobilization of all hexoses taken up by the cell. Huber & Akazawa (1985) reported an FK that utilizes UTP, while most other reported FKs show a relative specificity for ATP. In addition, FK shows an absolute requirement for magnesium (or manganese) and is strongly activated by potassium ions (Hers, 1953). Mg^{2+} has been shown to act by forming a complex with ATP, which appears to be the true substrate of the enzyme (Hers, 1953). When present in excess over fructose, it inhibits the kinase activity (German *et al.*, 2002).

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The characteristic structural feature of the enzymes belonging to this superfamily is a central nine-stranded sheet flanked by eight structurally conserved helices: five on one side and three on the other (Zhang *et al.*, 2004). The active site is located in a shallow groove along one edge of the sheet, with the phosphate-acceptor hydroxyl group and the γ -phosphate of ATP close together in the middle of the groove, while the substrate- and ATP-binding sites are at the ends (Zhang *et al.*, 2004; Chua *et al.*, 2010; Nocek *et al.*, 2011). The active-site groove is quite well conserved among a wide variety of bacterial and plant FKs (Chua *et al.*, 2010).

The *cscK* gene (UniProtKB accession code A5EZZ9) of *Vibrio cholerae* codes for an FK (*Vc*-FK) of 323 amino acids with a molecular weight of 35 kDa. To investigate the detailed molecular basis of the enzymatic behaviour of *Vc*-FK, we have crystallized *Vc*-FK and its cocrystal with fructose, adenosine diphosphate (ADP) and Mg^{2+} in the presence of polyethylene glycol 6000 by the hanging-drop vapour-diffusion method. Here, we report the cloning, expression, purification, crystallization and X-ray diffraction of *Vc*-FK.

2. Materials and methods

2.1. Cloning, expression and purification of fructokinase from *V. cholerae* O395

The *cscK* gene (accession No. A5EZZ9) encoding fructokinase (323 amino acids) was amplified by PCR with the chromosomal DNA of *V. cholerae* strain O395 using specific primers (forward, 5'-**GGA-ATTCATATGAAGGCTTTGGTTAGACTCAGC**-3'; reverse, 5'-**GGGGATCCTTATAATTCTAAAAAGGCATACAGCGCCG**-3'). The primers were designed with adaptors (bold) for the restriction enzymes *Nde*I and *Bam*HI (italics). The purified PCR products were ligated to the kanamycin antibiotic-resistant expression vector pET-28a(+) (Novagen), which adds six consecutive histidines to the N-terminus of the desired protein followed by a thrombin cleavage site. The clones were selected appropriately using *Escherichia coli* XL1-Blue cells with kanamycin resistance. The recombinant protein was overexpressed in *E. coli* BL21 (DE3) cells in the presence of kanamycin. For overexpression, a single colony was picked, transferred into 100 ml LB broth and grown overnight. 1 l LB broth was inoculated with 10 ml overnight culture and the culture was grown at 310 K until the OD₆₀₀ reached 0.6; the cells were then transferred to 293 K. The cells were induced with 0.2 mM IPTG and incubated for

16 h at 293 K following growth to mid-log phase. After induction, the cells were harvested at 4500g for 20 min and the pellet was resuspended in 25 ml ice-cold lysis buffer [50 mM Tris-HCl pH 8.0, 300 mM NaCl, 2 mM phenylmethanesulfonyl fluoride (PMSF), 5 mM β -mercaptoethanol]. The cell pellets were lysed by the addition of lysozyme followed by sonication on ice. The cell lysate was then centrifuged at 12 000g for 45 min. The supernatant was applied onto a nickel-nitrilotriacetic acid affinity-chromatography column (Qiagen) which had previously been equilibrated with buffer A (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM β -mercaptoethanol). The protein was eluted with a linear gradient of 5–150 mM imidazole in buffer A. The homogeneity of the purified *Vc*-FK protein was checked by 12% SDS-PAGE (Fig. 1).

2.2. Crystallization

For crystallization, the *Vc*-FK protein (in 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM β -mercaptoethanol) was concentrated to 12 mg ml⁻¹ using an Amicon Ultra centrifugation unit (10 kDa molecular-weight cutoff; Millipore). Crystallization trials were performed by the hanging-drop vapour-diffusion method at 293 and

Figure 1
The homogeneity of the purified *Vc*-FK protein was checked by 12% SDS-PAGE. Lane 1, *Vc*-FK; lane 2, molecular-mass markers (labelled in kDa).

(a)

(b)

Figure 2

(a) Crystals of *Vc*-FK in the native form grown in the presence of 5% PEG 6000 pH 6 at 293 K. The maximum dimensions of the crystals were $0.4 \times 0.2 \times 0.3$ mm. (b) Cocrystals of *Vc*-FK with fructose, ADP and $MgCl_2$ of $0.4 \times 0.3 \times 0.2$ mm in size appeared at 293 K when 5% PEG 6000 pH 6.0 was used as a precipitant.

Table 1

Data-collection and data-processing parameters for *Vc*-FK and *Vc*-FK-ADP-fructose crystals.

Values in parentheses are for the outermost resolution shell.

	<i>Vc</i> -FK	<i>Vc</i> -FK-ADP-fructose
Space group	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)	$a = 107.12$, $b = 99.86$, $c = 61.55$	$a = 107.01$, $b = 64.29$, $c = 41.76$
Resolution (Å)	2.45 (2.5–2.45)	1.75 (1.78–1.75)
Oscillation range (°)	1	1
Molecules per asymmetric unit	2	1
Matthews coefficient V_M (Å ³ Da ^{−1})	2.4	2.1
Solvent content (%)	48	40
No. of unique reflections	23435	30437
Multiplicity	2.73 (2.63)	3.15 (3.08)
Mosaicity (°)	0.38	0.40
Completeness (%)	95.1 (90.0)	99.0 (97.8)
R_{merge} † (%)	4.59 (21.40)	5.46 (26.38)
Average $I/\sigma(I)$	7.0 (1.8)	6.7 (1.6)

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity of reflection hkl calculated after scaling.

277 K using Grid Screen PEG 6000 (Hampton Research). Varying concentrations [20–40% (w/v)] of PEG 6000 in a buffer consisting of 100 mM Tris-HCl pH 8.0, 200 mM NaCl were used as the reservoir. A Hampton Research 24-well crystallization tray was used with 600 µl reservoir solution and 4 µl drop size (protein and precipitant in a 1:1 volume ratio). Crystals were obtained using conditions A3 (5% PEG 6000, 0.1 M MES pH 6.0) and A4 (5% PEG 6000, 0.1 M HEPES pH 7.0) of Grid Screen PEG 6000 as the precipitant with 35% (w/v) PEG 6000 in the reservoir after 15 d at 293 K (Fig. 2a).

For cocrystallization of *Vc*-FK with ADP and fructose, *Vc*-FK was mixed with 5 mM fructose, 5 mM ADP and 5 mM MgCl₂ and the mixture was incubated at room temperature for 1 h. Cocrystals of *Vc*-FK were obtained after 3 d at 293 K using conditions A3 (5% PEG 6000, 0.1 M MES pH 6.0) and A4 (5% PEG 6000, 0.1 M HEPES pH 7.0) of Grid Screen PEG 6000 as precipitant with 30% (w/v) PEG 6000 in the reservoir (Fig. 2b).

2.3. Data collection and preliminary X-ray diffraction

The fructokinase crystals were taken out of the mother liquor using a Hampton Research Cryo-Loop and flash-cooled to 100 K in a nitrogen stream. A diffraction data set was collected using an in-house MAR Research image-plate detector of diameter 345 mm and Cu $K\alpha$ radiation (1.54 Å) generated by a Bruker-Nonius FR591 rotating-anode generator equipped with Osmic MaxFlux confocal optics and operated at 50 kV and 50 mA. Data were processed and scaled using *iMOSFLM* (Battye *et al.*, 2011). Diffraction data for the apo *Vc*-FK crystals were collected to a resolution of 2.45 Å, whereas diffraction data for the cocrystals of *Vc*-FK bound with ADP and fructose were collected to a resolution of 1.75 Å. For the apo *Vc*-FK crystals, a 95.1% complete data set was obtained from 70 frames with 1° crystal oscillation. For the *Vc*-FK cocrystals, a 99.0% complete data set was obtained from 83 frames with 1° crystal oscillation. Data-collection statistics are given in Table 1.

3. Results and discussion

Apo crystals of *Vc*-FK diffracted to a resolution of 2.45 Å (Fig. 3a) and had symmetry consistent with the orthorhombic space group $P2_12_12$ and unit-cell parameters $a = 107.12$, $b = 99.86$, $c = 61.55$ Å. Packing considerations, based on the molecular weight of 35 kDa, indicated the presence of two molecules in the asymmetric unit,

corresponding to a Matthews coefficient V_M (Matthews, 1968) of 2.4 Å³ Da^{−1} and a solvent content of 48% (Table 1).

The cocrystal of *Vc*-FK bound with ADP and fructose produced excellent quality diffraction data to a 1.75 Å resolution limit (Fig. 3b) with symmetry consistent with the orthorhombic space group $P2_12_12$ and unit-cell parameters $a = 107.01$, $b = 64.29$, $c = 41.76$ Å. Packing considerations, based on the molecular weight of 35 kDa, indicated the presence of one molecule in the asymmetric unit, which corre-

Figure 3

(a) X-ray diffraction image of an apo *Vc*-FK crystal; the region in the black square is enlarged to show the diffraction at the edge, which corresponds to a resolution of 2.45 Å (black arrow). (b) X-ray diffraction image of a *Vc*-FK-ADP-fructose cocrystal; the region in the black square is enlarged to show the diffraction at the edge, which corresponds to a resolution of 1.75 Å (black arrow).

sponds to a Matthews coefficient V_M (Matthews, 1968) of $2.1 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 40%.

A *BLAST* (Altschul *et al.*, 1990) search for a homologous structure showed that *Vc*-FK exhibits the highest identity (42%) to aminoimidazole riboside (AIR) kinase from *Salmonella enterica* (PDB entry 1tyy; Zhang *et al.*, 2004), a member of the ribokinase superfamily. Therefore, the coordinates of AIR kinase were chosen as the initial model for structure solution of *Vc*-FK by the molecular-replacement (MR) method. The results of a sequence-similarity search between *Vc*-FK and AIR kinase indicated that the ATP-binding site of these enzymes is highly conserved but their substrate-binding site is semi-conserved. Based upon sequence alignment, the nonconserved residues were truncated to alanine from the coordinates of 1tyy (chain A) and the resulting model was used for MR in *CCP4* (Winn *et al.*, 2011). For the diffraction data from the *Vc*-FK crystal, two molecules of the modified model in the asymmetric unit produced a correlation coefficient of 56% with an *R* factor of 42.8% for data between 30.0 and 4.0 $\text{\AA}$ resolution using the program *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* package. The result obtained from the MR solution was further improved in *CNS* (v1.2; Brünger *et al.*, 1998) by a few cycles of rigid-body refinement and positional refinement using data between 30 and 2.8 $\text{\AA}$ resolution. The electron-density map calculated using this model showed continuous and unambiguous density for most of the polypeptide chain.

For the *Vc*-FK cocrystal data, the refined native *Vc*-FK structure was used as the search model and one molecule of the modified model in the asymmetric unit produced a correlation coefficient of 62% with an *R* factor of 40% for data between 30.0 and 4.0 $\text{\AA}$ resolution using the *MOLREP* program. Refinement of this structure

in *CNS* using the aforementioned procedure produced an electron-density map showing unambiguous density for fructose, ADP and a metal ion. Further refinement and detailed analysis are under way.

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