Crystallization and preliminary crystallographic analysis of human muscle phosphofructokinase, the main regulator of glycolysis

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Whereas the three-dimensional structure and the structural basis of the allosteric regulation of prokaryotic 6-phosphofructokinases (Pfk) have been studied in great detail, knowledge of the molecular basis of the allosteric behaviour of the far more complex mammalian Pfk is still very limited. The human muscle isozyme was expressed heterologously in yeast cells and purified using a five-step purification protocol. Protein crystals suitable for diffraction experiments were obtained by the vapour-diffusion method. The crystals belonged to space group $P6_22$ and diffracted to 6.0 Å resolution. The 3.2 Å resolution structure of rabbit muscle Pfk (rmPfk) was placed into the asymmetric unit and optimized by rigid-body and group B -factor refinement. Interestingly, the tetrameric enzyme dissociated into a dimer, similar to the situation observed in the structure of rmPfk.

1. Introduction

The main regulator of glycolysis, 6-phosphofructokinase (EC 2.7.1.11; Pfk), catalyzes the Mg-ATP-dependent formation of fructose 1,6-bisphosphate from fructose 6-phosphate in prokaryotic and eukaryotic cells. Pfk is regulated and fine-tuned by a wide range of effectors (Sols, 1981; Tejwani, 1978; Schöneberg *et al.*, 2013). Physiologically most relevant are AMP and fructose 2,6-bisphosphate (F-2,6-BP; Reinhart & Lardy, 1980; Zancan *et al.*, 2007) as activators as well as ATP and citrate as inhibitors (Zancan *et al.*, 2008; Usenik & Legiša, 2010; Schöneberg *et al.*, 2013). Recently, it was demonstrated that acyl-CoA modulates Pfk activity through both covalent and non-covalent interactions (Jenkins *et al.*, 2011). Furthermore, binding of Pfk to intracellular proteins such as calmodulin (Marinho-Carvalho *et al.*, 2006), actin (Liou & Anderson, 1980), microtubules (Vértessy *et al.*, 1997) and caveolin 1 (Vallejo & Hardin, 2005) modulates the activity, oligomeric equilibrium and localization of Pfk within the cell. Moreover, the equilibrium between monomer, dimers, tetramers and higher oligomers affects Pfk activity (Trujillo & Deal, 1977; Luther *et al.*, 1983). Mutations in the muscle Pfk gene can lead to type VII glycogen storage disease (Tarui disease; Toscano & Musumeci, 2007). More recently, post-translational modifications in terms of truncated C-termini were found in tumorigenic cell lines (Smerc *et al.*, 2011). A further post-translational modification of Pfk, namely *O*-GlcN-acylation, represents a mechanism for the regulation of metabolic pathways in cancer cells, suggesting a possible strategy to combat cancer (Yi *et al.*, 2012).

The enzyme from *Trypanosoma brucei* was the first eukaryotic representative to be structurally characterized. However, it is more similar to the bacterial diphosphate-dependent Pfk enzymes (Martinez-Oyanedel *et al.*, 2007; McNae *et al.*, 2009). Despite early reports of the crystallization of rabbit muscle Pfk (rmPfk; Parmeggiani *et al.*, 1966), the first structures of characteristic eukaryotic ATP-dependent Pfk became available only a few years ago *via* the Pfk structures from the yeasts *Pichia pastoris* (Sträter *et al.*, 2011) in the T-state and *Saccharomyces cerevisiae* in the R-state (Banaszak *et al.*, 2011) as well as that of rmPfk (Banaszak *et al.*, 2011). The crystals of rmPfk contain a homodimer which probably models one half of the physiological tetramer. Structural information on the allosteric switch of bacterial Pfk is based mainly on the two *Bacillus stearothermophilus* Pfk structures in the active state (PDB entry 4pfk) and in the

T-state (PDB entry 6pfk) (Evans *et al.*, 1981; Schirmer & Evans, 1990).

In this study, a preliminary crystallographic analysis of wild-type human muscle Pfk (hmPfk) is presented. This constitutes an important step towards our goal of characterizing the allosteric states, the binding of regulators and the conformational switch of human Pfk isozymes.

2. Materials and methods

2.1. Macromolecule production

Wild-type human muscle Pfk cDNA (UniProtKB P08237-1) was cloned into the plasmid pJH71 (Raben *et al.*, 1995; kindly provided by Professor Dr Jürgen J. Heinisch, University of Osnabrück). hmPfk was expressed in *S. cerevisiae* strain HD114-8D carrying a deletion in both yeast Pfk genes (Raben *et al.*, 1995). The protein was purified as described previously in Brüser *et al.* (2012). Macromolecule-production information is given in Table 1.

2.2. Crystallization

A buffer exchange was performed prior to crystallization using PD10 desalting columns (GE Healthcare) and sample buffer (Table 2). The eluted protein was concentrated to 6–11 mg ml⁻¹ by ultrafiltration (Vivaspin 6, Sartorius). Initial screening for crystallization conditions was performed with commercially available crystallization screens (from Hampton Research and Jena Bioscience) at 292 K using the sitting-drop vapour-diffusion technique in three-drop 96-well plates (Greiner). The volume of the reservoir solution was 90 µl. Equal volumes (0.2 µl) of reservoir and protein solution were dispensed using a MicroSys eight-channel dispensing system (Zinsser Analytic). Optimizations of the initial hits were performed by changing the pH value and the precipitant and protein concentrations, as well as the temperature, using the hanging-drop vapour-diffusion method. The same volumes of protein and reservoir solution (1 µl) were mixed and equilibrated against 500 µl reservoir solution in 24-well trays (Nelipak). Crystals of hexagonal shape (Fig. 1*a*) grew in 0.6 M LiNO₃. Crystals appeared within a few days and usually reached their final size within 5 days. For further optimization of crystal quality, microbatch-under-oil crystallization techniques, crystallization in agarose gel, additive screens (including Silver Bullets and Silver Bullets Bio, Hampton Research), *in situ* proteolysis, different seeding techniques and cross-linking were tested. The screening of cryoprotectants [glycerol, MPD, ethylene glycol (PEG)

Table 1
Macromolecule-production information.

Source organism	<i>Homo sapiens</i>
DNA source	hmPfk cDNA
Expression vector	pJH71Pfk (Raben <i>et al.</i> , 1995)
Expression host	<i>S. cerevisiae</i> strain HD114-8D
Complete amino-acid sequence of the construct produced	UniProtKB P08237-1: MTEEHAAKTLGIGKAIIVLTSGGDAQGMNAAVRAVVRV- GIFTGARVFFVHEGYQGLVDGGDHKEATWESVSMMLQLG- GTVIGSARCKDFREREGRLRAAYNLVKGITNLVIGGDDG- SLTGADTFRSEWSDLLSDLKAGKITDEEATKSSYLNIIVG- LVGSIDNDFCGTDMTIGTDSALHRIMEIVDAITTTAQSHQ- RTFVLEVMRHRCGYLALVTSLSGADWVFIECPDDDDWE- EHLCRRLSETRTRGSRNLIIIVAEGAIDKNGKPIITSEDIK- NLVVKRLGYDTRVTVLGHVQRGGTPSAFDRILGSRMGVEA- VMALLEGTDPDPACVVSLSGNQAVRLPLMECVQVTKDVT- AMDEKFKDEALKLRGRSFMNWEVYKLLAHVRPPVSKSGS- HTVAVMNVGAPAAAGMNAARSTVRIGLIQGNRLVVDHGF- EGLAKQIEEAGWSYVGGWTGGGSKLGTKRTLPPKSFQ- ISANITKFNIGQLVIIGGFEAYTGGLELMGRKQFDELICI- PFVVPATVSNVNVGDSFVSGADTALNTICTTCDRIKQSA- AGTKRRVFIETMGYCYLATMAGLAAGADAAYIFEEPF- TIRDLQANVEHLVQKMKTTVKRGLVLRNEKCNENYTTDFI- FNLYSEEGKIFDTSRKNVLGHMGGGSGPTPDRNFATKMG- AKAMNWSGKIKESYRNGRIFANTPDSCGVLGMRKRALVF- QPVAELKDQTDFEHRIPKEQWLLKLRPIILKILAKYEIDL- TSDHAHLEHITRKRSGEAAV

Table 2
Crystallization.

Method	Vapour diffusion
Plate type, screening	Three-drop 96-well plates, Greiner
Plate type, optimization	24-well PVC trays, Nelipak
Temperature (K)	292
Protein concentration (mg ml ⁻¹)	6
Buffer composition of protein solution	10 mM imidazole pH 7.0, 10% glycerol, 5 mM β-mercaptoethanol, 5 mM ATP, 100 µM fructose 1,6-bisphosphate
Composition of reservoir solution	0.6 M LiNO ₃ , 0.05 M NaF, 0.1 M sodium acetate pH 5.4
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (µl)	500

200, PEG 400, glucose, fructose, sucrose and fructose 6-phosphate] revealed PEG 200 to be the most suitable. Before data collection, crystals were cryoprotected by the stepwise addition of 10, 15, 20 and 25% PEG 200 to the reservoir buffer and were cooled in liquid nitrogen.

2.3. Data collection and processing

Data collection was performed at HZB (BESSY II, beamline 14.1). Data processing was performed with *XDS* (Kabsch, 2010). *POINT-*

Figure 1

Crystal (*a*) and diffraction image (*b*) of human muscle Pfk. (*a*) A polarization filter was used to enhance the visibility of the hexagonal hmPfk crystal. This results in the artificial purple colour of the crystals. The arrow in (*b*) marks a spot at 6.0 Å resolution.

LESS was used to determine the Laue group (Evans, 2006), and the reflections were scaled and merged with *AIMLESS* (Evans, 2006). Cell-content analysis with *MATTHEWS_COEF* indicated the presence of one monomer per asymmetric unit with 79.5% solvent content (Matthews, 1968; Kantardjieff & Rupp, 2003).

2.4. Structure solution and refinement

The structure of rmPfk (PDB entry 3o8n; Banaszak *et al.*, 2011) modified by *CHAINSAW* (Stein, 2008) served as a search model for molecular replacement with *Phaser* (McCoy *et al.*, 2007). The MR solution with an LLG score of 616 and a TFZ score of 25.5 indicates a definite solution. After rigid-body refinement of the rmPfk structure with *REFMAC5* (Murshudov *et al.*, 2011) the *R* and *R*_{free} factors

improved to 40.2 and 45.0%, respectively. An hmPfk model was generated by exchange of the differing residues and modelling in the most probable conformer that did not generate close contacts. Furthermore, the N- and C-termini were truncated (residues 8–755) based on the visible electron density. For further refinement, residues 8–394 and 395–755 were refined as independent rigid bodies. These regions correspond to the homologous N-terminal and C-terminal halves of eukaryotic Pfk that are related to a bacterial Pfk monomer. This resulted in *R* and *R*_{free} values of 38.7 and 41.3%, respectively. Finally, a group *B*-factor refinement (one *B* value per residue) was performed with *phenix.refine* (Adams *et al.*, 2010). *Phenix.autobuild* was used for the calculation of an iterative composite OMIT map (Terwilliger, Grosse-Kunstleve, Afonine, Moriarty, Adams *et al.*, 2008; Terwilliger, Grosse-Kunstleve, Afonine, Moriarty, Zwart *et al.*, 2008).

Figure 2

Crystal packing and electron-density maps of human muscle Pfk. The crystal packing (transparent grey cartoon) is viewed along the 6₂ symmetry axis (a) and the twofold symmetry axis (b). One hmPfk monomer is shown in blue and the second subunit of the dimer is coloured orange. The diameter of the solvent channel along the 6₂ axis is approximately 84 Å. (c) 2F_o - F_c (blue) and F_o - F_c (green/red) maps after rigid-body and group *B*-factor refinement. (d) Iterative composite OMIT map (blue).

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BESSY MX 14.1
Wavelength (Å)	0.91841
Temperature (K)	100
Detector	Rayonics MX-225 CCD
Crystal-to-detector distance (mm)	550.0
Rotation range per image (°)	1
Total rotation range (°)	100
Exposure time per image (s)	20
Space group	$P6_222$
Unit-cell parameters (Å)	$a = 229.7$, $c = 133.0$
Mosaicity (°)	0.17
Solvent content (%)	79.5
Resolution range (Å)	46.95–6.00 (6.71–6.00)
Total No. of reflections	64128
No. of unique reflections	5512 (1524)
Completeness (%)	99.8 (100)
Multiplicity	11.6 (12.1)
$\langle I/\sigma(I) \rangle$	12.5 (4.7)
$R_{\text{r.i.m.}}$ (%)	11.4 (46.2)
$CC_{1/2}$	99.1 (97.2)
Overall B factor from Wilson plot (Å ²)	268.6

Coot was used to mutate the initial model in order to generate the hmPfk model (Emsley & Cowtan, 2004). Molecular diagrams were drawn using PyMOL (Schrödinger).

3. Results and discussion

The diffraction limit of the first crystals of hmPfk was 12 Å and could be optimized to 6 Å. However, the crystals tended to break easily during the cryocooling step. Many crystals had to be tested at synchrotron beamlines, since the crystal quality after cryocooling generally differed considerably. The best data set obtained from these crystals extended to ~6.0 Å resolution (Table 3; Fig. 1b). All trials to improve the diffraction properties of the crystals by seeding, *in situ* proteolysis or the use of additives were not successful. Diffraction tests at room temperature without the cryocooling step also resulted in weak diffraction to a low resolution of >8 Å. Determination of an optimal cryoprotectant and cooling the crystals did not improve the resolution beyond 6 Å.

To analyze the packing and quaternary structure of hmPfk in the crystals, we used the structure of one monomer of rabbit skeletal

Table 4

Structure refinement.

Resolution range (Å)	46.9–6.0
Completeness (%)	99.7
No. of reflections, working set	4990
No. of reflections, test set	529
Final R_{cryst} (%)	23.9
Final R_{free} (%)	27.4
Total No. of non-H atoms	5715
Average B factor (Å ²)	194.5
Ramachandran plot (<i>MolProbity</i>)	
Favoured regions (%)	83.0
Additionally allowed (%)	13.2
Outliers (%)	3.8

muscle Pfk (PDB entry 3o8n), which has a sequence identity of 96%, as the search model in molecular replacement. One monomer was found in the asymmetric unit, resulting in a loose packing with large solvent channels along the 6_2 screw axes (Fig. 2a). The high solvent content of 79.5% probably contributes to the instability and weak diffraction properties of the nicely shaped hmPfk crystals (Fig. 1a).

The N- and C-terminal halves of hmPfk were refined as independent rigid bodies based on the rmPfk model obtained at 3.2 Å resolution, followed by a group B -factor refinement, resulting in R and R_{free} values of 23.9 and 27.4%, respectively. Refinement statistics are given in Table 4. Because of the limited resolution of the crystals, further refinement was not carried out. Fig. 2 shows the normal and iterative composite OMIT electron-density maps, which confirm the correctness of the MR solution. As in the structure of rmPfk, the physiological tetramer (Tsai & Kemp, 1973; Arechaga *et al.*, 2010) of hmPfk is dissociated into a dimer (Fig. 3). Although rmPfk (residues 1–762) was crystallized in a different hexagonal crystal form (space group $P6_122$, unit-cell parameters $a = 163.3$, $c = 356.9$ Å), the dimers are very similar (Fig. 3b). Superposition with the structures of the yeast Pfk from *Pichia pastoris* (Sträter *et al.*, 2011; PDB entry 3opy; Fig. 3a) and *Saccharomyces cerevisiae* Pfk (Banaszak *et al.*, 2011; PDB entry 3o8o) reveals that these dimer structures are part of the yeast Pfk tetramers and thus most likely model at least part of the physiological tetramer of mammalian Pfk. Interestingly, the dimers with the smaller protein–protein interface across the putative tetramerization interface are maintained, whereas the closer interacting dimers corresponding to the bacterial homotetramers are dissociated (Fig. 3a). This dissociation could be driven by the protonation of

Figure 3

Comparison of hmPfk, rmPfk and yeast Pfk. (a) Superposition of the Pfk core domains (N-terminal and C-terminal halves) of one $\alpha_2\beta_2$ heterotetramer of the PpPfk heterododecamer (chains A, B, C and D in orange, red, brown and yellow, respectively; PDB entry 3opy) with the hmPfk dimer (blue). The N-termini (glyoxalase I-like domains, residues 5–189) of PpPfk are omitted for clarity. (b) Superposition of the rmPfk dimer (orange; PDB entry 3o8n) onto the hmPfk dimer (blue).

His199 present at the tetramerization interface, which in its unprotonated form presumably interacts with the backbone NH group of the conserved residue Gly24 or Gly25. Therefore, we assume that the dissociation of hmPfk into dimers occurs owing to the low pH of the crystallization buffer, as previously suggested based on the rmPfk structure (Banaszak *et al.*, 2011). In summary, hmPfk and rmPfk have been crystallized as a dimer representing half of the physiologically active homotetramer. This dimer has now been observed in two independent crystal forms in different packing environments (not shown) and for two different (albeit highly identical) enzymes, indicating that this dimer structure may be the inactive dimer of mammalian Pfk discussed in previous studies to be involved in the regulation of Pfk activity (Mayr, 1984; Buschmeier *et al.*, 1987; Marinho-Carvalho *et al.*, 2006, 2009). These dimer structures may also help in the design of constructs for the crystallization of physiologically active homotetrameric mammalian Pfk.

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