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Expression, purification, crystallization and preliminary crystallographic analysis of *Trypanosoma brucei* phosphofructokinase

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Phosphofructokinase from *Trypanosoma brucei* (TbPFK) was purified from a recombinant expression system in *Escherichia coli* by metal-affinity chromatography *via* its N-terminal His tag. The yield was 15–20 mg of pure enzyme per litre of culture. M_r was shown to be 55 585 by mass spectrometry. Crystals suitable for X-ray diffraction analysis were obtained by the hanging-drop method of vapour diffusion with sodium formate as the precipitating agent. Monoclinic crystals of the apoenzyme grew within one week, as did orthorhombic crystals of PFK in the presence of enzymic reaction products or an active-site inhibitor. Initial attempts to solve the structure by molecular replacement with bacterial PFK structures as search models proved unrewarding, but a multiple-copy search with a polyalanine model was successful. In addition, heavy-atom soaking with platinum and mercury has yielded derivatives suitable for X-ray diffraction. A combination of the phase information from the molecular-replacement solution and the heavy-atom derivatives should allow structure solution of TbPFK. The availability of this first eukaryotic PFK structure will be of particular significance for structure-based drug design and will also provide important additional structural evidence for the allosteric control of PFK activity.

1. Introduction

Phosphofructokinase (PFK) is a key enzyme in glycolysis and catalyses the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. PFKs can be classified into ATP-dependent (EC 2.7.1.11) and PP_i-dependent (EC 2.7.1.90) enzymes; the former use ATP as a phospho-donor in a reaction that is essentially irreversible under physiological conditions, whereas the latter use PP_i in a reversible reaction that can be near equilibrium *in vivo* (Fothergill-Gilmore & Michels, 1993). PFKs from *Trypanosoma brucei* (TbPFK) and other Kinetoplastida have been shown to be ATP-dependent, despite possessing amino-acid sequences that more closely resemble the PP_i-dependent class of PFKs (Michels *et al.*, 1997). Moreover, PFKs from Kinetoplastida differ from the ATP-dependent PFKs of their mammalian hosts in the range of effector molecules to which they respond. In addition, the bloodstream form of *T. brucei* relies on glycolysis for survival and PFK can thus be considered a good target for the development of drugs to treat kinetoplastid-induced parasitic diseases such as African sleeping sickness, Chagas' disease and leishmaniases (Verlinde *et al.*, 2001).

However, drug-design studies have been hampered as no structure of a eukaryotic PFK at atomic resolution has yet been determined, although several structures of PFK have been solved from the prokaryotic species *Escherichia coli* (Shirakihara & Evans, 1988; Rypniewski & Evans, 1989), *Bacillus stearothermophilus* (Evans *et al.*, 1981; Schirmer & Evans, 1990) and *Borellia burgdorferi* (Moore *et al.*, 2002). Furthermore, PFK, in contrast to most other glycolytic enzymes, has been subject to considerable variation during evolution and bacterial, protozoal and mammalian enzymes show a progression toward larger size and greater complexity (Fothergill-Gilmore & Michels, 1993).

Tetrameric ATP-dependent PFK was one of the first enzymes to which the Monod–Wyman–Changeux model for allosteric control was first applied (Monod *et al.*, 1965; Blangy *et al.*, 1968) and the availability of structural information describing the enzyme from a eukaryotic source will be of particular interest.

Here, we report the overexpression, purification, crystallization and preliminary crystallographic analysis of TbPFK.

2. Methods and results

2.1. Overexpression

The gene coding for TbPFK was cloned as previously described, inserted into a pET15 expression vector containing an N-terminal His tag and transformed into BL-21 cells which had

been transformed previously to express the GroE chaperone proteins (Michels *et al.*, 1997). Single colonies grown on LB agar containing 100 µg ml^{-1} ampicillin, 30 µg ml^{-1} kanamycin and 28 µg ml^{-1} chloramphenicol were chosen and grown in LB medium containing the same concentrations of antibiotics.

Expression of TbPFK was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) when cell cultures containing 2.5 m*M* betaine and 1 *M* sorbitol and incubated at 310 K had attained late log phase (OD_{600} = 0.6). Expression conditions were optimized by examining the effect of IPTG concentration (0.1–2.0 m*M*), temperature (293–310 K) and time (6–48 h) on expression levels, as determined by SDS–PAGE analysis of total cell lysates and PFK activity (Claustre *et al.*, 2002) of soluble protein extracts. The best level of expression was achieved with 1.0 m*M* IPTG for 18 h at 303 K.

2.2. Purification of recombinant TbPFK

Cells collected from 1 l of cell culture by centrifugation at 2500*g* for 20 min at 277 K were frozen overnight and then suspended in 20 ml extraction buffer [buffer *A*, composed of 50 m*M* triethanolamine–HCl (TEA), 300 m*M* NaCl, 200 m*M* KCl, 1.0 m*M* KH_2PO_4, 5 m*M* $MgCl_2.6H_2O$, 0.1 m*M* disodium fructose 6-phosphate (F6P), 0.3 m*M* disodium glucose 6-phosphate and 10%(*v*/*v*) glycerol] in which two tablets of Complete Mini EDTA-free protease inhibitor mix (Roche) had been dissolved. Approximately 20 mg of lysozyme were then added and the mixture was incubated at room temperature for 15 min prior to sonication. After sonication, the cell lysate was centrifuged at 2500*g* for 30 min at 277 K and the precipitate was discarded. To the supernatant fraction were added 40 mg of RNaseA (Sigma) and 0.9 mg of DNaseI (Sigma) and the suspension was incubated at room temperature for 35 min. This was followed by the addition of 200 mg protamine sulfate and further incubation, with occasional stirring, at room temperature for 15 min. The precipitate was removed by centrifugation at 15 000*g* for 30 min at 277 K. Although the resulting soluble protein extract was usually purified immediately, samples stored at 277 K for up to two months did not show an appreciable loss in activity.

Purification was effected *via* the His tag by affinity chromatography at room temperature on a Talon column (Clontech). The column was loaded with sample at a flow rate of 30 ml h^{-1}. The column was then washed with 10 m*M* imidazole in buffer *A* and the protein was eluted from the column with 50 m*M* imidazole in buffer *A*, both at a flow rate of 50 ml h^{-1}. Fractions with PFK activity were pooled and buffer-exchanged using PD-10 size-exclusion columns (Amersham-Pharmacia) and 20 m*M* TEA buffer pH 8.0. The sample was then concentrated with a Vivaspin 20 ml centrifugal concentrator tube (Vivasciences) with a molecular-weight cutoff of 100 kDa. In this way, TbPFK was purified to apparent homogeneity as shown by SDS–PAGE and was purified tenfold over the crude extract, with a recovery of 15–20 mg per litre of culture. The M_r of the purified TbPFK was found to be 55 585 by MALDI-TOF mass spectrometry, compared with the M_r of 55 550 predicted from the sequence of TbPFK plus its N-terminal His tag (GSHHHHHHSS-GLVPRGSHM). No attempt was made to remove the His tag prior to crystallization.

2.3. Crystallization and data collection

The hanging-drop vapour-diffusion method was used to grow crystals of purified TbPFK in 24-well Linbro plates. Crystals of the free enzyme were initially obtained from preliminary screens using PEG 4000 over a wide range of concentration and pH, as well as from the Structure Screen I kit from Molecular Dimensions. These conditions were optimized by lowering the protein concentration, narrowing the range of precipitant and exploring the pH. In addition, better formed crystals were obtained by streak seeding. In optimized conditions, the well solution consisted of 1.7–1.8 *M* sodium formate in 0.1 *M* sodium acetate buffer pH 4.6–4.8. The hanging drop (3 µl) contained 1.5 µl of protein solution at 4–5 mg ml^{-1} in 20 m*M* TEA buffer pH 8.0 plus 1.5 µl of well solution.

TbPFK was also crystallized in the presence of 2,5-anhydro-1-deoxy-1-(*m*-nitro-D-mannitol-6-disodiumphosphate, an active-site inhibitor (Claustre *et al.*, 2002), referred to herein as JP-8. In these optimized conditions, the well solution consisted of 2.0–2.5 *M* sodium formate in 0.1 *M* sodium acetate buffer pH 4.6–5.0 and the protein solution (4–5 mg ml^{-1}) also contained 5 m*M* F6P, 1.25 m*M* JP-8 and 2.5%(*v*/*v*) DMSO. TbPFK was also crystallized in the presence of its enzymic reaction products. In these optimized conditions, the well solution consisted of 2.3–2.7 *M* sodium formate in 0.1 *M* sodium acetate buffer pH 4.6–5.0 and the protein solution (3–4 mg ml^{-1}) also contained 50 m*M* fructose 1,6-bisphosphate (F1,6P_2), 10 m*M* $MgCl_2$ and 10 m*M* Na_2ADP.

Heavy-atom derivatives were prepared by soaking crystals grown in the presence of enzymic reaction products in a solution consisting of well solution with the addition of 50 m*M* F1,6P_2, 10 m*M* $MgCl_2$, 10 m*M* Na_2ADP, 20%(*v*/*v*) glycerol and 10 m*M* $K_2Pt(CN)_4$ or 15 m*M* thiomersal (ethyl mercury thiosalicylate). After 15 min, the crystals were flash-frozen in liquid nitrogen.

Prior to data collection, crystals were briefly immersed in a cryoprotectant solution comprising 25% glycerol in mother liquor and flash-cooled by plunging them into liquid nitrogen. All data were collected at 100 K.

Data were collected using an in-house rotating-anode diffractometer (Nonius FR591; λ = 1.5418 Å) with a MAR345 detector using a φ scan with a step size of 1.00° and at the Daresbury SRS (station 9.6; λ = 0.870 Å) with an ADSC Q4 CCD detector using a φ scan with a step size of 1.00°. Data were indexed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

PFKs are enzymes of considerable interest as models for the conformational changes associated with allosteric control (Monod *et al.*, 1965; Blangy *et al.*, 1968). Previous work has provided descriptions of the crystal structures of bacterial PFKs (Shirakihara & Evans, 1988; Rypniewski & Evans, 1989; Evans *et al.*, 1981; Schirmer & Evans, 1990; Moore *et al.*, 2002), but the large more complex eukaryotic PFKs have so far proved recalcitrant to detailed structural study. Furthermore, a knowledge of the structure of trypanosomatid PFK is critical for the structure-based design of inhibitor drugs of therapeutic value for treatment of African sleeping sickness, Chagas' disease and leishmaniases.

We have crystallized TbPFK in the absence and presence of reaction products or an active-site inhibitor (Claustre *et al.*, 2002). Monoclinic crystals of the native PFK grew within one week at 290 K (Fig. 1*a*). For the JP-8 inhibitor complex, orthorhombic crystals grew within 9 d at 290 K (Fig. 1*b*). For the complex with the enzymic reaction products, orthorhombic crystals grew within one week at 290 K (Fig. 1*c*). Diffraction data and crystal parameters are shown in Table 1.

A self-rotation function search using the monoclinic apoenzyme data with the program *MOLREP* (Vagin & Teplyakov, 1997) showed the presence of two non-

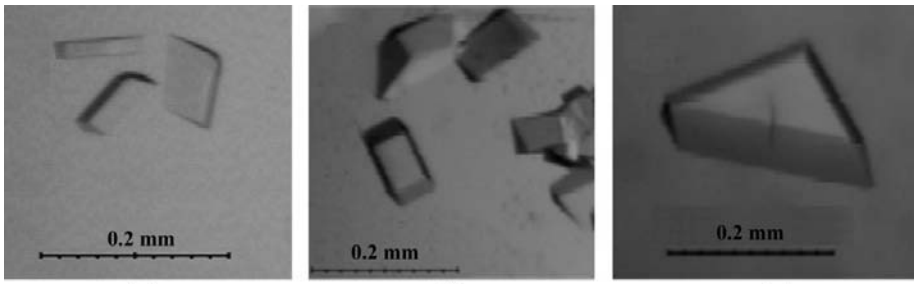

Figure 1
T. brucei PFK crystals. (*a*) Crystal of apoenzyme. (*b*) Enzyme co-crystallized with active-site inhibitor JP-8. (*c*) Enzyme co-crystallized with enzymic reaction products.

Table 1
Data-collection details and statistics.

Values in parentheses are for the highest resolution shell.

	Apoenzyme	TbPFK + JP-8	TbPFK + prods	TbPFK + prods, $K_2Pt(CN)_4$ soak	TbPFK + prods, thiomersal soak
Crystal dimensions (mm)	0.1 × 0.1 × 0.05	0.2 × 0.2 × 0.1	0.2 × 0.2 × 0.1	0.2 × 0.2 × 0.1	0.2 × 0.2 × 0.1
Space group	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters					
a (Å)	88.68	97.32	97.25	96.07	95.62
b (Å)	96.83	117.37	117.53	117.52	117.03
c (Å)	117.26	178.18	178.63	175.88	176.02
$\alpha = \gamma$ (°)	90.0	90.0	90.0	90.0	90.0
β (°)	89.7	90.0	90.0	90.0	90.0
V_M (Å^3 Da^{-1})	2.3	2.3	2.3	2.2	2.2
Solvent content (%)	45.2	45.9	45.6	44.6	44.6
Molecules per asymmetric unit	4	4	4	4	4
Diffraction source	SRS station 9.6	SRS station 9.6	SRS station 9.6	Rotating anode	Rotating anode
Wavelength (Å)	0.870	0.870	0.870	1.5418	1.5418
Resolution (Å)	2.9 (3.0–2.9)	2.9 (3.0–2.9)	3.2 (3.37–3.2)	3.2 (3.37–3.20)	4.0 (4.14–4.00)
No. of observations	467729	327564	360168	359834	364481
No. of independent reflections	44942 (3435)	37824 (3883)	32942 (4816)	25914 (1876)	15640 (1490)
R_{merge}† (%)	10.9 (42.6)	14.9 (65.7)	10.8 (23.1)	15.4 (44.3)	32.7 (61.9)
Completeness (%)	91.2 (76.8)	82.2 (85.5)	97.5 (97.3)	77.3 (75.1)	90.4 (87.8)
$I/\sigma(I)$	11.2 (2.7)	5.1 (1.1)	6.1 (3.1)	7.2 (2.3)	5.2 (2.5)

† $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$.

crystallographic twofold axes oriented perpendicular to the crystallographic twofold axis. This is consistent with TbPFK existing as a tetramer in the crystal. Attempts were made to find a solution through molecular replacement. The structure of a PFK from *E. coli* (PDB code 1pfk; Shirakihara & Evans, 1988), with a sequence identity of 31%, or the structure of PFK from *B. stearothermophillus* (PDB codes 3pfk and 6pfk; Evans *et al.*, 1981; Schirmer & Evans, 1990), with a sequence identity of 33%, were used as search models. *MOLREP* (Vagin & Teplyakov, 1997) and *AMoRe* (Navaza, 1994) were used unsuccessfully to try to find a molecular-replacement solution using polyalanine and polyserine models. There was no significant discrimination between the top ten molecular-replacement solutions for any of the various TbPFK X-ray data sets. However, for the orthorhombic data ('TbPFK + prods' in Table 1) the use of a multiple-copy search as implemented in *MOLREP* (Vagin & Teplyakov, 2000) using a polyalanine model provided a collision-free molecular-replacement solution consistent with the orientation of the three twofold axes determined from the self-rotation function. The *R* factor and correlation discriminators for this solution are 0.608 and 0.414, respectively, compared with values of 0.616 and 0.401 for the next best solution. X-ray data from platinum and mercury heavy-atom derivatives have also been collected (Table 1) and heavy-atom positions have been located. A combination of phase information from the molecular-replacement solution and the heavy-atom derivatives should provide the basis for the structure determination of TbPFK.

We are currently exploring co-crystallization conditions with a variety of other ligands, including substrate analogues and effector-site inhibitors. The availability of the detailed structure of TbPFK may then lead to the solution of a number of different conformational states of the enzyme, thereby allowing advancement of structure-based drug design and providing important structural evidence for the allosteric control of PFK activity.

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