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Crystallization and preliminary X-ray analysis of the 12S form of phosphofructokinase from *Saccharomyces cerevisiae*

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Abstract

The tetrameric 12S form of yeast phosphofructokinase, obtained by limited proteolytic cleavage of the native enzyme, was crystallized under a variety of conditions. The crystals have been characterized in the X-ray beam and are suitable for crystallographic studies.

1. Introduction

Phosphofructokinase 1 (PFK1, E.C. 2.7.1.11) is a key enzyme in glycolysis and catalyzes the reaction,

fructose 6-phosphate + MgATP $\xrightarrow{PFK1}$ fructose 1,6-bisphosphate + MgADP.

The enzyme is more interesting for its role in control of metabolism than for its enzymatic action. Like many important control enzymes PFK1 is allosteric, with a number of effectors, both activators and inhibitors. In bacteria, PFK1 is a homotetramer with a subunit molecular weight 37 kDa. Mammalian PFK1's are also functional as homotetramers but with each subunit twice the size of a bacterial PFK1 subunit. In baker's yeast (*Saccharomyces cerevisiae*), PFK1 is a hetero-octamer ($\alpha_4\beta_4$) of molecular weight approximately 800 kDa (Chaffotte *et al.*, 1984; Kopperschläger, Bär, Nissler & Hofmann, 1977).

As the result of an early gene duplication each eukaryotic subunit consists of two homologous parts, with each repeat similar to one prokaryotic subunit (Poorman, Randolph, Kemp & Heinrikson, 1984). In yeast, subunits α and β are also homologous and are the result of a second gene duplication event (Heinisch, Ritzel, von Borstel, Aguilera, Rodicio & Zimmermann, 1989). One of the repeats of a eukaryotic PFK1 subunit is thought to have lost catalytic function and acquired a regulatory role. As a result, the control of PFK1 in eukaryotes is considerably more complex than in prokaryotes. For example, fructose 2,6-bisphosphate is an effector unique to eukaryotes.

Extensive crystallographic studies have been carried out on bacterial PFK1's (Evans & Hudson, 1979; Evans, Farrants & Hudson, 1981; Shirakihara & Evans, 1988; Rypniewski & Evans, 1989; Schirmer & Evans, 1990) but until now no eukaryotic PFK1 has been crystallized in a form suitable for X-ray analysis (Parmeggiani, Luft, Love & Krebs, 1966; Erdmann, 1994).

On the most general level it is interesting to examine the quaternary structure of eukaryotic PFK1. The gene-duplication events in eukaryotes gave rise to very complex molecules. Yeast PFK1 octamer consists of 16 prokaryotic like units with large terminal regions responsible for subunit association. It is hard to imagine how all the units can pack together in the structure.

On a more detailed level it is interesting to examine how the various effectors interact with PFK1, especially fructose 2,6-bisphosphate which is thought to bind in the site which evolved from one of the fructose 6-phosphate/fructose 1,6-bisphosphate active sites which became redundant after the gene duplication (Heinisch, Boles & Timpel, 1996). A number of relevant mutants of yeast PFK1 are also available.

Apart from its merits for basic science, PFK1 is significant from a medical point of view. Mutant PFK1 is responsible for genetic disorders in some ethnic groups (Tarui, Kono, Kuwajima & Ikura, 1978; Raben *et al.*, 1995).

The 12S form of yeast PFK1 is obtained by limited proteolytic cleavage of the native enzyme by chymotrypsin in the presence of ATP. Under these conditions each native 12S octamer dissociates to form two 12S tetramers of identical size composed of partially degraded α and β subunits (Kopperschläger, Bär & Stellwagen, 1993). This truncated form, $\alpha''_2\beta'_2$, is catalytically active and possesses similar kinetic properties to the native enzyme.

2. Crystallization

12S PFK1 was prepared as described before (Bär, Huse, Kopperschläger, Behlke & Schulz, 1988). Crystals were obtained by vapour diffusion in hanging drops at 277 K. Before crystallization, the protein suspension was dialyzed overnight at 277 K against the corresponding buffer (see below) and concentrated using Centricon 30. The protein concentration was determined at 279 nm using the absorption coefficient 0.881 ml mg^{-1} cm^{-1}.

2.1. *Crystal form I*

20 m*M* HEPES buffer, pH 7.4, containing 1 m*M* EDTA, 0.2 *M* sodium acetate, 0.1 *M* ammonium sulfate, 2 m*M* dithiothreitol (DTT), 0.1 m*M* phenylmethyl sulfonyl fluoride (PMSF), 10 m*M* fructose-6-phosphate was used for dialysis. Then the protein solution (3 µl, 8 mg ml^{-1}) was mixed with an equal volume of the reservoir solution containing 6–10% PEG 4000, 0.2 *M* sodium acetate in 0.1 *M* MES buffer, pH 6.0. Long needles with a diameter 0.2 mm appeared within two weeks (Fig. 1*a*).

Similar crystals grew at pH 6.5 in the presence of 0.4 *M* sodium acetate. Crystallization from PEG 5000 MME gave more isometric crystals but smaller in size.

The influence of the different additives was investigated using 0.1–0.5% 1-*O*-octyl-β-D-glucopyranoside (βOG), 5% ethanol, 5% PEG 400, 2–5% dioxane. None were effective in improving crystal quality. Sometimes the addition of 3–5% 2-methyl-2,4-pentanediol (MPD) resulted in better crystals.

2.2. *Crystal form II*

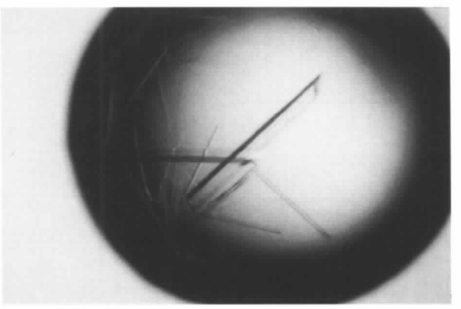
This crystal form was obtained under conditions similar to crystal form I, except that reservoir solution contained ammonium sulfate instead of sodium acetate and PEG 5000 monomethyl ether (MME) instead of PEG 4000. 5 m*M* fructose 2,6-bisphosphate and 2% MPD were used as additives in the protein solution (Fig. 1*b*).

2.3. *Crystal form III*

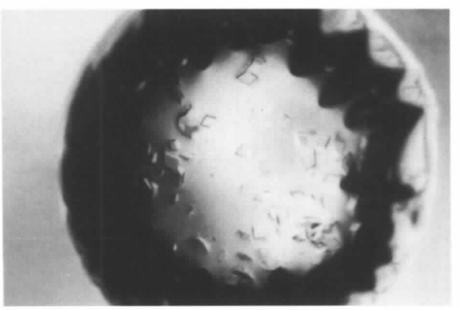
The protein solution was dialysed against 20 m*M* HEPES buffer, pH 7.4, containing 0.1 *M* ammonium sulfate, 0.1 m*M* PMSF, 2 m*M* DTT and 10 m*M* fructose-6-phosphate. For the crystallization 3 µl of the protein solution (8–10 mg ml^{-1}) were mixed with the equal volume of the reservoir solution containing 12–15% PEG 4000, 0.2 *M* ammonium phosphate in 0.1 *M* HEPES buffer pH 7.0. Small isometric crystals appeared after a few weeks. Often the crystals were twinned and intergrown. Sometimes the quality of crystals could be improved if 0.2 *M* ammonium phosphate was used in the dialyzing buffer instead of 0.1 *M* ammonium sulfate, or if PEG 5000 MME or PEG 2000 MME were used as precipitate, or by the addition of 2–5% of dioxane. In the presence of βOG, PEG 400, PEG 200, or 2-propane, small plates or intergrown crystals appeared.

The best results were obtained by macroseeding. Small seeds were washed and then introduced without equilibration into the protein droplets containing 0.1 *M* ammonium phosphate, 10% PEG 4000 in 20 m*M* HEPES buffer pH 7.0. Under these conditions crystals grew to maximum dimensions of 0.3–0.4 mm within a few days at 277 K.

All the crystal forms could also be obtained in the presence of 5 m*M* fructose-2,6-bisphosphate.



(*a*)



(*b*)

Fig. 1. Crystals of types I (*a*) and II (*b*).

3. X-ray diffraction studies

The crystals were tested on the EMBL beamlines (van Silfhout & Hermes, 1995) at the DORIS storage ring at DESY, Hamburg. Diffraction intensities were recorded with a MAR Research imaging-plate scanner. Images were indexed and evaluated with program *DENZO* (Otwinowski, 1993). Crystals mounted in quartz capillaries and cooled to 277 K diffracted initially to approximately 3 Å for type I crystals (Fig. 2) and to 4 Å for type II crystals. Unfortunately, they quickly deteriorated in the synchrotron beam because of radiation damage. Initial attempts at cryofreezing the crystals were unsuccessful because of crystal damage upon transferring them to cryoprotectant solution. However, the damage could be reduced if the crystals were transferred in several steps through solutions containing increasing concentrations of glycerol. Type I crystals could be best indexed on a primitive orthorhombic lattice. Axial reflections for one of the axes were recorded, showing no systematic absences. Thus, the space group cannot be $P2_12_12_1$ and is either $P222$ or $P222_1$. Type II crystals were indexed on a *C*-centred orthorhombic lattice. Type III crystals were either twinned or too small for reliable indexing of diffraction intensities and determination of unit-cell parameters. The crystal properties are summarized in Table 1.

4. Discussion

The size of yeast PFK1 makes the crystallography a challenge but structure solution is feasible using the crystals currently available, especially if suitable conditions can be found for cooling them to cryogenic temperatures. The use of synchrotron radiation is essential. Type I crystals are the most promising because they diffract to a higher resolution than the other crystal forms. Attempts are being made to further refine the cryoconditions and also to grow the crystals in the presence of a cryoprotectant. Initial experiments indicate that this may be

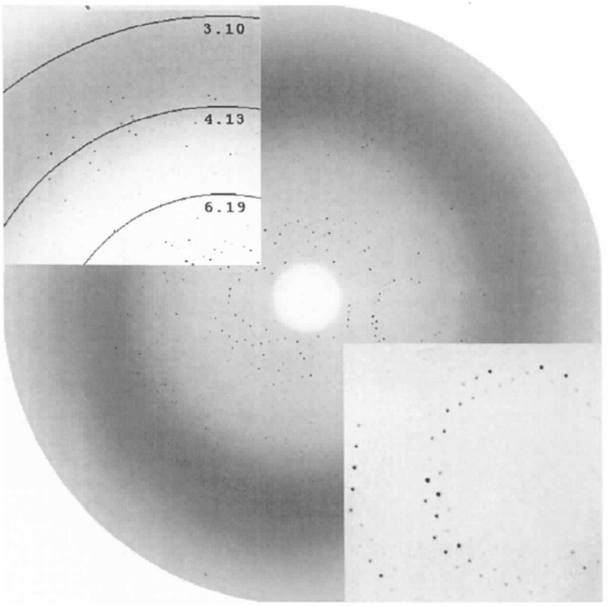


Fig. 2. Diffraction pattern of type I crystals, recorded on the EMBL BW7B wiggler beamline. The oscillation angle was 0.5° and the exposure time was 5 min.

Table 1. *Summary of crystals*

Crystal type	Morphology	Diffraction (Å)	Lattice	Cell parameters (Å)			V_M (Å^3 Da^{-1})†	V_{solv} (%)†
				a	*b*	*c*		
I	Needles	*ca* 3	*P*-orthorhombic	186	192	242	3.0	59
II	Blocks	*ca* 4	*C*-orthorhombic	262	453	151	3.1	61
III	Bipyramids	*ca* 5	?	?	?	?	?	?

† Assuming two teramers per assymetric unit.

achieved by using low-molecular weight PEG to replace high-molecular weight PEG. Further crystallization trials are also being carried out to obtain a crystal form with a smaller unit cell. Currently, both the known crystal forms have at least two PFK1 tetramers in the asymmetric unit which corresponds to a solvent content approximately 60%. This is not unusual for crystals of large proteins and explains the limited resolution and fragility of the crystals.

References

Bär, J., Huse, K., Kopperschläger, G., Behlke, J. & Schulz, W. (1988). *In. J. Biol. Macromol.* **10**, 99–105.

Chaffotte, A. F., Laurent, M., Tijane, M., Tardieu, A., Roucous, C., Seydoux, F. & Yon, J. M. (1984). *Biochemie (Paris)*, **66**, 49–58.

Erdmann, H. (1994). Personal communication.

Evans, P. R., Farrants, G. W. & Hudson, P. (1981). *Philos. Trans. R. Soc. London Ser. B*, **293**, 53–62.

Evans, P. R. & Hudson, P. (1979). *Nature (London)*, **279**, 500–504.

Heinisch, J. J., Boles, E. & Timpel, C. (1996). *J. Biol. Chem.* **271**, 15928–15933.

Heinisch, J., Ritzel, R. G., von Borstel, R. C., Aguilera, A., Rodicio, R. & Zimmermann, F. K. (1989). *Gene*, **78**, 309–321.

Kopperschläger, G., Bär, J., Nissler, K. & Hofmann, E. (1977). *Eur. J. Biochem.* **81**, 317–325.

Kopperschläger, G., Bär, J. & Stellwagen, E. (1993). *Eur. J. Biochem.* **217**, 527–533.

Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.

Parmeggiani, A., Luft, J. H., Love, D. S. & Krebs, E. G. (1966). *J. Biol. Chem.* **241**, 4625–4637.

Poorman, R. A., Randolph, A., Kemp, R. G. & Heinrikson, R. L. (1984). *Nature (London)*, **309**, 467–469.

Raben, N., Exelbert, R., Spiegel, R., Sherman, J. B., Nakajima, H., Plotz, P. & Heinisch, J. (1995). *Am. J. Hum. Genet.* **56**, 131–141.

Rypniewski, W. R. & Evans, P. R. (1989). *J. Mol. Biol.* **207**, 805–821.

Schirmer, T. & Evans, P. R. (1990). *Nature (London)*, **343**, 53–62.

Shirakihara, Y. & Evans, P. R. (1988). *J. Mol. Biol.* **204**, 973–994.

van Silfhout, R. G. & Hermes, C. (1995). *Rev. Sci. Instrum.* **66**(2), 1818–1820.

Tarui, S., Kono, N., Kuwajima, M. & Ikura, Y. (1978). *Mongr. Hum. Genet.* **9**, 42–47.