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Crystallization and preliminary crystallographic investigation of glycosomal pyruvate phosphate dikinase from *Trypanosoma brucei*

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The PP_i-dependent glycosomal enzyme pyruvate phosphate dikinase (PPDK) from *Trypanosoma brucei* is expressed in the insect stage of the parasite. Its precise function there is still unclear, but the enzyme may catalyze the 'reverse reaction' of transfer of phosphate from phosphoenolpyruvate (PEP) to generate pyruvate as a means of scavenging large amounts of pyrophosphate. This protein may represent a target for drug design against diseases caused by trypanosomes and related kinetoplastids. The recombinant protein is 918 amino acids long (predicted molecular mass $\simeq$ 100 kDa and pI = 8.9). Crystallization conditions for the recombinant PPDK are reported that result in crystals that diffract X-rays to better than 3.0 Å resolution. Their space group is $P2_12_12$, with unit-cell parameters a = 121.17, b = 153.5, c = 65.46 Å, $\alpha = \beta = \gamma = 90^\circ$. The crystals, like the protein in solution, are sensitive to temperature and fail to diffract or diffract only to low resolution after ageing for two weeks or longer.

1. Introduction and biological relevance

Trypanosomatids are composed of protozoa that belong to the order Kinetoplastida and are parasitic to mammals, including man, where infection can result in serious diseases (Lumsden & Evans, 1976). The African trypanosomes of the *T. brucei* sub-species (causative agents of nagana in cattle and sleeping sickness in human beings) are characterized by a life cycle with parasites that differentiate into several adaptive forms. The most predominant adaptative forms are the bloodstream form in the mammalian host and the procyclic form in the midgut of the tsetse fly vector. During life-cycle differentiation, the parasites have developed a concomitant adaptation of their metabolism to that of the host (Fairlamb & Opperdoes, 1986). The procyclic form uses amino acids as its energy source (Vickerman, 1985), while the bloodstream parasite is completely dependent on host-provided glucose to produce ATP through glycolysis (Opperdoes, 1987). In *T. brucei*, the Krebs cycle enzymes are totally repressed and pyruvate is the exclusive end product of glycolysis under aerobic (bloodstream form) conditions. In trypanosomatids, the first seven steps of glycolysis are carried out in peroxisome-like organelles called glycosomes (Opperdoes & Borst, 1977), where the corresponding glycolytic enzymes are sequestered. The glycosomes are surrounded by a single lipid bilayer and do not contain DNA. Consequently, all these glycosomal proteins are nuclear encoded, processed and transported to the glycosome.

The enzyme pyruvate phosphate dikinase (PPDK; Genbank accession number AF048689) isolated from *T. brucei* is the first reported PP_i-dependent enzyme found in trypanosomatids (Bringaud *et al.*, 1998). It can be detected by Western blot analysis and enzymatic assays in all the adaptative forms of trypanosomatids except the bloodstream form of *T. brucei*. The glycosomal localization of PPDK in the procyclic form of *T. brucei* was revealed by immunofluorescence staining and Western blot analysis on sucrose-gradient purified glycosome fractions. The reason for the glycosomal location of PPDK and its exact role in the parasite are still unknown, but the enzyme may function in the 'reverse direction' as a means of scavenging the pyrophosphate produced in the glycosomes by non-glycolytic pathways.

We report here work that has provided a set of crystallization conditions for the purified recombinant *T. brucei* PPDK and the characteristics of the resulting crystals.

2. Protein expression and purification

Glycosomal PPDK from *T. brucei* was expressed and purified as previously described (Bringaud *et al.*, 1998). Briefly, cultures of *Escherichia coli* containing the pET-PPDK15 plasmid that encodes for the single-copy full-length *T. brucei* PPDK protein were induced to

Table 1
Statistics of diffraction data processing.

Values in parentheses are for the highest resolution shell.

Resolution range (Å)	24.0–3.0 (3.1–3.0)
Completeness (%)	96 (88.6)
Multiplicity	5.3 (3.4)
Signal-to-noise ratio [$I/\sigma(I)$]	8.5 (2.8)
$R_{\rm merge}$† (%)	8.76 (28.0)

† $R_{\rm merge} = \sum_{hkl}\sum_{j=1,n}|\langle I_{hkl}\rangle - I_{hkl,j}|/\sum_{hkl}\sum_{j=1,n} I_{hkl,j}$.

express the poly(His)-tagged protein by the addition of IPTG. This material was then purified according to the standard instructions provided by Novagen Inc. (Madison, WI, USA). Purified PPDK was dialyzed against a storage buffer [composed of 20 m*M* imidazole pH 7.0, 100 m*M* NaCl, 100 m*M* $MgCl_2$, 20%(*v*/*v*) glycerol], concentrated to 50 mg ml^{-1} and stored in 100 µl aliquots at 193 K until used. During this low-temperature storage the enzyme gradually loses its activity.

3. Crystallization

All crystallization studies were performed with inactive aged PPDK obtained by storing the aliquots at 193 K for *ca* six months. Initial trials were carried out at the CDSB, Jena using the microbatch diffusion method under *n*-decane and paraffin oil (Chayen *et al.*, 1992) and the sparse-matrix crystallization screening conditions developed by Jancarik & Kim (1991) and others (Cudney *et al.*, 1994; Garman & Mitchell, 1996). Commonly used precipitants (*e.g.* ammonium sulfate, polyethylene glycols) were also screened. Screening kits from Hampton Research (Laguna Niguel, CA, USA) were used, with a microtip Impax 1-5 crystallization robot (Chayen *et al.*, 1990) from Douglas Instruments (East Garston, UK). The PPDK aliquots were thawed and dialysed against a buffer consisting of 20 m*M* imidazole pH 6.5, 250 m*M* NaCl, 10 m*M* $MgCl_2$, 5%(*v*/*v*) glycerol to obtain a protein concentration of ∼30 mg ml^{-1} for the crystallization experiments.

Examination of the initial crystallization trays led to the following observations: trials stored at 277 or 310 K gave protein precipitates for all conditions investigated. Therefore, only room-temperature (292 K) conditions were used for the crystallization of recombinant PPDK. Of the precipitants screened during the initial trials, it was observed that at the precipitant concentrations used all polyethylene glycols led to protein precipitation. A single condition at a much lower polyethylene glycol concentration [0.1 *M* Tris pH 8.5, 8%(*w*/*v*) PEG 8000] provided the first crystals of the PPDK enzyme.

It was therefore decided to screen lower concentrations of polyethylene glycols [1–7%(*w*/*v*) PEG 2000, PEG 4000 and PEG 5000 monomethyl ether] at several pH values ranging from 4.0 to 10.0. The hanging-drop vapour-diffusion crystallization method was used from this stage onwards. Myriads of small single crystals appear extremely rapidly (within seconds) at pH values between 8.0 and 9.0 and at low PEG concentrations [0.5–3%(*w*/*v*) PEG]. This pH is very close to the pI of the enzyme, 8.9. It was also possible to grow poorly shaped crystals from solutions devoid of precipitating agent, where the sole crystallization agent was the change in pH of the protein environment.

Further work focused on determining conditions that would slow crystal growth. The ability of glycerol to act as a crystal-growth retardant was tested, since the presence of this chemical in the buffer used to store the PPDK enzyme is essential to keep it in solution. For this reason, PPDK in its initial storage buffer [which contains 20%(*v*/*v*) glycerol and a protein concentration of ∼50 mg ml^{-1}] was used directly for these studies. The protein droplets were suspended over mother liquor composed of 0.1 *M* bicine pH 8.8, 0.5–3% PEG 5000 monomethyl ether and 10–30% glycerol. Crystals suitable for X-ray diffraction were obtained at 292 K by mixing 2 µl of protein solution with 2 µl mother liquor. Single rod-shaped crystals grew within 1–2 weeks from mother-liquor solutions containing 10%(*v*/*v*) glycerol and ∼1.5% PEG 5000 MME. The largest crystals have approximate dimensions of 0.2 × 0.2 × 0.3 mm. It has been noted that PPDK stability and crystal formation is sensitive to temperature. This suggests that *T. brucei* glycosomal PPDK may have a biologically active structure that is sensitive to temperature in a manner similar to that reported for other PPDKs (Usami *et al.*, 1995).

4. Crystal handling and diffraction data collection

The exposed surface of the crystallization droplets is covered by a very thin 'skin' (probably composed of deposited PPDK enzyme), from which the crystals grow and to which they adhere. Therefore, the crystals were first handled using crystal manipulation microtools from Hampton Research (Laguna Niguel, CA, USA) in order to remove this sticky skin. Care needs to be exercised at this stage, as the fragile crystals will shatter if touched with the tip of the microtool.

Attempts to find cryocooling conditions for our PPDK crystals were unsuccessful: the enzyme is crystallized from mother-liquor solutions that contain small amounts of precipitant [∼1.5%(*w*/*v*) PEG 5000 MME] and ∼10%(*v*/*v*) glycerol, *i.e.* an insufficient amount for this component to act as a cryoprotectant. In our hands, crystal formation and stability were sensitive to the amount of glycerol present in the mother liquor. Attempts to modify the glycerol concentration or to protect the crystals by coating them with a film of dry paraffin oil prior to data collection (Riboldi-Tunnicliffe & Hilgenfeld, 1999) only led to X-ray diffraction patterns that extend to very low resolution (between 20 and 10 Å) at cryotemperatures (∼100 K).

The method of mounting crystals in capillary tubes for data collection was therefore used. Under these conditions and with the mounted crystals cooled by a flux of cold air at 277 K, diffraction to ∼2.8 Å resolution is observed for the largest crystals. It has been noted that only freshly grown crystals will diffract X-rays; crystals do not diffract X-rays 20 d after they appear in the droplets.

A complete data set was recorded from five PPDK crystals on a MAR 345 image-plate device (MAR Research, Hamburg, Germany) at the DW32 beamline (Fourme *et al.*, 1992) of the LURE synchrotron source (Orsay, France). The crystals were oriented and aligned to ensure that a complete data set could be obtained. A total rotation range (Arndt & Wonacott, 1977) of 191° was collected, with the best crystals providing 50–60° each. The data frames (1° rotation per frame) were processed using the *XDS* software suite (Kabsch, 1993). Autoindexing and examination of the systematic absences in the data indicate that the space group is $P2_12_12$, with unit-cell parameters a = 121.17, b = 153.5, c = 65.46 Å, $\alpha = \beta = \gamma = 90°$. Data scaling and merging was carried out with programs from the *BIOMOL* suite (Groningen Protein Crystallography Group, unpublished program). The resulting data set is of adequate quality (see Table 1).

Using these data, attempts are being made to solve the structure of glycosomal PPDK by the molecular-replacement method (Rossmann, 1972) using the 2.3 Å resolution structure of *Clostridium symbiosium* PPDK (Herzberg *et al.*, 1996) as a search model. A convincing solution to the

molecular-replacement problem has recently been obtained and the structure determination is in progress.

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References

Arndt, U. W. & Wonacott, A. J. (1977). *The Rotation Method in Crystallography*. Amsterdam: North-Holland Publishing Company.

Bringaud, F., Baltz, D. & Baltz, T. (1998). *Proc. Natl Acad. Sci. USA*, **95**, 7963–7968.

Chayen, N. E., Shaw Stewart, P. D. & Blow, D. M. (1992). *J. Cryst. Growth*, **122**, 176–180.

Chayen, N. E., Shaw Stewart, P. D., Maeder, D. L. & Blow, D. M. (1990). *J. Appl. Cryst.* **23**, 297–302.

Cudney, R., Patel, S., Weisgraber, K., Newhouse, Y. & McPherson, A. (1994). *Acta Cryst.* D**50**, 414–423.

Fairlamb, A. H. & Opperdoes, F. R. (1986). *Carbohydrate Metabolism in Cultured Cells*, edited by M. J. Morgan, pp. 183–224. New York: Plenum.

Fourme, R., Dhez, P., Benoit, J.-P., Kahn, R., Dubuisson, J. M., Besson, P. & Frouin, J. (1992). *Rev. Sci. Instrum.* **63**, 982–987.

Garman, E. F. & Mitchell, E. P. (1996). *J. Appl. Cryst.* **29**, 584–587.

Herzberg, O., Chen, C. C. H., Kapadia, G., McGuire, M., Carroll, L. J., Noh, S. J. & Dunaway-Mariano, D. (1996). *Proc. Natl Acad. Sci. USA*, **93**, 2652–2657.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

Kabsch, W. (1993). *J. Appl. Cryst.* **26**, 795–800.

Lumsden, H. R. & Evans, D. A. (1976). *Biology of the Kinetoplastida.* New York: Academic Press.

Opperdoes, F. R. (1987). *Annu. Rev. Microbiol.* **41**, 127–151.

Opperdoes, F. R. & Borst, P. (1977). *FEBS Lett.* **80**, 360–364.

Riboldi-Tunnicliffe, A. & Hilgenfeld, R. (1999). *J. Appl. Cryst.* **32**, 1003–1005.

Rossmann, M. G. (1972). *The Molecular Replacement Method.* New York: Gordon & Breach.

Usami, S., Ohta, S., Komari, T. & Burnell, J. N. (1995). *Plant Mol. Biol.* **27**, 969–980.

Vickerman, K. (1985). *Br. Med. Bull.* **41**, 105–114.