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Crystallization and preliminary X-ray analysis of a bacterial psychrophilic enzyme, phosphoglycerate kinase

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The glycolytic enzyme phosphoglycerate kinase (PGK) from the Antarctic microorganism *Pseudomonas* sp. TACII18 is a cold-adapted enzyme that displays a high specific activity at low temperatures and decreased thermostability relative to its mesophilic counterpart. Herein, the preliminary crystallization and structure solution of psychrophilic PGK in its native form and cocrystallized with 3-phosphoglyceric acid (3-PGA) and the ATP analogue adenylyl imidophosphate (AMP-PNP) is reported. The complexed form of PGK crystallized in 2–3 d at 290 K, whereas the native form of the enzyme required 8–12 months. Morphologically, both crystal forms are similar and X-ray diffraction experiments indicate that the crystals are isomorphous. The crystals diffracted to a resolution of 2.0 Å and belong to the space group $P3_2$. with unit-cell parameters $a = b = 58.5$, $c = 85.4$ Å.

1. Introduction

Extremophilic microorganisms grow in a wide variety of environments that were once believed inhospitable to life. These environments include high-salinity, high-pressure, acidic and alkaline environments, and temperatures that range from Antarctic ice water to more than 388 K in some deep-sea hydrothermal vents. Psychrophilic bacteria have evolved molecular adaptations that enable them to survive at temperatures often below 273 K. At low and moderate temperatures, the enzymes of these psychrophiles display a higher specific activity than their mesophilic counterparts. For this reason, the study of psychrophilic enzymes is not only of basic scientific interest, but harbours potential biotechnological applicability (Gerday *et al.*, 2000). Some of the molecular adaptations of the enzymes of psychrophilic organisms include: fewer salt bridges, a smaller hydrogen-bonding network, higher interactions with the solvent and an increase in the relative hydrophobicity of the enzyme surface. These adaptations lead to an increase in the local or overall flexibility of cold-adapted catalysts (Jaenicke, 1990). Concomitant with the decreased rigidity of the enzyme is a decrease in its thermostability.

The glycolytic enzyme phosphoglycerate kinase (PGK) catalyzes the reversible transfer of a phosphate moiety from 1,3-bisphosphoglycerate (bPGA) to Mg-ADP to yield ATP and 3-PGA. The structure of horse muscle PGK (Banks *et al.*, 1979) revealed two domains, an N-terminal and a C-terminal domain, of approximately equal size, separated by a helical linker. The monomeric protein binds 1,3-bPGA in its N-terminal domain (based on the [3PGA-PGK] complex of Harlos *et al.*, 1992) and Mg-ADP in its C-terminal domain (Davies *et al.*, 1994); a 'hinge-bending' movement at the level of the linker region brings the two substrates into close proximity for phosphate exchange (Auerbach *et al.*, 1997; Bernstein *et al.*, 1997).

With the elucidation of the first psychrophilic PGK structure will come the possibility of analyzing and comparing the three-dimensional structures of psychro-, meso- (Watson *et al.*, 1982), thermo- (Davies *et al.*, 1993) and hyperthermophilic (Auerbach *et al.*, 1997) PGKs in order to develop a better understanding of the molecular adaptations to temperature.

2. Methods and materials

2.1. Expression and purification

Recombinant native (non-His-tagged) PGK was overexpressed in *Escherichia coli* BL21 (DE3) cells essentially as described in Bentahir *et al.* (2000). Briefly, the cells harbouring the expression vector pT22-pgk were grown in LB broth at 291 K with 100 mg l^{-1} ampicillin to an OD_{550} of 0.6. Subsequently, the cells were induced with 0.5 m*M* isopropyl-1-thio-β-galactopyranoside and incubated for an additional 20 h. The cells were then harvested by centrifugation at 20 000*g*, resuspended in buffer *A* (10 m*M* Tris–HCl, 0.1 m*M* EDTA, 1 m*M* dithiothreitol, 20 μ*M* phenylmethylsulfonyl fluoride, 20 μ*M* benzamidine pH 7.5), lysed by pressure at 193 MPa in a cell disrupter

Table 1
X-ray diffraction data.

Values in parentheses are for the highest resolution shell.

	(PGK–MgAMPPNP–3PGA)	Native
Space group	$P3_2$	$P3_2$
Unit-cell parameters (Å, °)	$a = b = 58.5$, $c = 85.4$, $\alpha = \beta = 90$, $\gamma = 120$	$a = b = 59.1$, $c = 87.0$, $\alpha = \beta = 90$, $\gamma = 120$
No. of observed reflections	61709	66233
No. of unique reflections	18955	23306
Redundancy	3.3	2.8
Resolution range (Å)	46.0–2.1	50–2.0
$I/\sigma(I)$	8.6 (1.7)	12 (1.9)
Completeness (%)	99.4 (99.4)	99.9 (99.9)
$R_{\rm merge}$	7.1 (43.3)	9.5 (44)

(Constant Systems Ltd, UK) and centrifuged for 30 min at 20 000*g*. The resulting supernatant was subjected to a protamine sulfate treatment [0.1%(*w*/*v*)] to remove nucleic acids and then to ammonium sulfate fractionation. Two chromatographic steps consisting of a phenyl Sepharose column and a Q-Sepharose column were required to purify the enzyme to homogeneity. For long-term storage at 277 K, the purified enzyme was placed in a solution of 3.2 *M* ammonium sulfate in buffer *B* (10 m*M* Tris–HCl at pH 7.5, 1 m*M* DTT and 0.1 m*M* EDTA). Prior to the crystallization trials, the enzyme was dialyzed against 4 l of buffer *B*.

2.2. Differential scanning calorimetry

Differential scanning calorimetry was performed, as previously described in Bentahir *et al.* (2000), in a MicroCal MCS-DSC instrument at a scan rate of 90 K h^{-1} and under 101 kPa nitrogen pressure. The protein samples were dialyzed extensively against 30 m*M* MOPS pH 7.5. The protein concentration was approximately 2 mg ml^{-1} in all experiments. The stability of the enzyme in the closed conformation was measured in the presence of 5 m*M* 3-PGA and 5 m*M* Mg-AMP-PNP. Thermograms were analyzed according to a non-two-state model using the MicroCal *Origin* 2.1 software.

2.3. Crystallization

Crystallization screening was carried out by the hanging-drop vapour-diffusion method (McPherson, 1999) in 24-well Linbro plates using crystallization screening kits I and II (Hampton Research, Laguna, CA, USA; Jancarik & Kim, 1991). Briefly, 2 µl of protein solution at 10 mg ml^{-1} was mixed with 2 µl of screening solution on a siliconized cover slip. This was placed over a 500 µl reservoir containing the respective screening solution and incubated at 290 K. For the cocrystallization, the protein at 10 mg ml^{-1} was mixed with 2.1 m*M* AMP-PNP, 2.5 m*M* $MgCl_2$, 4.2 m*M* 3-PGA, 1 m*M* DTT and 10 m*M* Tris–HCl at pH 7 prior to crystallization trials. Crystals of the complex grew in a mixture of 30% polyethylene glycol (PEG) 4000, 0.2 *M* $MgCl_2$ and 0.1 *M* Tris–HCl at pH 8.4 (condition 6, Screen Kit I). The native crystals grew in 20% PEG 10000 and 0.1 *M* HEPES at pH 7.5 (condition 38, Screen Kit II). For the cryo data collection, the crystals were soaked stepwise for 2 min per concentration in 5, 10 and 15% ethylene glycol plus the mother liquor. For the complex, the substrates were added to the cryosolution.

2.4. X-ray data collection

The X-ray diffraction experiment for the cocrystallization was performed in-house on a Nonius FU 581 Cu-anode generator and the Cu $K\alpha$ X-rays were focused with Osmic mirrors. For the native crystal, the data were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France on the beamline BM-30/FIP. The X-rays were focused with a combination of parabolic mirrors and a dual Si crystal monochromator at a wavelength of 0.90005 Å. The crystals were flash-frozen in supercooled N_2 gas produced by an Oxford Cryosystems Cryostream (600 series) and maintained at 100 K during data collection. The diffraction data were collected on a MAR345 image-plate detector (MAR Research) while the crystal was oscillated 1° per frame of data about the φ axis. The indexing and integration of the diffraction images, as well as the scaling and merging of the reflections, were accomplished with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystals belong to the trigonal space group $P3_2$, with unit-cell parameters $a = b = 58.5$, $c = 85.4$ Å (Table 1). Assuming one molecule in the asymmetric unit and a monomeric molecular weight of 40 110 Da, a Matthews coefficient ($V_{\rm M}$) of 2.1 $Å^3$ Da^{-1} and a solvent content of 39% were calculated for the crystal. These values fall in the range typical for protein crystals (Matthews, 1968).

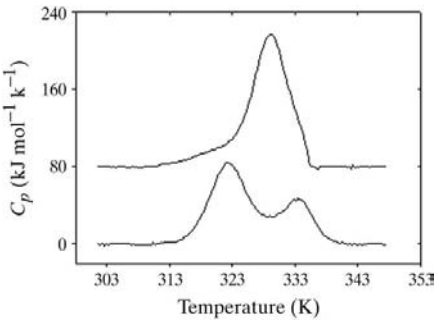

Figure 1
Thermal unfolding recorded by microcalorimetry of the psychrophilic PGK in the free state (lower curve) and in the presence of 5 m*M* 3-PGA and 5 m*M* Mg-AMP-PNP (upper curve, shifted for clarity) in 30 m*M* MOPS pH 7.5. Thermograms are baseline-subtracted and normalized for protein concentration (40 µ*M*).

3. Results and discussion

The stability of psychrophilic PGK in the absence and presence of 3-PGA plus Mg-AMP-PNP was analyzed by differential scanning calorimetry (DSC; Fig. 1) and used as a possible indicator of crystallization potential. In the thermograms, the top of the transitions corresponds to the melting point (Tm) of a domain and the surface area under the peak is a direct measurement of the total amount of heat absorbed during unfolding, *i.e.* the calorimetric enthalpy ($\Delta H_{\rm cal}$). This parameter increases when new interactions

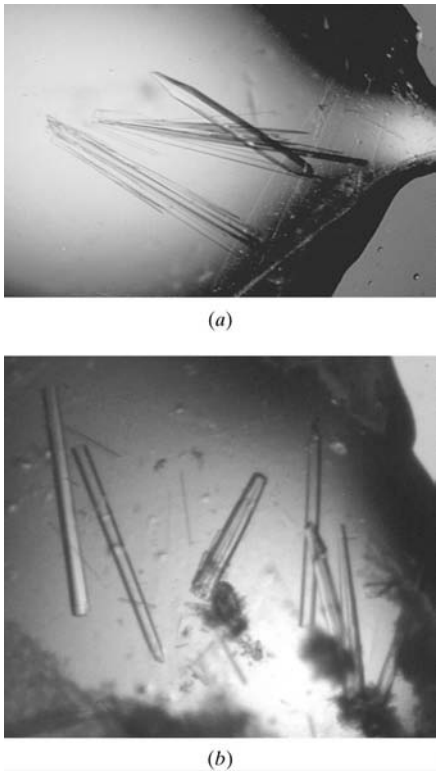

Figure 2
Crystals of the native (*a*) and 3-PGA + Mg-AMP-PNP complexed (*b*) forms of psychrophilic PGK from *Pseudomonas* sp. TACII18.

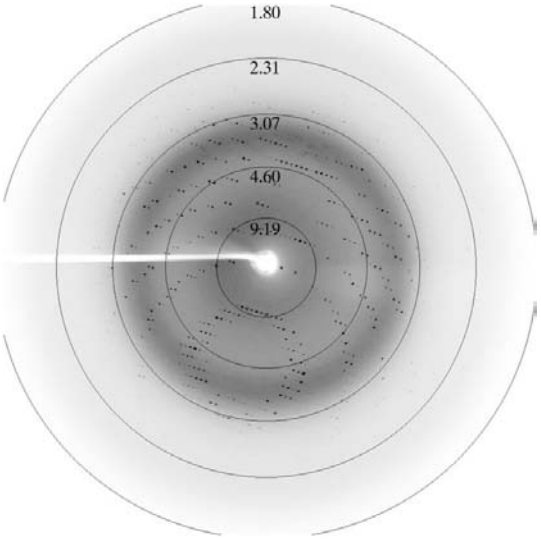

Figure 3
A 1° oscillation image collected at beamline BM-30 of ESRF at 100 K on a native PGK crystal.

are formed, for instance following ligand binding. In the free state, the psychrophilic PGK is composed of two domains having distinct stability. It has been proposed that the heat-labile domain provides the required flexibility around the active site for catalysis at low temperatures (Bentahir *et al.*, 2000). On the other hand, in the presence of 3-PGA and the substrate analogue Mg-AMP-PNP, the enzyme is more compact and only one transition is recorded. In addition, the $\Delta H_{\rm cal}$ value increases by a factor of two, suggesting that the substrate analogue is readily bound. These results open the perspective that the complexed conformation would more readily crystallize than the enzyme that exhibits two independent domains.

Crystals for the complex were observed within 2–3 d, whereas the native protein took between 8 and 12 months to crystallize (Fig. 2). Following the initial crystal growth, both the native and complex crystals could be grown within 24 h by streak-seeding techniques using crushed crystals (Stura, 1999). For seeding of the cocrystallization drops, the PEG concentration was lowered to 25% PEG 4000 to avoid rapid spontaneous overnucleation. The native and complex crystals grew as long rods and measured on average 0.8 × 0.1 × 0.1 mm.

Phases for the complex crystal diffraction data were obtained by molecular replacement using the program *AMoRe* (Navaza, 1987). The search model used was the PGK structure of *T. maritima* (PDB code 1vpe; Auerbach *et al.*, 1997), which has 45% sequence identity with the psychrophilic PGK. Owing to ambiguities in the space group, molecular-replacement solutions were sought in both $P3_1$ and $P3_2$. Since no molecular-replacement solution was found with the entire *T. maritima* structure, the model was divided into an N-terminal domain (residues 2–168) and a C-terminal domain (residues 185–399), with the helical linker between the two domains omitted from the search. Whereas in the translation search with the N-terminal domain no solutions were found in $P3_1$, a signal 50% above background was detected in the space group $P3_2$. With the N-terminal domain included and 'fixed' in place, a solution for the C-terminal domain was found; after rigid-body refinement of both domains a final correlation coefficient of 41.2% and an R factor of 47.8% for the molecular-replacement solution were obtained. The lack of a molecular-replacement solution with the intact 'closed' *T. maritima* PGK model puts into question the *true* conformational state of the complex PGK–3PGA–MPPNP. In addition, the isomorphism between the native and cocrystallized forms of PGK suggests there is very little, if any, difference between the two structures, an unexpected result for an enzyme that undergoes large conformational changes upon substrate binding and catalysis. The final refined models should clarify these discrepancies.

Currently, iterative rounds of model building with the program *TURBO-FRODO* (Roussel *et al.*, 1992) and high-temperature (5000 K) simulated-annealing refinements in *CNS* (Adams *et al.*, 1997) are under way for the psychrophilic PGK structure complexed with Mg-AMP-PNP and 3-PGA. When this model is completed, it will be used to phase the native data set by molecular replacement.

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