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Crystallization and preliminary X-ray analysis of inorganic pyrophosphatase from the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3

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Inorganic pyrophosphatase (PPase; EC 3.6.1.1) from the hyperthermophile *Pyrococcus horikoshii* was crystallized by the hanging-drop vapour-diffusion method at pH 5.0 using polyethyleneglycol 4000 as the precipitant. The crystal belongs to space group $P2_12_12$, with unit-cell parameters $a = 71.7$, $b = 86.5$, $c = 92.5$ Å, $\alpha = \beta = \gamma = 90^\circ$. There are two molecules in the asymmetric unit. The crystals were stable during exposure to X-rays and a full set of X-ray diffraction data was collected to 2.7 Å resolution in-house.

1. Introduction

Inorganic pyrophosphatase (PPase; EC 3.6.1.1) hydrolyzes inorganic pyrophosphate (PP_i) to inorganic phosphate (P_i). PP_i is formed principally as the by-product of many biosynthesis reactions, including tRNA charging DNA and protein syntheses that utilize ATP (Chen *et al.*, 1990). The very high level of PP_i that would result from the absence of PPase would certainly be toxic. Inorganic pyrophosphatases are therefore essential constitutive enzymes that are present in all living cells and have been purified and characterized from many eukaryotic and prokaryotic sources (Cooperman, 1982; Richter & Schafer, 1992).

Inorganic pyrophosphatases can be divided into two families on the basis of their primary sequences (Shintani *et al.*, 1998). Family I is the largest group and contains most of the currently known PPases. Family II PPases are relatively uncommon but include *Bacillus subtilis* PPase. The two families do not show any sequence similarity to each other (Shintani *et al.*, 1998; Young *et al.*, 1998). Of the family I enzymes, *Saccharomyces cerevisiae* PPase (Y-PPase) has been the most widely studied in eukaryotic organisms and was the first three-dimensional structure of a PPase to be solved (Terzyan *et al.*, 1984). In prokaryotic organisms, *Escherichia coli*PPase (E-PPase) has also been studied extensively. The metal-binding sites in *E. coli* PPase have been crystallographically identified at 2.2 Å resolution (Oganessyan *et al.*, 1994; Kankare *et al.*, 1996). These studies have provided interesting data on the structural and catalytic properties of PPase. Inorganic pyrophosphatase is becoming an important structural model for enzymatic hydrolysis and synthesis of polyphosphates.

PPases from archaebacteria show different structural and catalytic properties to those of their prokaryotic and eukaryotic counterparts (Richter & Schafer, 1992; Hansen *et al.*, 1999). In recent years, the structural basis of protein thermophilicity and thermostability in archaeal PPases has also come to light (Salminen *et al.*, 1996). Archaeal PPases are relatively thermostable, especially in the presence of divalent metal cations (Ichiba *et al.*, 1998). The structures of the archaebacterial inorganic pyrophosphatases from *Thermus thermophilus* (T-PPase; Teplyakov *et al.*, 1994) and *Sulfolobus acidocaldarius* (S-PPase; Leppanen *et al.*, 1999) have been determined at high resolution.

As a step towards understanding the general mechanism of catalysis by PPase, the structural basis of the thermostability of these proteins and the evolutionary relationships between the members of this family, we have isolated an inorganic pyrophosphatase from the hyperthermophilic archaea *Pyrococcus horikoshii* (Pho PPase). Pho PPase can be grown at up to 386 K and has an optimum growth temperature of 268 K (Gonzalez *et al.*, 1998), which is significantly higher than those of S-PPase (348–353 K), T-PPase (348–353 K) and E-PPase (310 K). Studies of the hyperthermophilic proteins should provide further information about the structural determinants of protein thermostability. The full enzymatic properties of inorganic Pho PPase will be presented elsewhere (Feng & Gao, 2004). To obtain detailed structural and catalytic information on PPases from hyperthermophilic archaea and to obtain a more thorough explanation of some of the functional analyses of Pho PPase, we have obtained crystals of Pho PPase and are analyzing its three-dimensional structure.

2. Materials and methods

2.1. Expression and purification

The gene encoding inorganic pyrophosphatase from the archaea *P. horikoshii* was cloned into pET15b (Novagen) and expressed in *E. coli* strain BL21. Briefly, the cells were incubated with vigorous shaking at 110 K until

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses correspond to the outer shell.

Space group	$P2_12_12$
Unit-cell parameters (Å, °)	a = 71.8, b = 86.5, c = 92.5, $\alpha = \beta = \gamma = 90$
V_M (Å^3 Da^{-1})	2.7
Solvent content (%)	35.7
Resolution (Å)	50–2.7
No. observations	118041
No. unique reflections	17030
Redundancy	6.93
Average $I/\sigma(I)$	5.8
R_{merge}† (%)	12.7 (30.9)
Completeness (%)	98.3

† $R_{merge} = \sum |I - \langle I \rangle| / \sum I$.

OD_{600} reached ∼0.8 and then induced by adding IPTG to a final concentration of 1 m*M* at 298 K for ∼6–8 h. The harvested cells were suspended in 50 m*M* Tris–HCl pH 8.0 and lysed by sonication. The lysate was centrifuged at 23 000*g* for 20 min after being heated at 358 K for 30 min. The supernatant was subjected to Ni-chelation chromatography and the unbound proteins were washed out with washing buffer (25 m*M* Tris–HCl pH 8.0, 10% glycerol, 500 m*M* NaCl, 20 m*M* imidazole, 1 m*M* PMSF). Pho PPase was eluted with the above buffer containing 500 m*M* imidazole. The collected fractions were buffer-exchanged to 25 m*M* Tris–HCl pH 8.0 using a Sephadex G-25 column (Amersham Pharmacia Biotech). The desalted sample was applied onto a Resource Q column (Amersham Pharmacia Biotech) and eluted with a linear gradient of 25 m*M* Tris–HCl pH 8.0 and 600 m*M* NaCl. The peak containing the Pho PPase protein was further applied onto a Superdex 75 column (Amersham Pharmacia Biotech) and eluted with 25 m*M* Tris–HCl pH 8.0 and 50 m*M* NaCl. Purity was determined by SDS–PAGE.

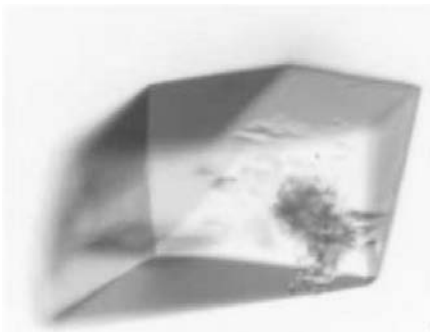

Figure 1
A typical crystal of Pho PPase. Maximum crystal dimensions are about 0.5 × 0.3 × 0.27 mm.

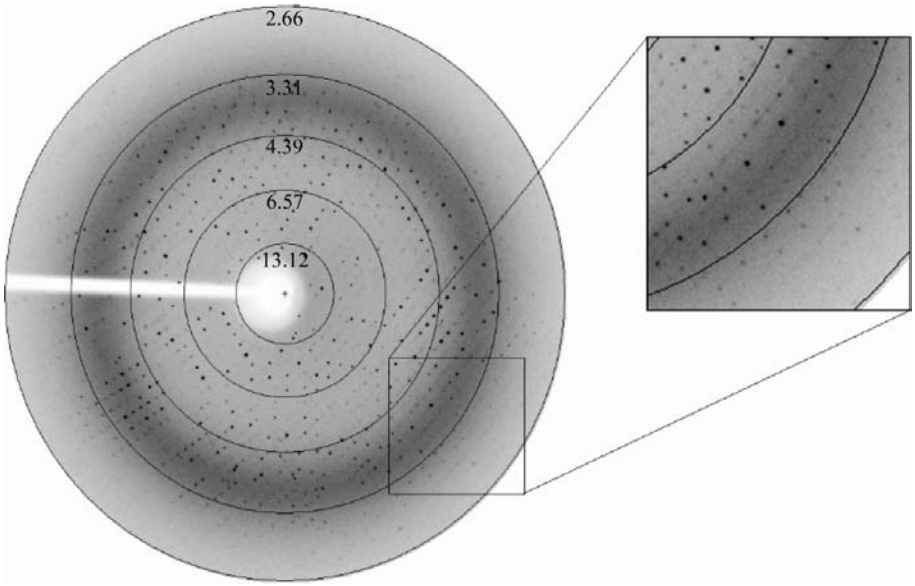

Figure 2
A representative diffraction image collected at 100 K with 1.5° oscillation per frame. The enlarged image shows diffraction to 2.7 Å resolution at the detector edge.

2.2. Crystallization

The purified protein was concentrated to ∼20 mg ml^{-1} using a 10 kDa molecular-weight cutoff ultrafiltration membrane (Amicon) in a solution containing 5 m*M* Tris–HCl pH 8.0 and 50 m*M* NaCl. Initial crystallization trials were performed at 291 K using the hanging-drop vapour-diffusion method and sets of screening reagents were supplied by Hampton Research (Riverside, CA, USA). Drops consisting of 1 µl protein solution and 1 µl reservoir solution were equilibrated against 0.5 ml reservoir solution.

Needle-shaped crystals were obtained by equilibration against 8% PEG 4000 and 0.1 *M* sodium acetate pH 4.5 within several days. On the basis of the preliminary results, further refinements were carried out varying the concentrations of protein and precipitant. Crystals large enough for X-ray diffraction experiments were grown in ∼3.8% PEG 4000 and 0.1 *M* sodium acetate pH ∼5.0–5.2 within several days, but most of them were twinned. It was difficult to find a single crystal that showed Bragg diffraction beyond 5 Å. When 0.02 *M* $MgCl_2$ was added, however, crystals suitable for X-ray analysis were obtained within several weeks. The crystals reached dimensions of 0.5 × 0.3 × 0.27 mm (Fig. 1).

3. Data collection and preliminary X-ray crystallographic analysis

Data collection was carried out at 110 K using a MAR 345 image plate with an in-house Rigaku rotating Cu-anode X-ray generator operating at 48 kV and 98 mÅ (λ = 1.5418 Å). Paraffin oil was used as the cryoprotectant. Prior to data collection, the crystal was soaked in the paraffin oil for a few seconds. The crystal was then flash-cooled in a nitrogen-gas stream at 110 K. When the crystals were exposed to X-rays, diffraction spots were observed to a Bragg spacing of at least 2.7 Å (Fig. 2). A data set was collected at 2.7 Å with an oscillation range of 1.5° per image. The crystal-to-detector distance was 250 mm. All diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The crystal belongs to space group $P2_12_12$, with unit-cell parameters a = 71.7, b = 86.5, c = 92.5 Å, $\alpha = \beta = \gamma = 90°$.

There are two Pho PPase molecules per asymmetric unit. Crystal data and data-collection statistics are listed in Table 1. Molecular replacement is now in progress using the *S. acidocaldarius* structure (Leppanen *et al.*, 1999) as a starting model.

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