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Cloning, purification, crystallization and preliminary crystallographic analysis of acylphosphatase from *Pyrococcus horikoshii* OT3

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Acylphosphatase is one of the smallest enzymes and catalyzes the hydrolysis of the carboxy–phosphate bond. An extremely thermostable acylphosphatase from a hyperthermophilic archaea, *Pyrococcus horikoshii* OT3, has been cloned, expressed in *Escherichia coli*, purified and crystallized using the sitting-drop vapour-diffusion method with potassium/sodium tartrate as the precipitant at pH 5.5. X-ray diffraction data have been collected to a highest resolution of 1.72 Å on a synchrotron-radiation source. The crystals belong to space group $P3_221$, with approximate unit-cell parameters $a = b = 86.6$, $c = 75.4$ Å and two monomers in the asymmetric unit.

1. Introduction

Acylphosphatase (ACP; EC 3.6.1.7) is one of the smallest known enzymes and catalyzes the hydrolysis of acylphosphates such as acetylphosphate, carbamoylphosphate, 1,3-bisphosphoglycerate, succinylphosphate, β-aspartylphosphate and benzoylphosphate. This enzyme is widespread in all vertebrate tissues and has been implicated in control of the glycolytic pathway, pyrimidine biosynthesis and ion-pump activity (Stefani & Ramponi, 1995; Liguri *et al.*, 1994; Tadini-Buoninsegni *et al.*, 2003). The three-dimensional structure of acylphosphatase has been determined by NMR (from horse muscle; Pastore *et al.*, 1992) and X-ray crystallography (from bovine tissue; Thunnissen *et al.*, 1997). Regardless of their source, the structures are similar to each other, consisting of one very compact globular α/β fold with two α-helices and a five-stranded β-sheet.

The stability of horse muscle acylphosphatase has previously been studied by denaturing the protein under various conditions of temperature, pH and urea concentration (Taddei *et al.*, 1994). CD and NMR data show horse muscle acylphosphatase to be 50% denatured at approximately 328 K.

Pyrococcus horikoshii OT3 is a hyperthermophilic archaea that was isolated from a hydrothermal fluid. *P. horikoshii* OT3 is an obligate anaerobe that grows at temperatures between 361 and 377 K, with an optimal temperature of 371 K, in the presence of sulfur. The proteins produced by this hyperthermophilic archaea have outstanding heat resistance. Determining the structures of such proteins can reveal the intramolecular interactions involved in maintaining protein structure and function under such extreme conditions. *P. horikoshii* OT3 genome data indicate that this hyperthermophilic archaea contains an acylphosphatase gene (ORF PH0305a; Kawarabayasi *et al.*, 1998). The protein encoded by this ORF consists of 91 amino-acid residues with a molecular weight of 10 260 Da.

To determine the first structure of archaeal acylphosphatase and to analyze its thermostability by comparison with other acylphosphatases, we cloned, expressed and crystallized *P. horikoshii* OT3 acylphosphatase.

2. Methods and results

2.1. Expression and purification

The gene encoding the acylphosphatase homologue (PH0305a) was identified in the *P. horikoshii* OT3 genome. The acylphosphatase gene fragment was amplified by PCR from genomic DNA of *P. horikoshii* OT3. The genomic DNA was isolated from a *P. horikoshii* OT3 cell pellet using the DNeasy Plant Mini Kit (Qiagen). Primers were designed to create *Nde*I and *Bam*HI sites upstream from the initiation and stop codons, respectively. The amplified fragment was digested with *Nde*I and *Bam*HI and was cloned into a pET-26b(+) T7 RNA polymerase-based expression vector (Novagen). The plasmid was transformed into *Escherichia coli* Rosetta (DE3) (Novagen).

The transformants were cultivated at 310 K in Luria–Bertani medium containing 100 µg ml^{-1} ampicillin until the optical density at 600 nm reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was added to induce protein expression and cell cultivation was continued for 16 h at 298 K. Cells were harvested by centrifugation, resuspended in buffer *A* (50 m*M* Tris–HCl pH 8.0, 100 m*M*

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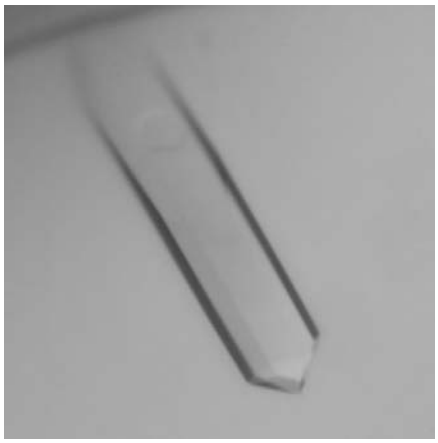

Figure 1
A crystal of *P. horikoshii* acylphosphatase grown from 0.8 *M* potassium/sodium tartrate, 100 m*M* citrate buffer pH 5.5 and 10 mg ml^{-1} protein at 293 K.

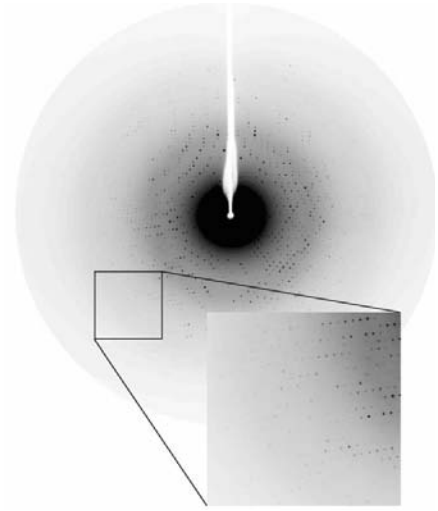

Figure 2
A 1° oscillation frame of a cryocooled crystal of acylphosphatase. Diffraction spots are observed to 1.72 Å.

NaCl) and lysed by sonication. After centrifugation, the supernatant was incubated at 353 K for 30 min. The supernatant after centrifugation was purified and concentrated by the ammonium sulfate fractionation method. The precipitant was dissolved in buffer *A* and dialyzed against buffer *A*. The resulting solution was loaded onto a Resource S 6 ml cation-exchange column (Amersham Bioscience). The proteins were eluted with a 120 ml linear gradient of 0–0.5 *M* NaCl in the same buffer. Pooled fractions were dialyzed against buffer *B* (10 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl) and concentrated to 10 mg ml^{-1} for crystallization.

Table 1
Crystal parameters of *P. horikoshii* OT3 acylphosphatase.

Values in parentheses are for the highest resolution shell.

X-ray source	SPring-8 BL41XU
Wavelength (Å)	1.000
Space group	$P3_221$
Unit-cell parameters (Å)	a = 86.6, b = 86.6, c = 75.4
Resolution range (Å)	30.0–1.72 (1.81–1.72)
Observed reflections	492961
Unique reflections	34672
Data completeness (%)	100 (100)
Redundancy	12.0 (3.8)
$R_{\rm merge}$†	0.063 (0.248)
$\langle I\rangle/\langle\sigma(I)\rangle$	14.6 (8.5)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average.

2.2. Crystallization

The initial crystallization conditions were screened by the sparse-matrix method using Crystal Screen HT (Hampton Research) and Wizard I and Wizard II (Emerald Biostructures). All crystallization experiments were performed using the sitting-drop vapour-diffusion method in Corning 96-well crystallization plates (Hampton Research) at 293 K. In initial trials, the drops were made up of equal amounts (1 µl) of reservoir solution and protein solution (10 mg ml^{-1} in buffer *B*). Initial crystals of the protein appeared from Wizard II No. 48 containing 1.0 *M* potassium/sodium tartrate and 100 m*M* MES buffer pH 6.0. The conditions were refined by varying the potassium/sodium tartrate concentration and buffer pH. The best crystals were obtained after 2 d using the following reservoir composition: 0.7–0.9 *M* potassium/sodium tartrate and 100 m*M* citrate buffer pH 5.5 (Fig. 1).

2.3. Data collection and processing

The native crystals were transferred into a cryoprotectant solution containing 0.8 *M* potassium/sodium tartrate, 0.1 *M* citrate buffer pH 5.5 and 20% ethylene glycol before being picked up and flash-cooled in a nitrogen stream. Diffraction data were collected to a resolution of 1.72 Å on BL41XU at SPring-8 at 100 K using a MAR CCD detector system (Fig. 2). Data were processed with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). The crystals belonged to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 86.6$, $c = 75.4$ Å. Consideration of the values of $V_{\rm M}$ suggests that these crystal have three, four or five molecules per asymmetric unit ($V_{\rm M}$ = 2.7, 2.0 and 1.6 Å^3 Da^{-1}, respectively; Matthews, 1968). The data statistics are given in Table 1. Structure determination was performed by molecular replacement using *MOLREP* v.7.3 (Vagin & Teplyakov, 1997) from the *CCP*4 v.4.2 software suite (Collaborative Computational Project, Number 4, 1994), using the coordinates of acylphosphatase from bovine tissue (PDB code 2acy; Thunnissen *et al.*, 1997) as a search model. The results of molecular replacement suggest these crystals contain two monomers per asymmetric unit and belong to space group $P3_221$. The Matthews coefficient and solvent content were 3.9 Å^3 Da^{-1} and 68%, respectively. Refinement of structural details is now in progress.

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