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Purification, crystallization and initial X-ray crystallographic analysis of the putative GTPase PH0525 from *Pyrococcus horikoshii* OT3

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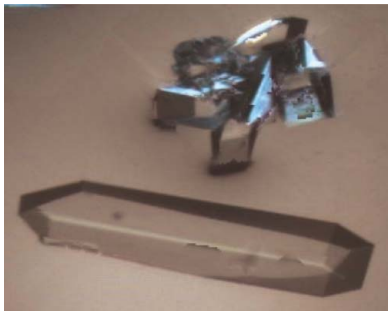


GTPases are involved in diverse cellular functions including cell proliferation, cytoskeleton organization and intracellular traffic. The putative GTPase PH0525 from *Pyrococcus horikoshii* OT3 has been overexpressed in *Escherichia coli* and purified. Two distinct crystal forms were grown by the microbatch method at 291 K using a very high protein concentration (80 mg ml^{-1}). Native data sets extending to resolutions of 2.3 and 2.4 Å have been collected and processed in space groups $P2_1$ and $C222_1$, respectively. Assuming the presence of one monomer per asymmetric unit gives V_M values of 2.6 and 2.4 Å^3 Da^{-1} for the $P2_1$ and $C222_1$ forms, respectively, which is consistent with dynamic light-scattering experiments, which show a monomeric state of the protein in solution.

1. Introduction

Proteins that bind and hydrolyze nucleoside triphosphates are crucial for almost all aspects of life. Guanosine triphosphatases (GTPases) are widely used as binary switches for cellular processes including signal transduction, protein translocation/trafficking and cell-cycle control (Boume *et al.*, 1990, 1991). Families of GTPases are widely distributed and include heterotrimeric GTP-binding proteins, small Ras-like GTP-binding proteins and translation-elongation GTP-binding factors. These proteins can be regarded as a molecular switch that is turned on by GTP binding and turned off by hydrolysis of GTP to GDP. Generally, a GTPase has different conformations depending on whether it is bound to GTP or GDP. Usually, bound GTP stabilizes the active conformation. In the GTP-hydrolysis reaction, the γ-phosphate group acts as a general base in abstracting a water proton; subsequently the generated nucleophilic hydroxide ion attacks the protonated γ-phosphate group to generate the penta-covalent reaction intermediate (Sondek *et al.*, 1994; Schweins *et al.*, 1997; Coleman *et al.*, 1994). This function is facilitated or inhibited by the action of three effectors: guanine-activating factor, guanine-exchange factor and guanine-dissociation inhibitors (Geyer & Wittinghofer, 1997; Yu & Schreiber, 1995; Barrett *et al.*, 1997).

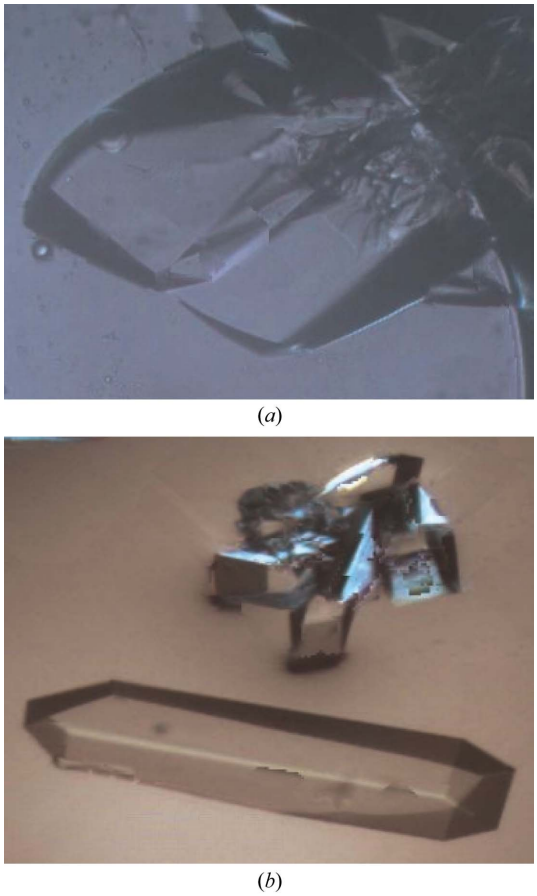
In terms of molecular evolution, the GTPases are defined as a monophyletic superclass within the P-loop NTPase fold. GTPases share three conserved sequence motifs (Leipe *et al.*, 2003): G*XXXX*GKT/S (where *X* denotes any amino acid), known as the Walker A nucleotide-binding motif, the Walker B motif hhhhD/E*XX*G, which binds a water-bridged magnesium ion (Walker *et al.*, 1982), and the GTP-specificity motif N/TK*X*D. On the basis of shared structural and sequence features, the GTPase superclass has been divided into two classes. The first class TRAFAC includes enzymes involved in translation (initiation, elongation and release factors), signal transduction (extended Ras-like family), cell motility and intracellular transport. The TRAFAC members share a characteristic antiparallel β-strand topology at the Walker B motif and typically function as monomers. The second class SIMBI is involved in protein localization, chromosome partitioning and membrane transport and includes a group of metabolic enzymes with kinase or related phosphate transferase activity. In contrast to TRAFAC, the SIMBI members share parallel β-strand topology at the Walker B motif and normally function as dimers (Leipe *et al.*, 2002).

In the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3, the PH0525 protein has been listed as a conserved hypothetical protein which possesses signature sequence motifs that are conserved across the family of GTP-binding proteins. PH0525 contains Walker A, Walker B and GTP-specificity motifs. Judging from sequence similarity, PH0525 may belong to the monomeric TRAFAC class of GTPases (28–34% identity). In order to understand its function and GTP-hydrolysis activity and as part of a structural genomics project, we intended to determine the crystal structure of PH0525 protein. Here, we report the purification, crystallization and preliminary crystallographic analysis of the putative GTPase PH0525 from *P. horikoshii* OT3.

2. Experimental

2.1. Protein expression and purification

The putative GTPase PH0525 from *P. horikoshii* OT3 used in this study has a molecular weight of 44.4 kDa and consists of 397 amino-acid residues. The plasmid encoding this protein, provided by RIKEN Genomic Sciences Center, was digested with *Nde*I and *Bgl*II and the fragment was inserted into the expression vector pET-11a (Novagen) linearized with *Nde*I and *Bam*HI. *Escherichia coli* BL21 Codon Plus (DE3)-RIL cells were transformed with the recombinant plasmid and grown without IPTG induction at 310 K in Luria–Bertani medium containing 50 µg ml^{-1} ampicillin for 20 h. Chloramphenicol was not added. The cells were harvested by centrifugation at 4500*g* for 5 min at 277 K and were subsequently suspended in 20 m*M* Tris–HCl pH 8.0 containing 0.5 *M* NaCl and 5 m*M* 2-mercaptoethanol. They were finally disrupted by sonication and heated at 363 K for 10 min. The cell debris and denatured protein were removed by centrifugation (18 000*g* for 30 min). The supernatant solution was used as the crude extract for purification.



(*a*)

(*b*)

Figure 1
Crystals of the putative GTPase PH0525. Crystals of the two distinct forms were grown in space groups $P2_1$ (*a*) and $C222_1$ (*b*), with approximate dimensions of 0.3 × 0.2 × 0.2 and 0.5 × 0.2 × 0.2 mm, respectively.

The crude extract was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) and applied onto a Super Q Toyopearl 650M (Tosoh) column equilibrated with 20 m*M* Tris–HCl pH 8.0 (buffer *A*). After elution with a linear gradient of 0–0.3 *M* NaCl, the fraction containing PH0525 was desalted using a HiPrep 26/10 desalting column (Amersham Biosciences) with buffer *A*. The sample was loaded onto a Resource Q column (Amersham Biosciences) equilibrated with buffer *A*. After elution with a linear gradient of 0–0.2 *M* NaCl, the fraction containing the protein was desalted using a HiPrep 26/10 desalting column with 10 m*M* sodium phosphate pH 7.0. The sample was then applied onto a Bio-Scale CHT-20-I column (Bio-Rad) equilibrated with 10 m*M* sodium phosphate pH 7.0 and eluted with a linear gradient of 10–150 m*M* sodium phosphate pH 7.0. The sample was concentrated by ultrafiltration (Vivaspin) and loaded onto a HiLoad 16/60 Superdex 200 prep-grade column (Amersham Biosciences) equilibrated with buffer *A* containing 0.2 *M* NaCl. The homogeneity and identity of the purified sample were assessed by SDS–PAGE (Laemmli, 1970) and N-terminal sequence analysis. Finally, the purified PH0525 was concentrated by ultrafiltration to 80 mg ml^{-1} in buffer *A* containing 0.2 *M* NaCl. The oligomeric state of purified PH0525 was examined by a dynamic light-scattering experiment using a DynaPro MS/X instrument (Protein Solutions), which was performed at a protein concentration of 20 mg ml^{-1} in 20 m*M* Tris–HCl pH 7.6 with 0.2 *M* NaCl. Several measurements were taken at 291 K and analyzed using the *DYNAMICS* software v.3.30 (Protein Solutions). A bimodal analysis resulted in a molecular weight of 47 kDa, which is consistent with the monomeric state of the protein in solution.

2.2. Crystallization

All crystallization trials were carried out using the microbatch method at 291 K. Initial crystallization conditions were established using the TERA (automatic crystallization) system (Sugahara & Miyano, 2002) from 144 independent conditions. Two initial conditions were identified and optimized by varying the protein concentration, the buffer concentration and the use of additives. Optimal conditions for crystal growth were obtained by mixing 1.0 µl protein solution (80 mg ml^{-1}) with 1.0 µl of a precipitant solution comprising 7.5%(*w*/*v*) PEG 20 000 and 0.1 *M* MES pH 6.7 (condition 1). Crystals suitable for X-ray analysis grew within three weeks. X-ray quality crystals were also grown by mixing 1.0 µl protein solution (80 mg ml^{-1}) with 1.0 µl of a precipitant solution comprising 27.5%(*w*/*v*) PEG 4000, 0.1 *M* HEPES pH 7.5 and 10%(*v*/*v*) dioxane (condition 2). Crystals suitable for X-ray analysis grew within 14–18 d (Fig. 1).

2.3. Data collection and analysis

Initial tests for the diffraction quality of the crystals were performed using a Rigaku R-AXIS V image-plate detector and Cu $K\alpha$ X-rays (Rigaku, Japan). Crystals of both forms 1 and 2 were flash-cooled in a cryoprotectant solution consisting of the respective precipitant solution containing 30%(*v*/*v*) glycerol. Crystals were cryocooled in a nitrogen-gas steam at 100 K. X-ray diffraction data for crystal form 1 were collected on a Rigaku R-AXIS V image-plate

Table 1
Data-collection statistics for the two crystal forms of the putative GTPase PH0525.

Values in parentheses are for the highest resolution shell.

	Form 1	Form 2
Space group	$P2_1$	$C222_1$
Unit-cell parameters (Å, °)	a = 43.86, b = 67.82, c = 77.21, β = 100.2	a = 74.96, b = 143.00, c = 78.93
Resolution range (Å)	40.0–2.30 (2.38–2.30)	30.0–2.40 (2.49–2.40)
Total observations	70619	49871
Unique reflections	18561	16622
Redundancy	3.8 (3.8)	3.0 (3.0)
Completeness (%)	93.7 (94.0)	97.6 (97.5)
Mean $I/\sigma(I)$	17.9 (5.8)	12.8 (5.2)
$R_{\rm merge}$† (%)	4.7 (24.6)	9.5 (21.3)

† $R_{\rm merge} = \sum_{hkl}\sum_j |I_j(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j \langle I(hkl)\rangle$, where $I_j(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity of measurement j and the mean intensity for reflections with index hkl, respectively.

detector (Rigaku, Japan) using Cu $K\alpha$ radiation from a Rigaku rotating-anode X-ray generator (50 kV, 70 mA). A complete data set consisting of 180 frames was collected with a crystal-to-detector distance of 200 mm, an oscillation angle of 1° and an exposure time of 10 min per frame. The X-ray source was equipped with an Osmic confocal mirror assembly. X-ray diffraction intensity data for crystal form 2 were collected at SPring-8 beamline BL26B1 using a Rigaku R-AXIS V image-plate detector. 120 frames were collected with 1° oscillation and 25 s exposure time per frame. The wavelength of the synchrotron radiation was 0.9791 Å and the distance between the crystal and the detector was 350 mm. The intensity data were indexed, integrated and scaled with *DENZO* and *SCALEPACK* implemented in the *HKL*2000 program package (Otwinowski & Minor, 1997).

3. Results and discussion

The growth of X-ray quality crystals of the putative GTPase PH0525 from *P. horikoshii* OT3 was highly dependent on the concentration of the protein. A protein concentration of 30 mg ml^{-1} does not produce any crystals. Increasing the protein concentration to 80 mg ml^{-1} greatly increased the size and quality of the crystals. Crystallization condition 1 yielded crystals belonging to the monoclinic space group $P2_1$, with unit-cell parameters a = 43.86, b = 67.82, c = 77.21 Å, β = 100.2° (form 1). Crystals grown under condition 2 belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 74.96, b = 143.00, c = 78.93 Å (form 2). Complete data sets were collected for both crystal forms and the data-collection statistics are summarized in Table 1. Assuming a protomer of PH0525 in the asymmetric unit, the Matthews coefficient $V_{\rm M}$ (Matthews, 1968) for the $P2_1$ form crystals was calculated to be 2.6 Å^3 Da^{-1}, corresponding to a solvent content of 52%. Similarly, the $V_{\rm M}$ value for the $C222_1$ form crystals was calculated to be 2.4 Å^3 Da^{-1}, corresponding to a solvent content of 48%. A dynamic light-scattering experiment gives a result consistent with a monomeric state of this protein in solution (see §2). From these observations, the asymmetric unit is most likely to contain a monomer of PH0525 in both forms. Recently, the crystal structure of YchF GTPase, which shows the highest sequence identity (30%) to PH0525, has been reported (Teplyakov *et al.*, 2003). The sequence similarity between these two proteins is evenly distributed. However, we failed to solve the structure of PH0525 by the molecular-replacement method using the YchF protein coordinates, indicating that there is substantially different folding in these two proteins. Therefore, we intend to solve the structure of PH0525 using the multiple anomalous dispersion method (Hendrickson *et al.*, 1990). The study of this putative GTPase will be particularly focused on rational enhancement of its thermostability on the basis of its three-dimensional structure and its detailed GTP-hydrolysis/catalytic mechanism.

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