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Purification, crystallization and preliminary X-ray crystallographic analysis of *Pyrococcus furiosus* DNA polymerase

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Abstract

DNA polymerase gene from the hyperthermophilic Archaeon *Pyrococcus furiosus* has been cloned and the protein over-expressed in *Escherichia coli* to produce an active enzyme. The purified protein was crystallized from 0.08 *M* ammonium sulfate, 0.05 *M* Na-cacodylate, pH 6.5, 0.15%(*v*/*v*) NP40, 0.05%(*v*/*v*) Tween 20 and 4.5%(*w*/*v*) polyethylene glycol 6000 by the vapour-diffusion method. The orthorhombic crystals had unit-cell dimensions of a = 92.5, b = 125.4, c = 192.1 Å; $\alpha = \beta = \gamma = 90°$. The crystals diffracted beyond 4 Å on a 1.08 Å synchrotron radiation source.

1. Introduction

DNA polymerases have the important roles of replication and repair of genetic material. Many laboratory techniques, including the polymerase chain reaction (PCR), utilize these enzymes and in addition, they are important pharmacologically since they are the targets of several antiviral and anti-tumor drugs. DNA polymerase genes from many organisms have been cloned and their deduced amino-acid sequences have been compared. Based on similarities in their amino-acid sequences and drug sensitivities, DNA polymerases have been classified into three major groups: the *Escherichia coli* DNA polymerase I (Pol I) family, the eukaryotic DNA polymerase α family, and the RNA-dependent DNA polymerase family (Delarue *et al.*, 1990). In the last few years, several DNA polymerases from thermophilic archaebacteria have been identified (Perler *et al.*, 1992; Bergseld *et al.*, 1992; Cline *et al.*, 1992; Pisani *et al.*, 1992) and all are found to belong to the Pol I family of enzymes. The crystal structures of three thermostable DNA polymerases, *Thermus aquaticus* DNA polymerase (Kim *et al.*, 1995), Klentaq1 (Konolev *et al.*, 1995), a fragment analogous to Klenow fragment and *Bacillus stearothermophilus* large fragment DNA polymerase I (Kiefer *et al.*, 1997), have been solved at 3.0, 2.4 and 2.1 Å resolution, respectively.

Here we present the cloning, purification and crystallization of DNA polymerase from *Pyrococcus furiosus* (*Pfu*), a hyperthermophilic archeon that has an optimum growth temperature of 373 K. *Pfu* DNA polymerase has significant sequence homology to the eukaryotic α-like DNA polymerases represented by human DNA polymerase a and *E. coli* DNA polymerase II (Uemori *et al.*, 1993), showing a homology in 37 out of 57 residues in a conserved region (Mathur *et al.*, 1991). We would like to further our understanding for the structural basis of hyperthermostability of this enzyme. *Pfu* DNA polymerase has 5′ to 3′ polymerase activity as well as 3′ to 5′ proofreading exonuclease activity. *Pfu* polymerase has the lowest error rate of any thermostable DNA polymerase so far identified, thus making it the preferred DNA polymerase for techniques that require high-fidelity DNA synthesis by the polymerase chain reaction (Lundberg *et al.*, 1991; Scott *et al.*, 1994). This intact DNA polymerase consists of 769 amino acids with an N-terminal poly-histidine tag with a calculated molecular weight of 92.5 kDa. The purified enzyme yielded long rod-like single crystals. Optimization of crystallization conditions are in progress.

2. Experimental

2.1. *Bacterial strain and plasmid*

Pfu genomic DNA was generously donated by Dr Robert Kelly of North Carolina State University. For cloning and expression of the DNA polymerase gene we used *E. coli* K-12 strain BL21 (DE3) {F$^-$ompT [lon] hsdSβ ($r_B^-m_B^-$)} carrying a derivative of plasmid pACYC184 (gift of Dr David Wemmer, University of California, Berkeley). This plasmid, pACYC184A, has an origin of replication that allows it to co-exist with vectors that carry the ColE1 origin and a gene that allows for overexpression of the lactose repressor and is maintained by kanamycin selection (50 μg ml^{-1}). The presence of this plasmid allowed high levels of expression of the pET16b plasmid containing the *Pfu* DNA polymerase gene sequence. The expression vector pET16b was obtained from Novagen (Madison, WI). The *Pfu* polymerase gene was cloned into the *Nde*I and the *Xho*I sites of vector pET16b. This vector carries the His-Tag sequence which codes for a stretch of ten consecutive histidine residues that are expressed at the N-terminal end of the protein and a total of 21 residues are added to the 5′ end of the protein.

2.2. *Cloning and sequencing of the P. furiosus DNA polymerase gene*

Standard molecular biological techniques were carried as described in Maniatis *et al.* (1982). A lambda genomic DNA library was prepared and the DNA polymerase gene was cloned using primers synthesized against the gene based on the sequence published by the Yoshizumi Inhino Biotechnology Research Laboratory in Japan (Genebank accession number D12983). The upstream primer (5′-GCGGCCGCATATGATTTTAGATGTGGATTACATAACTGAAGAAGG-3′) and the downstream primer sequence (5′-GTCGAGCTCGAGCTAGGATTTTTTAATGTTAAGCCAGGAAGTTAGG-3′) were synthesized by Genset (La Jolla, CA, USA).

2.3. *Expression, purification and assay*

The *E. coli* BL21 (DE3)/pACYC184 A cells harboring pET16b/*Pfu* DNA pol plasmid were grown at 310 K in 1 l of

LB medium supplemented with ampicillin (50 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). At an OD_{600} of 1.0, the cells were induced with IPTG at a final concentration of 0.5 m*M* for 3 h. Cells were harvested and resuspended in 20 ml of 20 m*M* Tris–HCl, pH 8.0, 25%(*w*/*v*) sucrose, 1 m*M* EDTA, 1 m*M* PMSF (phenyl methyl sulfonyl fluoride), 1 m*M* DTT (dithiothreitol), 0.01%(*v*/*v*) NP-40 and lysozyme at a final concentration of 100 μg ml^{-1}. After disruption by sonication, the crude extract was spun for 20 min at 15 000*g* and the supernatant was heated at 353 K for 30 min to precipitate heat unstable proteins from *E. coli*. Approximately 50% of the DNA polymerase expressed was soluble. The denatured *E. coli* proteins were removed by centrifugation at 15 000*g* for 20 min. The heat-treated extract was then applied to a His–Bind metal chelation resin (Novagen) which had been equilibrated in 20 m*M* Tris–HCl, pH 7.4, 0.5 *M* NaCl. After washing away the unbound proteins, DNA polymerase was eluted with 100 m*M* EDTA. After buffer exchange, the *Pfu* DNA polymerase was applied onto a S-Sepharose column (Pharmacia) previously equilibrated with 50 m*M* Tris, pH 8.7, 50 m*M* NaCl, 0.5 m*M* EDTA, 1 m*M* DTT (buffer *A*). A 20 column volume linear gradient of 0–1 *M* NaCl in buffer *A* was applied. *Pfu* DNA polymerase eluted at 150 m*M* NaCl. The protein was further purified through a Superdex 200 (Pharmacia) column in 50 m*M* Tris–HCl, pH 8, 0.5 *M* NaCl, 0.5 m*M* EDTA, 1 m*M* DTT. All the purification steps were performed at room temperature. The yield was 3–10 mg l^{-1} of cell culture. Enzyme purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE, Laemmli, 1970). The single band on SDS–PAGE corresponded to a molecular weight of 92.5 kDa. *Pfu* DNA polymerase was concentrated to 12.5 mg ml^{-1} and activity assays were carried out using PCR (Cline *et al.*, 1995). The enzyme routinely had an activity of 2.5–7.5 units μg^{-1} of enzyme.

2.4. *Crystallization*

Crystallization conditions were tested using the sparse-matrix sampling method (Jancarik & Kim, 1991) using the hanging-drop vapour-diffusion method with 2 μl drops (1 μl of protein and 1 μl of mother liquor) equilibrated against 500 μl of reservoir solution at room temperature. Crystals were obtained in a solution of 0.08 *M* ammonium sulfate, 0.05 *M* Na-cacodylate, pH 6.5, 0.15%(*v*/*v*) NP40, 0.05%(*v*/*v*) Tween 20 and 4.5%(*w*/*v*) polyethylene glycol 6000 (Fig. 1).

3. Results and discussion

The crystals grew within 14 d at room temperature with dimensions of 0.2 × 0.05 × 0.05 mm. They diffracted to beyond 4 Å resolution at Stanford Synchrotron Radiation Laboratory beamline VII-I with 1.08 Å synchrotron X-ray source. The crystals of *Pfu* polymerase are of *C*-centered orthorhombic lattice with cell parameters a = 92.5, b = 125.4, c = 192.1 Å; $\alpha = \beta = \gamma = 90^\circ$ (Fig. 2). Assuming one molecule of *Pfu* polymerase per asymmetric unit, the calculated V_m is 3.0 Å^3 Da^{-1} corresponding to a solvent content of approximately 59%. Because crystals decayed rapidly under X-ray exposure, crystallization buffer containing 30%(*w*/*v*) PEG 6000 was used as a cryoprotectant to allow low-temperature data collection. Frozen crystals of *Pfu* DNA polymerase diffracted to 5.5 Å at 100 K on an RAXIS IIC imaging-plate system with Cu $K\alpha$ X-ray from a Rigaku rotating-anode X-ray generator.

Further low-temperature data collection and heavy-atom screening will be conducted at synchrotron radiation facilities.

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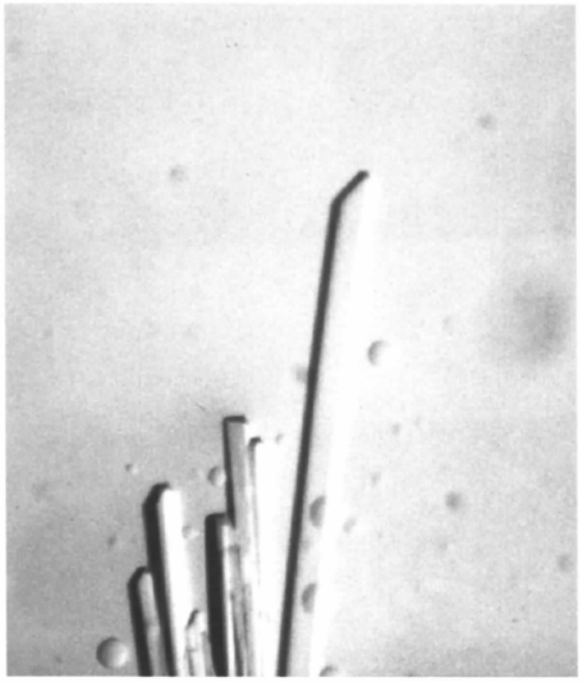

Fig. 1. Crystals of *Pfu* DNA polymerase from *Pyrococcus furiosus*. The largest dimension is 0.2 mm.

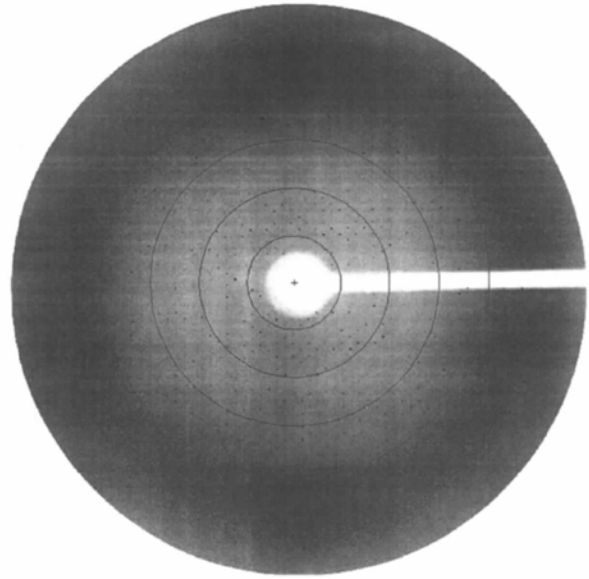

Fig. 2. Data were collected on a MAR imaging-plate detector with 1.08 Å synchrotron radiation at SSRL beamline VII-1. The oscillation angle was 1°. The outer circle indicates resolution at 4 Å.

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