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Crystallization and Preliminary X-Ray Analysis of a DNA Primase from Hyperthermophilic Archaeon *Pyrococcus horikoshii*[1]

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At the initiation of chromosomal DNA replication, DNA primases synthesize short RNA primers, which are subsequently elongated by DNA polymerases. To understand the structural basis for the primer synthesis by archaeal/eukaryotic-type primases, the gene of the DNA primase from hyperthermophilic archaeon *Pyrococcus horikoshii* was cloned and overexpressed in *Escherichia coli* as a fusion protein with a hexa-histidine tag at its amino terminus. The recombinant DNA primase was purified and crystallized by the hanging-drop vapor diffusion method at 293K, with polyethylene glycol 8000 as the precipitant. The crystals belong to the $P3_221$ space group with unit-cell parameters $a = b = 77.8$, $c = 129.6$ Å, and $\alpha = \beta = 90°$, $\gamma = 120°$. Crystals of the selenomethionine derivative were obtained by means of a cross-seeding method using native crystals. The data for the native and selenomethionine-substituted crystals were collected to 1.8 and 2.2 Å resolution, respectively, with synchrotron radiation at SPring-8 under flash-frozen conditions at 100K. The four wavelength MAD data provided a phase to determine the structure of the primase at 2.2 Å resolution.

Key words: crystallization, DNA primase, DNA replication, hyperthermophilic archaeon, X-ray crystallography.

Primase is an essential enzyme of the cellular DNA replication machinery (*1*). On single-stranded DNA, eukaryotic DNA primase synthesizes the first dinucleotide and then elongates it to approximately 10 nucleotides in length (*2*). In addition to the amino acid sequence homology among the primases, their very limited processivity and low fidelity distinguish them from other replicative polymerases (*3*). The primases involved in chromosomal DNA replication can be divided into two families, bacterial-type and archaeal/eukaryotic-type primases (*4*). Recently, the crystal structures of both types of primases, from *Escherichia coli* and *Pyrococcus furiosus*, were reported, which revealed that the catalytic domains of bacterial and archaeal/eukaryotic primases have distinct architectures (*4–8*). While the initiation activity of a primase is essential to start DNA replication *de novo*, the initiation mechanisms as well as those of the binding with the template DNA have not yet been determined for these primases. In order to elucidate the catalytic mechanism of the archaeal/eukaryotic-type of DNA primase, we crystallized a DNA primase from hyperthermophilic archaeon *P. horikoshii*, and determined the phase by means of a multiple anomalous dispersion method using a selenomethionine (SeMet)-substituted crystal. Here, we report the crystallization and preliminary X-ray crystallographic study of the DNA primase from *P. horikoshii* (PhPri).

An open reading frame encoding a protein homologous to the eukaryotic DNA primase catalytic subunit was identified in the genomic DNA sequence database of *P. horikoshii* (*9*). The length of the open reading frame is 1041 nucleotides, and it encodes a 346 amino acid protein with a calculated molecular mass of 40,270 Da. The PhPri cDNA was amplified by the polymerase chain reaction from *P. horikoshii* genomic DNA (purchased from the Japan Collection of Microorganisms; RIKEN) using an upstream primer (5′-CAT ATG CTC CTA AGA GAG GTA ACA AGA-3′) and a downstream primer (5′ GGA TCC TTA AGT TCC GAG ACT TTC CAG GAA-3′). For subcloning, artificial *Nde*I and *Bam*HI sites were included in the primers (indicated by underlines). The amplified fragment was cloned into the *Sma*I site of pBluescript KS(+) to verify the nucleotide sequence. The resultant plasmid, pBS-PhPri, was digested with *Nde*I and *Bam*HI, and the resultant fragment was subcloned into 6xHis-pET11d. This DNA construct, termed 6xHis-pETPhPri, was used for the expression of PhPri, which has an amino terminal 20 amino acid extension con-

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taining a hexa-histidine tag and the sequence for thrombin digestion (MGSSHHHHHHSSGLVPRGSH).

To overproduce the DNA primase homolog from *P. horikoshii*, an *E. coli* strain, BL21 codon plus (DE3) (Stratagene), was transformed with 6xHis-pETPhPri, and the precultured at 37°C in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Ten milliliters of the overnight culture was added to 1.5 liters of LB medium containing the same antibiotics, and then the culture was incubated at 37°C until the absorbance at 600 nm reached 0.6. The cells were induced by adding isopropyl 1-thio-β-D-galactoside (IPTG) to 0.5 mM, and the cultivation was continued at 30°C for 3 h. The induced cells were collected by low speed centrifugation, and the cell pellet was stored at –80°C before use. For extraction of the proteins, the thawed pellet was suspended in sonication buffer [50 mM Hepes-NaOH (pH 7.8), 10% glycerol, 0.01% Nonidet P-40, 5 mM 2-mercaptoethanol] containing 0.5 M NaCl, sonicated in an ice-water bath, and then clarified by centrifugation (lane 2 in Fig. 1). All of the following chromatographic steps were carried out at 4°C, using an FPLC system (Amersham Pharmacia Biotech). The resultant extract containing the overexpressed protein was loaded onto a nickel ion–charged HiTrap-chelating column (Amersham Pharmacia Biotech; 1 ml), and then the column was washed with sonication buffer containing 0.5 M NaCl. The protein was eluted with 20 ml of a linear gradient of 0.2 M imidazole and 0.1 M NaCl in sonication buffer. The fractions containing the 6xHis-tagged PhPri were identified by SDS-PAGE (lane 4 in Fig. 1). The peak fractions were collected and loaded onto a HiTrap heparin column (Amersham Pharmacia Biotech; 5 ml) equilibrated with 10 mM Hepes-NaOH (pH 7.8) containing 0.1 M NaCl. After a 0.3 M NaCl wash, the bound proteins were eluted with 20 ml of a linear gradient of NaCl, from 0.3 to 1 M. PhPri was eluted at around 0.6–0.8 M NaCl. At this step, PhPri appeared to be homogeneous on a SDS–polyacrylamide gel (lane 5 in Fig. 1).

To verify the primase activity of the purified protein, *de novo* primer synthesis was assayed by the formation of oligoribonucleotide products on a single stranded M13 DNA (M13 sscDNA) template. The assay conditions were as follows. The heparin fraction (176 ng) was incubated in a 25 μl reaction mixture comprising 18 mM Tris-HCl buffer (pH 8.0) containing 3 mM 2-mercaptoethanol, 7.2 mM $MgCl_2$, 0.18 mg/ml bovine serum albumin, 1 unit/μl RNase inhibitor (Takara Shuzo), 1.8 mM ATP, 0.18 mM CTP, 0.18 mM GTP, 10 μM [α-^{32}P]UTP (6 μCi), and 1 μg of M13 sscDNA. The mixture was incubated for 15 min at 37 or 60°C. The reaction was terminated by the addition of 20 mM Na_3EDTA (pH 8.0), and the resultant mixture was treated with bacterial alkaline phosphatase (Takara Shuzo). The mixture was precipitated by the addition of 2 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2) at –20°C. The precipitate was dissolved in 5 μl of sample buffer (97% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol), and then heated for 5 min at 95°C. These samples were loaded onto a 20% acrylamide gel containing 7 M urea, 89 mM Tris borate (pH 8.0), and 1 mM Na_3EDTA, and then electrophoresed at 2,000 V for 2.5 h. Autoradiography was performed at –80°C with Kodak XAR-5 X-ray film. Oligoribonucleotides, which had been labeled with [α-^{32}P]UTP, were efficiently produced when the primase was incubated at 37°C (lane A in Fig. 2). However, the primase activity was very low at 60°C (lane B in Fig. 2), in striking contrast to in the case of the *P. furiosus* primase (*10*). Furthermore, while Bocquier *et al.* recently reported that the presence of $MnCl_2$ in the reaction mixture enhanced the primase activity of *P. furiosus* at 60°C, the primase activity at 60°C of PhPri under our assay conditions was not enhanced by the replacement of 8 mM $MgCl_2$ with 4 mM $MnCl_2$ (data not shown). On the other hand, the oligoribonucleotide primers produced by *P. furiosus* and *P. horikoshii* are usually much longer than the primers synthesized by both the eukaryotic and *M. jannaschii* primases (*10, 11*) (lane A in Fig. 2). However, there are no plausible explanations for the differences in the lengths of the primers produced by the primases.

For crystallization, a micro-concentrator (Sartørius; Centrisart cutoff 20,000) was used to reduce the NaCl concentration to 0.1 M as well as to concentrate the protein up to 10 mg/ml. For initial crystallization trials, the hanging-drop vapor-diffusion method was used with a commercially available screening kit (from Hampton Research), the original

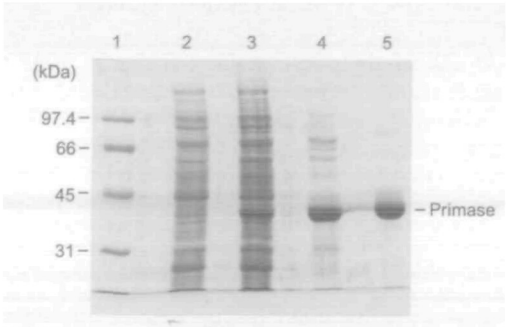

Fig. 1. **Expression of the *P. horikoshii* DNA primase.** A 10% SDS–polyacrylamide gel (CBB stain) showing expression of the *P. horikoshii* DNA primase and the subsequent purification steps. Lane 1, low molecular weight markers (BioRad); lane 2, uninduced BL21 codon plus (DE3) carrying 6xHis-pETPhPri; lane 3, IPTG-induced BL21 codon plus (DE3) carrying 6xHis-pETPhPri; lane 4, Ni^{2+}-chelating-Sepharose purification; lane 5, heparin-Sepharose purification.

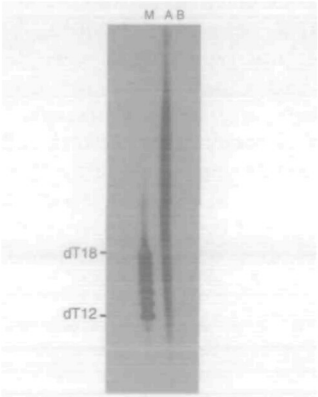

Fig. 2. **Primase activity of the recombinant *P. horikoshii* DNA primase.** The DNA primase (176 ng) was incubated in a reaction mixture containing 6 M [α-^{32}P]UTP and M13 DNA. The incubation was carried out at 37°C (lane A) or 60°C (lane B), followed by resolution by denaturing polyacrylamide gel electrophoresis as described in the text. 5′-Monophosphorylated oligo $d(T)_{12\text{-}18}$ were used as molecular markers.

kit solutions being diluted 2-fold before use. The protein solution (1 μl of 10 mg/ml) was mixed with an equal volume of the reservoir solution, and each hanging drop was then vapor-equilibrated against 0.4 ml of the reservoir solution supplemented with 0.1 M NaCl at 20°C. Since PhPri easily aggregated at a low salt concentration within several hours, storage of the protein solution after desalting and concentration is quite difficult. Therefore, it is necessary to purify a sample just before the crystallization experiment. On initial screening, crystals appeared in 12 h under six sets of conditions at 20°C, the largest crystal being obtained with 10% PEG8000 and 25 mM potassium phosphate (pH 7) (Crystal Screen™ #42). The crystals diffract X-ray up to 6 Å with the use of the in-house diffractmeter. However, the crystals formed under these conditions were not large enough for further structural determination. Therefore, to optimize the crystallization conditions, we mixed a one-ninth volume of each solution of the Crystal Screen™ kits 1 and 2 with the reservoir solution, a drop being formed as described above. Three kit solutions were found to improve the crystal size, Crystal Screen™ #15 [0.2 M ammonium sulfate, 30% PEG8000, and 0.1 M sodium cacodylate (pH 6.5)] being the best. Finally, the best crystals of 0.1 mm × 0.1 mm × 1 mm were obtained under the conditions of 10.5% PEG8000, 10 mM ammonium sulfate, 22.5 mM potassium phosphate (pH 7), and 5 mM sodium cacodylate (pH 6.5) (Fig. 3A). However, to our surprise, when we harvested the crystals with a 1.5 times concentration of the reservoir solution, no diffraction was observed in the in-house X-ray diffraction experiment (data not shown). Therefore, we harvested the crystals with a 1.5 times concentration of the basal crystallization solution [15% PEG8000 and 37.5 mM potassium phosphate (pH 7)], without ammonium sulfate or sodium cacodylate used for the crystallization. The crystals diffracted X-rays up to 4 Å, as described below, but suffered serious radiation damage. These results suggest that the additives used in the crystallization are good for crystal growth but are not good for X-ray diffraction. Furthermore, our findings suggest that the removal of the chemicals nonessential for the preservation of crystals can be applied to improve the diffraction of other crystals.

Preliminary X-ray data were collected at room temperature on a Rigaku R-AXIS IV imaging-plate system, using Cu*K*α radiation from a Rigaku RU300 rotating-anode generator operated at 40 kV and 100 mA. In this in-house experiment at room temperature, the crystals diffracted up to 4 Å resolution. Then, to collect high-resolution data, we examined some chemicals as anti-freezing reagents for cryo-crystallography at the synchrotron. Crystals were damaged when we used glycerol, ethylene glycol, trehalose, and 2-methyl-2,4-pentanediol, but not polyethylene glycol 400. Therefore, we used polyethylene glycol 400 as the cryo-protectant for data collection at cryogenic temperatures with liquid nitrogen gas. Prior to the collection of high resolution data, the crystals were transferred to a micro dialysis button, and the button was dialyzed against the harvest buffer containing 10 and 20% PEG400 for 30 min, respectively.

Autoindexing and data processing were performed with the *DENZO* and *SCALEPACK* programs (*12*). The autoindexing option of *DENZO* suggested a trigonal space group, which was further characterized as $P3_121$ or $P3_221$ during data scaling. The unit-cell dimensions, $a = b = 77.8$, $c = 129.6$ Å, give a unit-cell volume of 679,332 Å^3, which can accommodate 6PhPri molecules, giving a reasonable V_m value of 2.8 Å^3 Da^{-1}, indicating one PhPri molecule in the asymmetric unit. By flash-freezing the native crystals, we collected data up to 1.8 Å resolution using the mar-CCD detector at the beamline 41XU at SPring-8 (SP-8 BL41XU) (Harima, Hyogo). Although the amino acid sequence similarity between the *P. horikoshii* and *P. furiosus* DNA primases is as high as 87.8%, the space groups and unit cell parameters of the two protein crystals are different. This is probably because the crystallizing precipitants were different, which changes the manners of crystal packing of the two protein crystals.

Since phase determination by the multiple isomorphous replacement method was unsuccessful, due to the signifi-

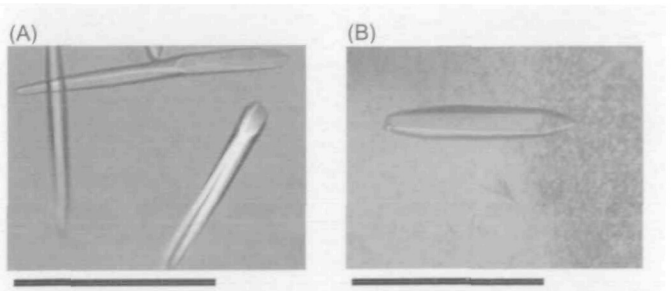

Fig. 3. **Crystals of the *P. horikoshii* DNA primase.** (A) Native crystals. (B) A selenomethionine derivative crystal. The scale bars correspond to 0.5 mm.

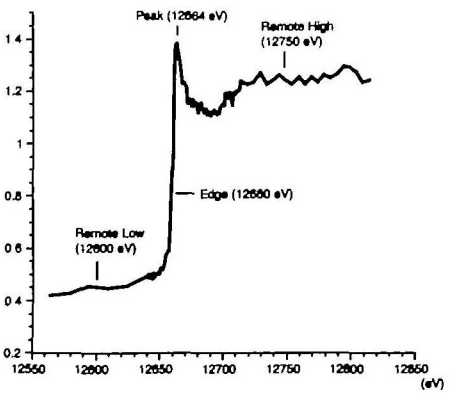

Fig. 4. **X-ray fluoresence spectra for a selenomethionine derivative crystal.** The four wavelengths chosen for diffraction measurements in the MAD experiments are indicated. The data were collected from BL41XU at SPring-8.

TABLE I. **Crystallographic data.**

X-ray source	SPring-8 BL41XU
Wavelength (Å)	0.9790
Resolution (Å)	50–2.2
Unique reflections	23,387
Redundancy	9.1
Completeness (%)[a]	99.3 (99.2)
I/σ (I)[a]	13.8 (5.2)
R_{merge} (%)[a, b]	4.7 (23.8)

[a]Numbers in parentheses correspond to the values in the highest resolution shell. [b]$R_{merge} = \Sigma_h\Sigma_i|I_{hi}-\langle I_h\rangle|/\Sigma_h\Sigma_i|I_{hi}|$, where h represents unique reflection indices, and i indicates symmetry equivalent indices.

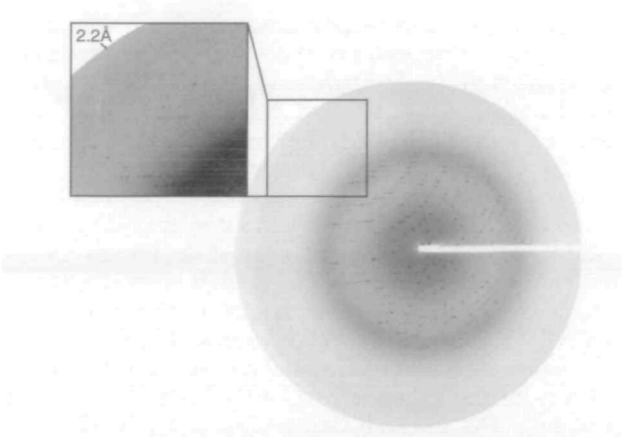

Fig. 5. **An oscillation image of a selenomethionine-substituted crystal of the primase with synchrotron radiation at beamline 41XU of SPring-8.** The resolution of the highest-order reflection is 2.2 Å.

cant non-isomorphism of the crystals, we used the MAD method with a SeMet-substituted crystal for structure determination. The SeMet-substituted protein was prepared by growing the *E. coli* methionine-auxotroph B834 (DE3) strain (Novagen) transformed with 6xHis-pETPhPri in LeMaster's medium, in which the methionine was replaced by 30 mg/liter L-SeMet (Sigma) (*13*). The SeMet crystals were obtained by the cross-seeding method, with half the concentration of the precipitant used for the native protein (Fig. 3B). The SeMet derivative crystals diffracted beyond 2.2 Å resolution at SP-8 BL41XU, and a clear X-ray absorption fluorescence spectrum (XAFS) of the SeMet derivative crystal was observed (Fig. 4), from which the measurement wavelengths for the MAD data were determined. Four wavelength MAD data were collected at SP-8 BL41XU (Table I). The phase determined from the MAD data was sufficient for structure determination, and so we determined the structure of the primase at 2.2 Å resolution (data not shown). Furthermore, when we soaked the native crystals in a fresh preparation of 10 mM UTP and UTP derivatives for 6 h, we observed clear electron density of the triphosphate moiety and definitely identified the enzyme active site. The details of the structure of the *P. horikoshii* primase and that in a complex with nucleotides will be discussed elsewhere (Ito, N. *et al.*, in preparation).

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