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Crystallographic Studies on a Family B DNA Polymerase from Hyperthermophilic Archaeon *Pyrococcus kodakaraensis* Strain KOD1[1]

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A hyperthermostable family B DNA polymerase from the hyperthermophilic archaeon, *Pyrococcus kodakaraensis* strain KOD1, has been crystallized by the hanging-drop vapor diffusion method at 293 K with 2-methyl-2,4-pentanediol as the precipitant. The diffraction pattern of a crystal extends to 3.0 Å resolution, and two full sets of 3.0 Å resolution diffraction data for native crystals were successfully collected at 290 K and 100 K upon exposure to synchrotron radiation at KEK-PF, Japan. The crystals belong to the space group, $P2_12_12_1$, with unit-cell dimensions of $a=112.8$, $b=115.4$, and $c=75.4$ Å at 290 K, and $a=111.9$, $b=112.4$, and $c=73.9$ Å at 100 K. Structural analysis by means of the multiple isomorphous replacement method is now in progress.

Key words: crystallization, family B DNA polymerase, hyperthermophilic archaeon, polymerase chain reaction, X-ray crystallography.

The DNA polymerases are a group of enzymes that use single-stranded DNA as a template for the synthesis of the complementary DNA strand. These enzymes have multifunction, synthetic mode (polymerase) and one or two degradative modes (5′-3′ and/or 3′-5′ exonucleases), and play an essential role in nucleic acid metabolism, including in the process of DNA replication, repair and recombination. More than 50 DNA polymerase genes have been cloned and sequenced. The amino acid sequences deduced from their nucleotide sequences can be classified into four major types: *Escherichia coli* DNA polymerase I (family A), *E. coli* DNA polymerase II (family B), *E. coli* DNA polymerase III (family C), and others (family X) (*1*). The DNA polymerases in family B are called α-like DNA polymerases because they have the amino acid sequence of conserved eukaryotic DNA polymerase α (*2*).

In this paper, we report the crystallization and preliminary X-ray diffraction analysis of a family B DNA polymerase obtained from the hyperthermophilic archaeon, *Pyrococcus kodakaraensis* strain KOD1 (KOD DNA polymerase). *P. kodakaraensis* KOD1 was isolated from a sulfataric hot spring on Kodakara Island, Kagoshima. This strain is one of the most thermostable organisms known, with an optimum growth temperature of 95°C (*3*). Enzymes produced by this strain were reported to be extremely thermostable and to have eukaryotic characteristics (*4*). Crystallization of O^6-methylguanine-DNA methyltransferase, a DNA repair protein, and the crystal structure of aspartyl-tRNA synthetase obtained from this strain have been reported (*5, 6*). The enzymes from hyperthermophiles are extremely thermostable and thus are of industrial importance. The optimum temperature (75°C) and mutation frequency (3.5×10^{-3}) of KOD DNA polymerase are similar to those of the DNA polymerase obtained from *Pyrococcus furiosus* (*Pfu* DNA polymerase), however, KOD DNA polymerase exhibits a 5 times higher extension rate (100 to 130 nucleotides/sec) and 10 to 15 times higher processivity than those of *Pfu* DNA polymerase (*7*). KOD DNA polymerase is, therefore, an enzyme of considerable biological, technological, and economic importance. Thermostable DNA polymerases are widely used in polymerase chain reaction (PCR) experiments.

The crystal structures of three thermostable DNA polymerases, *Thermus aquaticus* DNA polymerase (*taq* polymerase)(*8*), Klentaq1 (*9*), and a fragment analogous to Klenow fragment and *Bacillus stearothermophilus* large fragment DNA polymerase I (*10*), and DNA polymerase α (family B) from bacteriophage RB69 (*11*) have been solved at 3.0, 2.4, 2.1, and 2.7 Å resolution, respectively. Except for RB69 DNA polymerase, these enzymes exhibit no similarity with KOD DNA polymerase. RB69 DNA polymerase shares conserved amino acid regions with archaeal DNA polymerases (*11*). But no information on the three-dimensional structures of archaeal DNA polymerases has ever been reported. The structure of KOD DNA polymerase may provide useful information for increasing the

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thermostability of enzymes already in use, or for designing new hyperthermostable proteins. Furthermore, structural comparison of DNA polymerases from various species may provide evolutional knowledge, because DNA polymerase is essential for DNA replication and all organisms have their own DNA polymerases. Most recently, three archaeal DNA polymerases were crystallized (*12-14*). Structural comparison of these archaeal DNA polymerases may reveal the evolutional relationships among these species.

KOD DNA polymerase was overexpressed in *E. coli* strain BL21(DE3) and purified by the previously reported method (7). KOD DNA polymerase was concentrated to about 25 Abs cm^{-1} at 280 nm in a Tris buffer, pH 8.5, containing 0.1 mM EDTA and 1 mM DTT using a Centricon-50 concentrator (Amicon, Beverly, MA). All the crystallization experiments were carried out by the hanging drop vapor diffusion technique. A droplet (typically 4 μl) was prepared by mixing equal volumes (2 μl) of the protein and reservoir solutions. Preliminary screening of crystallization conditions was performed with a Crystal Screen kit (Hampton Research, Laguna Hills, CA, USA). Then, a number of precipitants including salts, polyethylene glycol solutions, and organic solvents were examined over a broad

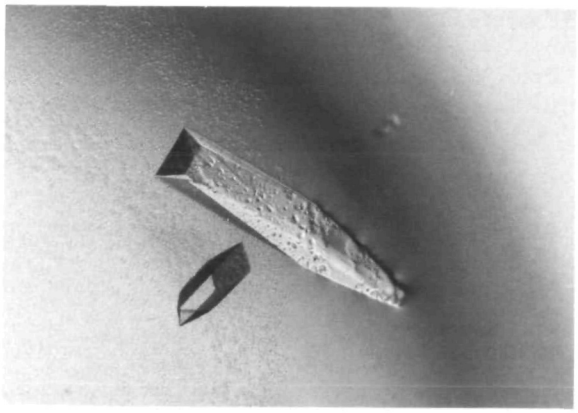

Fig. 1. **Crystals of KOD DNA polymerase with maximum dimensions 0.4×0.1×0.1 mm.**

TABLE I. **Diffraction data statistics of a KOD DNA polymerase crystal at 290 K (a) and 100 K (b), processed and scaled with *DENZO* and *SCALEPACK*.**

Resolution (Å)	No. of reflections (unique)	Completeness (%)	R_{merge}
(a) At 290 K			
40.00-6.46	2,123	97.0	0.039
6.46-5.13	2,041	98.4	0.074
5.13-4.48	2,006	98.5	0.082
4.48-4.07	1,989	97.5	0.114
4.07-3.78	1,922	96.0	0.165
3.78-3.56	1,904	94.2	0.228
3.56-3.38	1,824	91.1	0.290
3.38-3.23	1,733	87.3	0.342
3.23-3.11	1,717	85.5	0.389
3.11-3.00	1,667	83.7	0.411
Overall	18,926	93.0	0.094
(b) At 100 K			
40.00-6.46	1,902	91.4	0.042
6.46-5.13	1,819	92.9	0.071
5.13-4.48	1,817	93.8	0.070
4.48-4.07	1,777	92.6	0.089
4.07 3.78	1,769	91.9	0.117
3.78-3.56	1,718	90.0	0.153
3.56-3.38	1,712	90.5	0.191
3.38-3.23	1,687	89.0	0.230
3.23-3.11	1,650	87.5	0.279
3.11 3.00	1,632	86.1	0.316
Overall	17,483	90.6	0.085

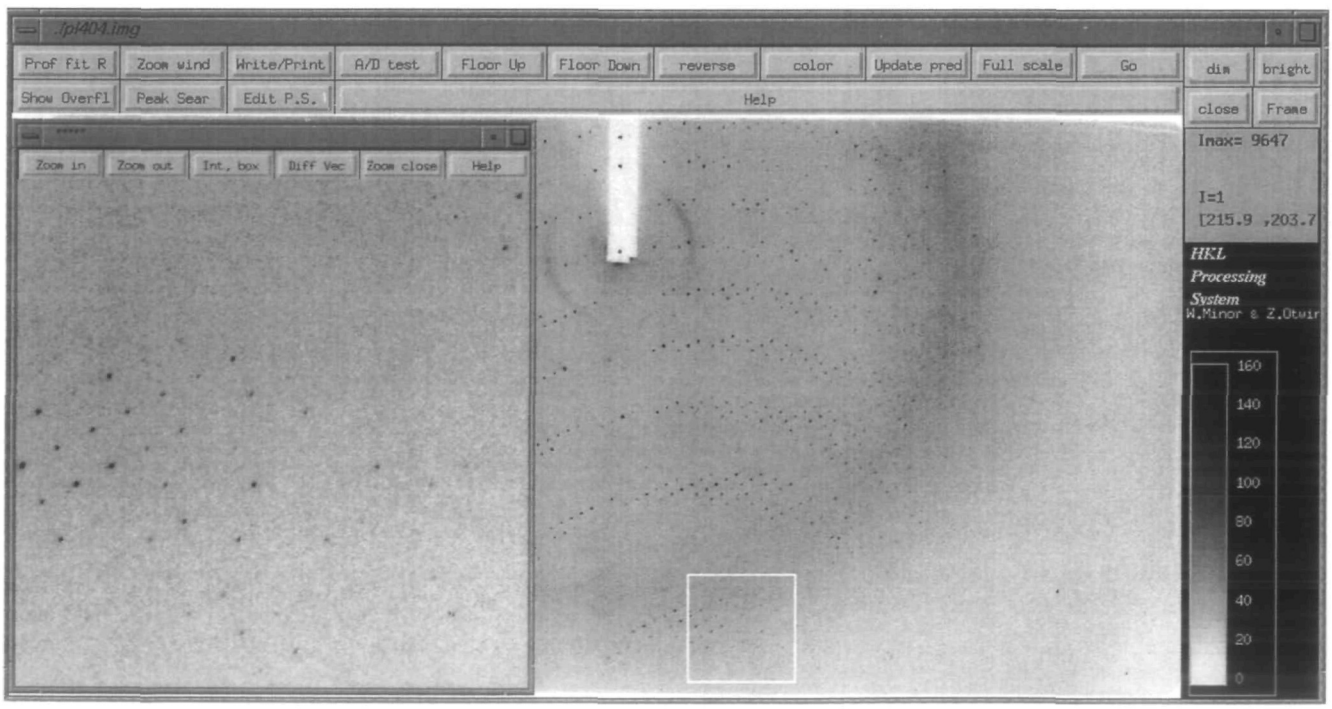

Fig. 2. **Diffraction image of KOD DNA polymerase at 100 K with synchrotron radiation X-rays at Beamline 18B at KEK-PF, Japan.**

pH range, from 4.0 to 10.0.

Crystals of KOD DNA polymerase suitable for diffraction experiments were obtained at 293 K with hanging drops formed from 2 μl of the protein solution and 2 μl of a reservoir solution comprising 100 mM Na citrate buffer, pH 5.5, and 25-30% (v/v) 2-methyl-2,4-pentanediol (MPD), equilibrated against the reservoir solution. The crystals were thin plates and reached a maximum size of $0.4\times0.1\times0.1$ mm in several months (Fig. 1). Several crystals were washed, dissolved, and analyzed by SDS-PAGE. The analysis showed that these crystals were of a protein with a molecular weight of about 90 kDa, resulting in a protein free from the unexpected proteolysis.

All the X-ray diffraction measurements were carried out with $\lambda = 1.00$ Å synchrotron X-rays monochromated with a Si(111) double mirror coupled with a Weissenberg camera for macromolecules and a BAS2000 imaging-plate system (Fuji Film Company) at KEK-PF, Japan (*15, 16*). The full X-ray diffraction data were processed and scaled with *DENZO* and *SCALEPACK* (*17*), and systematic extinctions in the intensity data were checked with *HKLPLOT* (Eleanor Dodson, unpublished; Collaborative Computational Project, Number 4, 1994). The diffraction data statistics are summarized in Table I.

Normally, when a KOD DNA polymerase crystal was mounted in a glass capillary and then exposed to X-rays on synchrotron radiation, the diffraction spots observed exhibited Bragg spacings up to 3.0 Å. The space group was determined to be $P2_12_12_1$, with unit-cell parameters of $a=$ 112.8, $b=115.4$, and $c=75.4$ Å. An asymmetric unit contains a single molecule with a mass of 89,500, giving a crystal volume per protein mass (V_m) of 2.74 Å^3·Da^{-1} and a solvent content of 54.8%, by volume (*18*). As listed in Table Ia, a full data set was collected for a single crystal, although the crystal was slightly decayed in later frames. There were 73,785 measurements of 18,926 unique observed reflections with an overall R_{merge} of 9.4% ($R_{merge} = \Sigma|I-\langle I\rangle|/\Sigma I$) and an overall $I/\mathrm{sig}I$ of 6.6. This represents 93.0% of the theoretically observable reflections at 3.0 Å resolution. The outermost shell of data between 3.11 and 3.00 Å is 83.7% complete.

Fortunately, the crystallization conditions for KOD DNA polymerase involve 25-30% MPD as the precipitant. MPD is widely used as a conventional anti-freezing reagent in cryogenic protein crystallography. In this case, crystals can be cooled with liquid N_2 without dialysis against a buffer solution containing anti-freezing reagents. Each KOD DNA polymerase crystal was directly picked up with a nylon fiber loop from a drop of mother liquid, and then rapidly transferred to the liquid N_2 gas stream. Diffraction intensities at 100 K were observed beyond 3.0 Å resolution (Fig. 2). The crystal did not decay at all during data collection with synchrotron radiation X-rays and diffracted more than at 290 K. The unit-cell parameters were determined to be $a=$ 111.9, $b=112.4$, and $c=73.9$ Å, with the space group of $P2_12_12_1$. The cell parameters were slightly decreased by the cooling. The unit-cell parameters gave a Matthew's coefficient of 2.60 Å^3·Da^{-1} and a solvent content of 52.2%, by volume. As listed in Table Ib, a full data set was collected for one crystal. There were 61,509 measurements of 17,483 unique reflections with an overall R_{merge} of 8.5% and an overall $I/\mathrm{sig}I$ of 7.6. This represents 90.6% of the theoretically observable reflections at 3.0 Å resolution. The outermost shell of data between 3.11 and 3.00 Å is 86.1% complete. During this experiment, diffraction from the frozen crystal did not decay and the variation in the mosaicity of a crystal was small. Therefore, the data set at 100 K was used for the next step. Attempts were made to determine the KOD DNA polymerase structure by molecular replacement using the structure of DNA polymerase from bacteriophage RB69 (*11*) and the partial structure of a polymerase from bacteriophage T4 (*19*). But no consistent set of rotation function solutions could be obtained because of the low sequence identity. Structure determination of KOD DNA polymerase by means of multiple isomorphous replacement methods is underway.

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