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Crystallization and preliminary diffraction analysis of a hyperthermostable DNA polymerase from a *Thermococcus* archaeon

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Abstract

The hyperthermostable DNA polymerase from a marine *Thermococcus* archaeon has been crystallized in space group $P2_12_12_1$, with unit-cell dimensions $a = 94.8$, $b = 98.2$, $c = 112.2$ Å with one molecule per asymmetric unit. Conditions for data collection at 98 K have been identified, and a complete data set was collected to 2.2 Å resolution. Strategies employed here may facilitate crystallization of other hyperthermostable proteins. The structure of this enzyme will provide the first structural data on the archaeal and hyperthermostable classes of DNA polymerases. Sequence homology to human polymerase α (polymerase B family) may make it a model for studying eukaryotic and viral polymerases and for the development of anti-cancer and anti-viral therapeutics.

1. Introduction

Modern phylogeny classifies all life into one of three classes (Bacteria, Archaea and Eukarya), all of which originated from a common ancestor. Archaea are thought to have split off of the evolutionary line which lead to the eukaryotes (Olsen & Woese, 1997). DNA polymerases are grouped into families based on sequence homology, and most eukaryotic replication proteins share greater sequence similarity with their archaeal homologues than with their bacterial ones (Edgell & Doolittle, 1997). Every archaeal DNA polymerase sequenced thus far belongs to the family B of polymerases (Edgell & Doolittle, 1997), which contains nearly all of the genomically encoded DNA polymerases of eukaryotes (Braithwaite & Ito, 1993).

An 89.6 kDa DNA polymerase (9N7) has recently been purified from a hyperthermophilic *Thermococcus* sp. 9° N-7, which was isolated from a hydrothermal vent at 9° N latitude off of the East Pacific Rise (Southworth *et al.*, 1996). The primary sequence of the enzyme (775 amino acids), which encodes both polymerase and 3′–5′ proofreading exonuclease activities, contains all consensus domains (data not shown) previously identified for family B polymerases (Wong *et al.*, 1988). The 9N7 polymerase shares significant sequence homology with human polymerase α, another family B polymerase. Polymerase α is one of the principal replicative DNA polymerases in eukaryotes (Fry & Loeb, 1986; Tsurimoto & Stillman, 1991) and shares 33% identity and 60% similarity at the amino-acid level in its polymerase domain with 9N7 (data not shown). Biochemical (Fry & Loeb, 1986) and mutagenesis (Copeland *et al.*, 1995) studies on polymerase α have sought to characterize eukaryotic polymerases. Placing these results in a structural context has been hampered by the lack of a crystal structure of polymerase α or a family B homologue. Co-crystal structures of 9N7 with nucleotide analogs used for treating certain cancers or to inhibit strand elongation by polymerase α (Zittoun *et al.*, 1989; Huang *et al.*, 1995; Gandhi *et al.*, 1995) should illustrate how these inhibitors interact with polymerases and may provide a rationale for anti-cancer drug design.

The structure of 9N7 polymerase would be the first of a hyperthermostable DNA polymerase. The enzyme has a half-life of 6.7 h at 368 K (RBK, unpublished results). By contrast, the thermostable polymerase from *Thermus aquaticus* has a half-life of only 1.6 h at 368 K (Kong *et al.*, 1993). The structure of 9N7 polymerase may provide useful information for increasing the thermal stability of enzymes already in use, or for designing new thermostable proteins.

Here we describe the crystallization and preliminary diffraction analysis of wild-type 9N7 polymerase and a mutant lacking detectable 3′–5′ exonuclease activity (Southworth *et al.*, 1996). The mutant (9N7exo-) polymerase was obtained by making two point mutations (D141A, E143A) in the Exo I (DIE) motif highly conserved among the 3′–5′ exonuclease domains of many DNA polymerases (Derbyshire *et al.*, 1995). Working with 9N7exo- polymerase will simplify co-crystal studies with DNA by eliminating exonucleolytic degradation of the DNA.

2. Results and discussion

Wild-type 9N7 and 9N7exo- mutant polymerase were overexpressed in *Escherichia coli* and purified as described previously (Southworth *et al.*, 1996). Both proteins were filtered through 0.22 μm filters and concentrated in a buffer containing 10 m*M* Tris–HCl (pH 7.5), 50 m*M* NaCl, and 0.1 m*M* EDTA using Centricon-10 concentrators (Amicon, Beverly, MA). Both proteins spontaneously formed showers of needle crystals in their storage buffer when concentrated to 2–9 mg ml^{-1} protein. Even when diluted to concentrations below 1 mg ml^{-1}, the protein precipitated or formed showers of microscopic crystals during initial crystallization trials. Spontaneous nucleation was eliminated by heating the concentrated protein for 5 min at 345 K and centrifuging it at 2000*g* immediately prior to setting up hanging-drop trays, or by storing the protein in the following glycerol-containing buffer: 100 m*M* KCl, 10 m*M* Tris–HCl (pH 7.4), 1 m*M* dithiothreitol, 0.1 m*M* EDTA, 0.1% Triton-X 100, and 50% glycerol (Southworth *et al.*, 1996).

A preliminary screen of crystal growth conditions was performed with the Crystal Screen kit (Hampton Research, Laguna Hills, CA, USA) by the hanging-drop vapor-diffusion method. Screening was initially performed using the wild-type protein but later continued with the exo- mutant. Given the prevalence in hydrothermal vents of divalent cations such as nickel and tungsten (Seyfried & Mottl, 1995), these metals

Table 1. *Diffraction data statistics*

Unit-cell parameters (Å)	a = 94.8, b = 98.2, c = 122.2
Space group	$P2_12_12_1$
Resolution (Å)	25.0–2.2
No. of measured reflections	138533
No. of unique reflections	44293
Completeness (%)	82.3
R_{sym}† (%)	5.01
Multiplicity	3.13

Resolution (Å)	No. of measured reflections	No. of unique reflections	Completeness (%)	R_{sym}† (%)	Multiplicity
25.0–4.73	19550	5535	94.2	2.9	3.66
4.73–3.76	19147	5309	97.3	3.8	3.61
3.76–3.28	20020	5375	99.4	6.4	3.72
3.28–2.98	20682	5367	99.8	8.3	3.85
2.98–2.77	20594	5350	99.8	11.0	3.85
2.77–2.61	15428	5152	96.6	13.9	2.99
2.61–2.48	10026	4547	85.3	12.7	2.20
2.48–2.37	6665	3635	68.5	12.9	1.83
2.37–2.28	4202	2633	49.3	14.9	1.60
2.28–2.20	2221	1590	30.1	22.1	1.40

† $R_{sym} = \sum_h \sum_i |I_{ih} - \langle I_h \rangle| / \sum_h \langle I_h \rangle$, where $\langle I_h \rangle$ is the mean intensity of the i observations of reflection h.

were tried as additives in crystallization screening trials. Nickel ions were found to be essential for good crystal quality. Diffraction-quality crystals were obtained at room temperature with hanging drops of 4 µl of H_2O and 4 µl of 3–4 mg ml^{-1} protein, equilibrated against a reservoir solution of 90 m*M* glycine–NaOH (pH 8.6–9.0), 10 m*M* $NiCl_2$, and 8–12%(*v*/*v*) PEG 1500.

These crystals diffracted to high resolution but were often small and showed numerous satellite crystals. Crystallization at elevated temperatures (303–313 K) had no positive effect on crystal quality. Microdialysis crystallization (Drenth, 1994), however, produced larger crystals than the hanging-drop method, and mostly eliminated the formation of satellite crystals. Crystals that diffract beyond 2.2 Å resolution grow when 25 µl of 3.9 mg ml^{-1} protein (in the glycerol-containing buffer) are dialyzed at 291 K against 2 ml of 100 m*M* glycine–NaOH (pH 9.0 or 9.1), 10 m*M* $NiCl_2$, and 4–7% PEG 1500. Crystals reach full size (about 250 × 250 × 600 µm) in about two weeks.

After testing various cryoprotectant solutions, conditions that enabled data collection at 98 K were identified for 9N7exo- mutant polymerase. The crystals grown by the dialysis method are transferred from the mother liquor to a stabilization solution of 15% PEG 1500, 10 m*M* $NiCl_2$, and 100 m*M* glycine–NaOH (pH 9.0). The crystals are then stepped through three stabilization solutions supplemented with 3, 9 and 15% 2-methyl-2,4-pentanediol. The incubations in each solution are 10, 10 min and overnight, respectively.

A native data set has been collected at 98 K from a single crystal using an R-AXIS IIC phosphor imaging plate system (Molecular Structure Corporation, The Woodlands, TX, USA) mounted on a Rigaku rotating-anode X-ray generator. The diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Table 1 summarizes the characteristics of the reduced data set. Analysis of the native protein and a heavy-atom derivative show that one molecule is present per asymmetric unit. This gives a Matthews number of 2.9 Å^3 Da^{-1} and a solvent content of 57.7% (Matthews, 1968). The structure solution is in progress by the method of multiple isomorphous replacement, and co-crystallization studies of 9N7exo- mutant polymerase with duplex DNA are under way.

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