

Crystallization and preliminary X-ray crystallographic analysis of NAD⁺-dependent DNA ligase from *Thermus filiformis*

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A highly thermostable DNA ligase from *Thermus filiformis* has been crystallized at room temperature using methoxypropylglycol 5000 as a precipitant. The crystal belongs to the monoclinic space group $P2_1$, with unit-cell parameters $a = 90.63$, $b = 117.80$, $c = 98.65$ Å, $\beta = 115.56^\circ$. Two molecules of DNA ligase are present in the asymmetric unit, giving a crystal volume per protein mass (V_m) of $3.1 \text{ \AA}^3 \text{ Da}^{-1}$ and a solvent content of 61%. A native data set extending to 3.0 \AA resolution has been collected at 100 K using synchrotron X-rays.

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1. Introduction

DNA ligases (polydeoxyribonucleotide synthases, E.C. 6.5.1) play essential roles in the replication and repair of DNA and in genetic recombination by catalyzing the formation of phosphodiester bonds at single-stranded or double-stranded breaks between adjacent 3'-hydroxyl and 5'-phosphate termini (Lehman, 1974; Kornberg & Baker, 1991; Lindahl & Barnes, 1992). Enzymes from eukaryotic organisms as well as from bacteriophages of the T series and from archaeobacteria utilize ATP as a cofactor, while eubacterial ligases are NAD⁺-dependent. The two classes of DNA ligase share low overall sequence homology; however, a conserved sequence motif (KxDG) is common to their active sites. The higher specificity and thermostability of NAD⁺-dependent DNA ligases from *Thermus* species have been exploited in the ligase chain reaction (LCR) to detect a single-base mutation associated with genetic diseases (Barany, 1991). For further application of thermostable DNA ligases to LCR, it will be useful to understand the structural determinants for thermostability and specificity.

The NAD⁺-dependent DNA ligase from *T. filiformis* (*Tfi* ligase) is a monomeric protein of 667 amino-acid residues ($M_r = 75\,936$ Da; Kim & Kwon, 1998). Until now, the crystal structure of only the NAD⁺-binding domain of an NAD⁺-dependent DNA ligase from *Bacillus stearothermophilus* has been reported (Singleton *et al.*, 1999), whereas the whole structure of an ATP-dependent DNA ligase from bacteriophage T7 has been determined (Subramanya *et al.*, 1996). *Tfi* ligase is an interesting target for structural analysis, because its structure determination will shed light on the structural similarities to and differences from the ATP-dependent DNA ligase and will also provide a structural basis

for better understanding the catalytic action as well as the thermostability and specificity. Here, we report the crystallization of *Tfi* ligase and preliminary X-ray crystallographic data.

2. Experimental

2.1. Protein expression and purification

The cloning, nucleotide sequence and expression of *Tfi* ligase have been reported previously (Kim & Kwon, 1998). *Escherichia coli* BL26 Blue cells harbouring the plasmid pTFL were grown in Luria-Bertani media containing 50 mg l^{-1} ampicillin and 10 mg l^{-1} tetracycline. Protein expression was induced with 0.3 mM IPTG. After 5 h expression, the cells were harvested, suspended in the lysis buffer (10 mM Tris-HCl pH 7.6, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride) and lysed with an ultrasonic processor. The crude lysate was centrifuged at $36\,000g$ for 30 min at 277 K and the supernatant was heated and kept between 348 and 353 K for 10 min, taking 5 min to reach 348 K from room temperature, in order to denature contaminating heat-labile proteins. After being chilled on an ice bath, it was centrifuged at $36\,000g$ for 1 h at 277 K. The recombinant protein in the supernatant fraction was purified by three chromatography steps involving a Q-Sepharose anion-exchange column (Amersham-Pharmacia) and affinity columns (heparin-Sepharose and Blue-Sepharose).

2.2. Crystallization and X-ray data collection

The purified enzyme was concentrated to about 20 mg ml^{-1} concentration using a YM30 ultrafiltration membrane (Amicon) and was dialyzed against 20 mM Tris-HCl pH 8.3 for crystallization. The protein concentration was estimated by measuring the absorbance at 280 nm, assuming a correspondence of

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell (3.11–3.00 Å).

X-ray source	PF (BL-6A)
Wavelength (Å)	1.000
Resolution range (Å)	50–3.0
Number of observations	86261
Unique reflections	33838
Space group	$P2_1$
Unit-cell parameters	
<i>a</i> (Å)	90.63
<i>b</i> (Å)	117.80
<i>c</i> (Å)	98.65
β (°)	115.56
Completeness (%)	90.1 (80.5)
$I/\sigma(I)$	16.5 (3.2)
R_{merge}^\dagger (%)	7.7 (34.8)

$^\dagger R_{\text{merge}} = \sum_h \sum_i |I(h)_i - \langle I(h) \rangle| / \sum_h \sum_i I(h)_i$, where $I(h)_i$ is the intensity of reflection h , \sum_h is the sum over all reflections and \sum_i is the sum over i measurements of reflection h .

0.83 mg ml⁻¹ concentration to the unit absorbance for 1.0 cm path length. Crystallization was performed by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates (Hampton Research) at 297 K. Each hanging drop was prepared by mixing 2 µl each of the protein solution and the reservoir solution and was placed over 1 ml of the reservoir solution. Results from the initial searches for crystallization conditions performed using Crystal Screen I (Jancarik & Kim, 1991) and Crystal Screen II kits (Hampton Research) were optimized. The optimized reservoir solution consisted



Figure 1
Monoclinic crystals of *Tfi* DNA ligase. Largest crystals have approximate dimensions of 0.35 × 0.25 × 0.1 mm.

of 5% methoxyPEG 5000, 100 mM sodium citrate at pH 5.6, 5 mM calcium chloride. Initially, crystals were too small and therefore the microseeding technique was used to grow larger crystals. A stock solution of microseeds was prepared by crushing approximately 50–100 microcrystals (~0.05 mm) in 0.10 ml of the reservoir solution and diluting the suspension serially by a factor of 1000–5000. Each hanging drop was prepared by mixing the protein solution, the reservoir solution and the microseed solution in a ratio of 2:2:0.2. Crystals grew reproducibly to dimensions of approximately 0.35 × 0.25 × 0.1 mm within a week.

A crystal of *Tfi* DNA ligase was transferred to a solution of 10% methoxyPEG 5000, 100 mM sodium citrate pH 5.6, 5 mM calcium chloride with 25% glycerol within a minute in eight steps before being flash-frozen. Native data were collected to 3.0 Å resolution at 100 K using a Weissenberg camera for macromolecular crystallography at the BL-6A experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). The wavelength of the synchrotron X-rays was 1.000 Å and a 0.1 mm collimator was used. The image plate (20 × 40 cm, Fuji BAIII) was placed at a distance of 429.7 mm from the crystal. The oscillation range per image plate was 4.5°, with a speed of 1.0° s⁻¹ and a coupling constant of 1.5° mm⁻¹. An overlap of 0.5° was allowed between contiguous image plates. The diffraction patterns recorded on the image plates were digitized by the off-line scanner Fuji BA100. The raw data were processed and scaled using the programs *DENZO* and *SCALE-PAK* (Otwinowski & Minor, 1997).

3. Results

Crystals of *Tfi* ligase have been obtained using methoxyPEG 5000 as precipitant (Fig. 1). They diffract to ~3.3 Å using conventional X-rays but are not stable in the X-ray beam at room temperature. Therefore, native diffraction data have been collected to 3.0 Å at 100 K using synchrotron X-rays. Table 1 summarizes the data-collection statistics. The autoindexing

procedure performed with *DENZO* indicated that the crystals belong to a primitive monoclinic space group, with unit-cell parameters $a = 90.63$ (20), $b = 117.80$ (8), $c = 98.65$ (15) Å, $\beta = 115.56$ (3)°. The space group was determined to be $P2_1$ on the basis of systematic absences.

The self-rotation function $R(\varphi, \psi, \kappa)$ was calculated using the program *POLARRFN* from the *CCP4* suite (Collaborative Computational Project, Number 4, 1994). It showed a strong peak (80% of the origin peak) at $\varphi = 65^\circ$ and $\psi = 0^\circ$ in the $\kappa = 180^\circ$ section, while the $\kappa = 120^\circ$ section did not show any significant peaks. This indicates that two copies of *Tfi* ligase molecule are present in the asymmetric unit, with a corresponding crystal volume per protein mass (V_m) of 3.1 Å³ Da⁻¹ (Matthews, 1968) and a solvent content of 61%.

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