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Crystallization and preliminary X-ray diffraction studies of nucleoside diphosphate kinase from *Thermus thermophilus* HB8

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Nucleoside diphosphate (NDP) kinase contributes to the maintenance of cellular pools of all nucleoside triphosphates (NTPs) by catalyzing the transfer of the γ-phosphoryl group from an NTP donor to an NDP acceptor. NDP kinase from the extreme thermophilic bacterium *Thermus thermophilus* HB8 was overexpressed in *Escherichia coli* and crystallized at 297 K using polyethylene glycol 8000 as the precipitant by means of the hanging-drop vapour-diffusion procedure. The crystals belong to the hexagonal system, space group $P6_322$, with unit-cell parameters $a = b = 124.0$, $c = 104.9$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Assuming the asymmetric unit to contain two subunits, the calculated V_M value was 3.7 Å^3 Da^{-1}. The crystals diffracted X-rays generated by the synchrotron at SPring-8 to at least 1.9 Å resolution and were suitable for high-resolution X-ray crystal structure determination.

1. Introduction

Nucleoside diphosphate (NDP) kinases (EC 2.7.4.6) are ubiquitous enzymes which catalyze the transfer of the γ-phosphate of a nucleoside triphosphate (NTP) donor to the β-phosphate of an NDP acceptor (Parks & Agarwal, 1973). The catalytic reaction follows a two-step ping-pong mechanism in which the enzyme is transiently phosphorylated on a histidine residue that is conserved in all NDP kinases. NDP kinases are crucial for the homeostasis of cellular NDP and NTP compositions. These enzymes can utilize purines, pyrimidines, ribonucleotides and deoxyribonucleotides as donor and acceptor nucleotides. However, the main role of the NDP kinase reaction is to supply NTPs other than ATP, as ATP is abundant in cells.

Extensive studies have been carried out to determine the three-dimensional structures of NDP kinases from several eukaryotes (*Dictyostelium discoideum*, human, bovine and *Drosophila melanogaste*) and one prokaryote (*Myxococcus xanthus*) (Williams *et al.*, 1993). The eukaryotic enzymes are homohexamers, while the bacterial enzymes are homotetramers. Structural studies together with site-directed mutagenesis and kinetic studies have contributed to our understanding of the catalytic mechanism of the phosphotransfer reaction by visualizing individual steps of the reaction pathway at the atomic resolution level (Janin & Deville-Bonne, 2002). However, such detailed images have not thrown any light on new biological roles for NDP kinases. In humans, NDP kinases (Nm23s) are known to play roles in cell proliferation, differentiation and tumour-associated mechanisms (Otero, 2000). Among these enzymes, Nm23-H2/NDP kinase B functions as a transcriptional regulator *via* binding and cleavage of DNA (Postel *et al.*, 2000). Recently, DNA-binding and DNA-cleavage activities of *Escherichia coli* NDP kinase have been identified (Levit *et al.*, 2002). Several lines of study have suggested that NDP kinases interact with other proteins and DNA (Bernard *et al.*, 2000; Postel *et al.*, 2002) and indicate that NDP kinase functions as a multifunctional enzyme *in vivo*.

In order to understand the cellular functions of NDP kinase in detail, we started to analyze the NDP kinase (ttNDPK) from *Thermus thermophilus* HB8 (Oshima & Imahori, 1974). *T. thermophilus* HB8 is an aerobic rod-shaped non-sporulating Gram-negative eubacterium which can grow at temperatures in excess of 348 K. This is the most thermophilic bacterium of the thermophiles for which gene-manipulating systems have been established. Its proteins are heat-stable and easily crystallized and are therefore suitable for detailed analysis of structure–function relationships.

Based on the DNA-sequence information supplied by the RIKEN Structural Genomics Initiative (Yokoyama *et al.*, 2000), an ORF (project code 0088) coding for a protein homologous to other NDP kinases was found in the genome sequence of *T. thermophilus* HB8. The ttNDPK consists of 137 amino acids with a molecular mass of 15 kDa and shares sequence similarity (56–62%) with other NDP

kinases deposited in the Protein Data Bank. Here, we describe the overproduction, crystallization and preliminary X-ray diffraction analysis of ttNDPK.

2. Materials and methods

2.1. Cloning and overexpression

The ttNDPK gene was amplified by the polymerase chain reaction using the primers 5′-ATATCATATGGAACGGACCTTCGTCATGATCAA-3′ and 5′-ATATAGATCTTTATTAAAGGAGCTCCTCGGGACGGA-3′ and cloned into the plasmid pT7Blue (Novagen). After confirmation of the nucleotide sequence, the ttNDPK gene was ligated into the expression vector pET-11a (Novagen) at the *Nde*I/*Bam*HI sites. The resulting expression plasmid was used to transform *E. coli* strain BL21(DE3)pLysE (Novagen). The transformant was cultured in Luria broth containing 50 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol at 310 K until the density of the culture reached 4×10^8 cells ml^{-1}. The cells were then incubated for a further 4 h in the presence of 50 µg ml^{-1} isopropyl-β-D-thiogalactopyranoside and were harvested by centrifugation.

2.2. Protein purification

Frozen cells (25 g) were thawed, suspended in 140 ml buffer I (20 m*M* MES pH 6.0) and sonicated on ice for 10 min with an ultrasonic disrupter; 140 ml pre-warmed buffer I was then added to the suspension. The cell extract was incubated at 343 K for 10 min and then centrifuged (40 000*g*) for 60 min at 277 K. After this step, most of the *E. coli* proteins had been excluded. The supernatant was divided into two halves which were loaded onto an SP Sepharose FF column (Amersham Biosciences) equilibrated with buffer I. The column was washed with 40 ml buffer I and the fraction that passed through was collected. The pH of this fraction was adjusted to 8.0 by adding 1 *M* Tris and this sample was loaded onto a Super Q-Toyopearl 650M column (Tosoh) equilibrated with buffer II (20 m*M* Tris–HCl pH 8.0). The column was washed with 100 ml buffer II and the proteins were eluted with a linear gradient of 0–1 *M* NaCl in 200 ml buffer II. The fractions containing ttNDPK were loaded onto a hydroxyapatite CHT10 column (Bio-Rad) equilibrated with buffer III (10 m*M* sodium phosphate pH 7.0). The column was washed with 100 ml buffer III and the proteins were eluted with a linear gradient of 10–250 m*M* sodium phosphate pH 7.0 in a total volume of 200 ml. The fractions containing ttNDPK were desalted and loaded onto a HiTrap Blue column (Amersham Biosciences) equilibrated with buffer I. The column was washed with 10 ml buffer I and the proteins were eluted with a linear gradient of 0–2.0 *M* NaCl in 100 ml buffer I. The fractions containing ttNDPK were concentrated and loaded onto a HiLoad 16/60 Superdex 75pg column (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl and 150 m*M* NaCl pH 8.0. The purity of the protein was confirmed by SDS–PAGE, which showed the presence of a single band corresponding to the calculated molecular weight of the ttNDPK sequence.

2.3. Crystallization

For the crystallization experiments, the purified protein solution was desalted in buffer II and concentrated to 8.5 mg ml^{-1} by ultrafiltration. The initial screening for ttNDPK crystallization conditions was performed using the sitting-drop vapour-diffusion method at 293 K and Crystal Screen Cryo (Hampton Research). Drops were prepared by mixing 2 µl protein solution with 1 µl reservoir solution and were equilibrated against 500 µl reservoir solution. Further optimization of the crystallization conditions was performed using the hanging-drop vapour-diffusion method at 293 K and drops prepared using twice the above volumes.

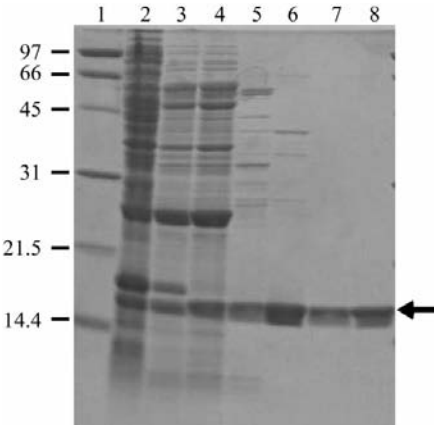

Figure 1
Overproduction and purification of ttNDPK. At each step, the proteins were analyzed by staining the SDS–PAGE gel (15% polyacrylamide) with Coomassie brilliant blue. Lane 1, size markers with the indicated molecular weights (kDa); lane 2, total cell extract induced by IPTG; lane 3, supernatant after heat treatment; lanes 4, 5, 6, 7 and 8, HiTrap SP Sepharose FF, Super Q-Toyopearl 650M, hydroxyapatite CHT10, HiTrap Blue HP and HiLoad 16/60 Superdex 75pg chromatography fractions, respectively. The arrow indicates ttNDPK.

2.4. Data collection

Each crystal was mounted on a CryoLoop (Hampton Research) and then flash-cooled in a nitrogen-gas stream at 100 K. The concentrations of polyethylene glycol 400 and glycerol in the crystallization media were sufficient for cryoprotection of the crystals, so no cryoprotectant was added. X-ray diffraction data for two crystallization conditions were collected at 100 K on the BL44B2 and BL45PX stations at SPring-8 (Hyogo, Japan; Adachi *et al.*, 2001; Yamamoto *et al.*, 1998, 2001). All data were processed, scaled and merged using the *HKL* package (Otwinowski & Minor, 1997).

3. Results and discussion

The ttNDPK gene product without an affinity tag was successfully overexpressed and purified to homogeneity with a yield of 1 mg per gram of cells (Fig. 1). In the gel-filtration experiment, ttNDPK eluted at a retention time corresponding to about 60 kDa, which suggests that ttNDPK exists as a tetramer in solution. The purified ttNDPK catalyzed the phosphorylation of guanosine or cytidine diphosphates to the corresponding triphosphates with ATP ($k_{\rm cat,\ GDP}$ = 38 s^{-1}, $K_{\rm m,\ GDP}$ = 20 µ*M* at pH 7.5 and 298 K).

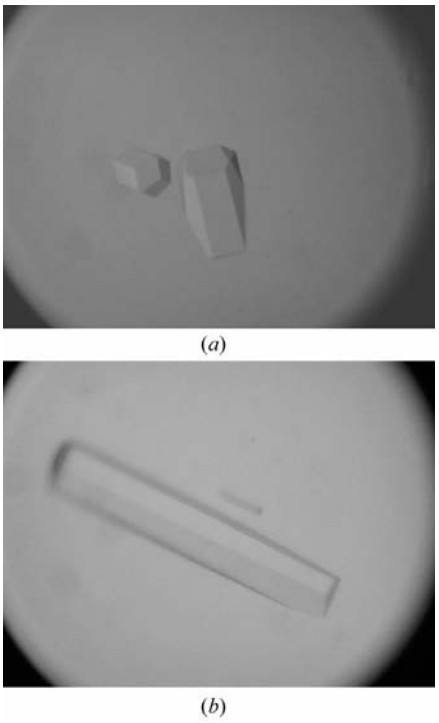

Figure 2
(*a*) Hexagonal crystal of ttNDPK after optimization of the crystallization conditions corresponding to Crystal Screen Cryo No. 14 (crystal 1). (*b*) Hexagonal crystal of ttNDPK after optimization of the crystallization conditions with solutions containing polyethylene glycol 8000 (crystal 2).

Table 1
Crystal data and diffraction measurement results.

Values in parentheses are for the outermost shell.

	Crystal 1	Crystal 2
Space group	$P6_322$	$P6_322$
Unit-cell parameters (Å)	$a = b = 122.85$, $c = 104.32$	$a = b = 124.00$, $c = 104.89$
Resolution range (Å)	50.0–2.4 (2.49–2.40)	50.0–1.9 (1.97–1.90)
No. of measured reflections	766520	702359
No. of unique reflections	18728	37747
Completeness (%)	99.7 (100.0)	99.4 (100.0)
$R_{\rm merge}$† (%)	7.7 (27.2)	8.6 (34.0)
Average $I/\sigma(I)$	21.5 (4.9)	40.5 (10.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I\rangle| / \sum_{hkl}\sum_i I_{hkl,i}$, where I is the observed intensity and $\langle I\rangle$ is the averaged intensity for multiple measurements.

Initial crystallization trials using Crystal Screen Cryo produced several crystal forms using solutions containing polyethylene glycol. Optimization of the crystallization conditions corresponding to Crystal Screen Cryo No. 14 produced hexagonal crystals suitable for X-ray diffraction experiments by mixing 4 µl protein (8.5 mg ml^{-1}) with 2 µl reservoir solution consisting of 24–28%(*v*/*v*) polyethylene glycol 400, 200 m*M* $CaCl_2$ and 100 m*M* HEPES pH 7.5–8.0 (Fig. 2*a*). Using synchrotron radiation from BL44B2 at SPring-8, X-ray diffraction data were collected from the crystals to 2.4 Å resolution (crystal 1 in Table 1). These crystals belong to space group $P6_322$, with unit-cell parameters $a = b = 122.85$, $c = 104.32$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Assuming the presence of two and four ttNDPK subunits in the asymmetric unit, the respective $V_{\rm M}$ and solvent-content values are 3.7 and 1.85 Å^3 Da^{-1} and 0.67 and 0.34, respectively. The $V_{\rm M}$ value and the tetrameric state in solution suggest the presence of four subunits in the asymmetric unit, although the possibility of two subunits cannnot be excluded. Under these conditions, many small crystals appeared in the drops within 1 d and reached their maximum size within a few days. Further optimization and modification of the conditions did not improve the resolution limit. In order to collect X-ray diffraction data at a higher resolution, alternative crystallization conditions were optimized. The best crystals were obtained with 14%(*w*/*v*) polyethylene glycol 8000, 100 m*M* sodium acetate, 15%(*w*/*v*) glycerol and 100 m*M* MES pH 6.0 (Fig. 2*b*). The crystals usually appeared within one week and continued to grow for several more weeks. Using synchrotron radiation at BL45XU-PX, SPring-8, X-ray diffraction data were collected from these crystals to 1.9 Å resolution (crystal 2 in Table 1). These crystals belong to space group $P6_322$, with unit-cell parameters similar to those of crystal 1. Using these data, structural determination by molecular replacement and refinement is in progress.

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