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Purification, crystallization and preliminary structural analysis of nucleoside diphosphate kinase from *Bacillus anthracis*

Gauri Misra, Anita Aggarwal, Sonia Mittal, Yogendra Singh and Ravishankar Ramachandran

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Gauri Misra,^a Anita Aggarwal,^b
Sonia Mittal,^a Yogendra Singh^b
and Ravishankar
Ramachandran^{a*}

^aMolecular and Structural Biology Division,
Central Drug Research Institute, PO Box 173,
Chattar Manzil, Mahatma Gandhi Marg,
Lucknow 226 001, India, and ^bInstitute of
Genomics and Integrative Biology, Mall Road,
Delhi 110 007, India

Correspondence e-mail:
r_ravishankar@cdri.res.in

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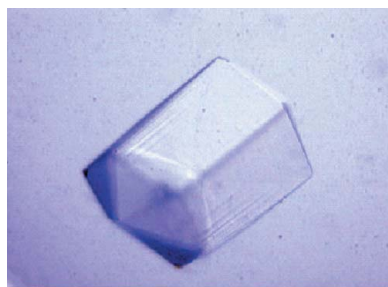
Purification, crystallization and preliminary structural analysis of nucleoside diphosphate kinase from *Bacillus anthracis*

Bacillus anthracis nucleoside diphosphate kinase (BaNdk) is an enzyme whose primary function is to maintain deoxynucleotide triphosphate (dNTP) pools by converting deoxynucleotide diphosphates to triphosphates using ATP as the major phosphate donor. Although the structures of Ndks from a variety of organisms have been elucidated, the enzyme from sporulating bacteria has not been structurally characterized to date. Crystals of the *B. anthracis* enzyme were grown using the vapour-diffusion method from a hanging drop consisting of 2 μ l 10 mg ml⁻¹ protein in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA equilibrated against 500 μ l reservoir solution consisting of 2.25 M ammonium formate and 0.1 M HEPES buffer pH 7.25. Diffraction data extending to 2.0 Å were collected at room temperature from a single crystal with unit-cell parameters $a = b = 107.53$, $c = 52.3$ Å. The crystals are hexagonal in shape and belong to space group $P6_322$. The crystals contain a monomer in the asymmetric unit, which corresponds to a Matthews coefficient (V_M) of 2.1 Å³ Da⁻¹ and a solvent content of about 36.9%.

1. Introduction

Nucleoside diphosphate kinases (Ndks) are a highly conserved group of enzymes. The primary function of the enzyme is to maintain deoxynucleotide triphosphate (dNTP) pools by converting deoxynucleotide diphosphates to triphosphates using ATP as the major phosphate donor. The reaction proceeds by the formation of a covalent intermediate in which the enzyme is phosphorylated at a catalytic histidine residue. The dNTPs thus formed serve as precursors for DNA and RNA synthesis (Lascu & Gonin, 2000). Ndk is postulated to be involved in numerous other physiological processes that are independent of its metabolic functions (Chakrabarty, 1998; Hartsough & Steeg, 2000; Bernard *et al.*, 2000; Postel, 2003; Shen *et al.*, 2004; Wallet *et al.*, 1990; Biggs *et al.*, 1990; Miller *et al.*, 2002; Munoz-Dorado *et al.*, 1990). Human Ndk/NM23 homologues have been identified as tumour suppressors with implications in tumour metastasis and pathogenesis (Hartsough & Steeg, 2000; Leone *et al.*, 1991). Another isoform of the same enzyme appeared to act as a transcription factor, with the *c-myc* gene as one of its targets (Postel, 2003; Postel *et al.*, 1993). DNA-binding and DNA-cleavage activities have also been reported for *Mycobacterium tuberculosis* Ndk, with implications in dissemination of the intracellular pathogen (Saini *et al.*, 2004). Ndks from various organisms share primary, secondary and tertiary structural similarity, but differ in their quaternary structure. Ndks from eukaryotes, halophilic archaea, *Bacillus* species and *M. tuberculosis* form hexamers, although the axial symmetry and stability of the hexamer is species-dependent. Some bacterial enzymes have been reported to also have tetrameric structures (Mock & Fouet, 2001; Singh *et al.*, 2004). Ndk oligomerization has been correlated with its ability to interact with other molecules and carry out its function. However, the diverse cellular functions of Ndk from a sporulating bacterium have not been studied.

B. anthracis, the causative agent of anthrax, forms metabolically dormant spores in response to nutrient deprivation and high cell density similar to other members of the genus *Bacillus* (Setlow, 2003;



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Setlow *et al.*, 2001). The spores of *B. anthracis* are resistant towards extreme temperature, fluctuations in pH, hazardous radiation and noxious chemicals. These properties allow *B. anthracis* spores to be used as a potent biological weapon for mass destruction. These spores can persist in their habitat for years and can germinate in response to favourable conditions. The genome sequence of *B. anthracis* revealed that it has 'a large metabolic tool kit', similar to those of other soil bacteria, that gives the bacterium the versatility to import nutrients and respond to signals from its environment (Setlow *et al.*, 2002).

We have purified, crystallized and collected high-resolution data from *B. anthracis* Ndk and have solved the structure using molecular replacement with human nucleoside diphosphate kinase (PDB code 1jxv; 56% identity) as a template. The structure, when refined, will form the basis for a structure-based mutational analysis of catalytically important residues to probe the structure–function relationship in the enzyme.

2. Experimental methods

2.1. Protein expression and purification

B. anthracis genomic DNA was used as a template for the amplification of the gene encoding Ndk (BAS1425). The nucleotide sequences of the two primers were 5'-CCATGGAAAAACA-TTTCTAATGG-3' to introduce a *Bam*HI site at the 5' end and 5'-CTCGAGTGGAAATCTTAGTAAATCCATTCA-3' with an *Eco*RI site at the 3' end. The amplified product was digested with *Bam*HI and *Eco*RI and was ligated into *Bam*HI–*Eco*RI-digested pPROEX-HTc expression vector (Invitrogen). The resultant plasmid carrying *ndk* was designated pndk.

Recombinant protein was expressed and purified from *Escherichia coli* BL21 cells. In brief, the culture was harvested and suspended in 50 mM Tris pH 8.5, 5 mM 2-mercaptoethanol and 1 mM PMSF at 277 K, which was followed by sonication at 2.5 W with 30 s pulses for 10 min with a microtip probe (Misonix 2000). The sonicated sample was centrifuged, the supernatant was incubated with Ni-NTA (trinitriloacetate) resin (Qiagen) and the protein was purified according to the manufacturer's instructions. Fractions containing the desired protein were pooled after analysis on 15% SDS-PAGE and were dialyzed against PBS containing 20% glycerol. The concentration of protein was determined using Bradford's reagent (Bradford, 1976) with BSA as a standard and stored in aliquots until further use. The purified protein gave a single band corresponding to a molecular weight slightly above the expected ~21 kDa on SDS-PAGE. Size-

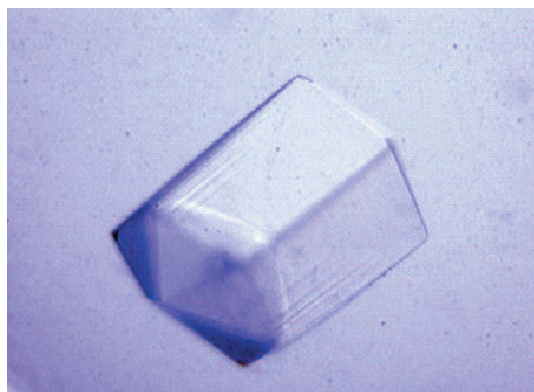


Figure 1
Crystal of *B. anthracis* nucleoside diphosphate kinase.

Table 1

Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	<i>P</i> 6 ₃ 22
Unit-cell parameters	<i>a</i> = 107.53, <i>b</i> = 107.53, <i>c</i> = 52.3
Resolution range (Å)	26.7–2.0 (2.11–2.0)
No. of measured reflections	79277 (11305)
No. of unique reflections	12490 (1784)
Multiplicity	6.3 (6.3)
<i>I</i> / σ (<i>I</i>)	14.9 (3.4)
Completeness (%)	99.9 (100.0)
<i>R</i> _{merge}	0.087 (0.55)
<i>R</i> _{r.i.m.} †	0.095 (0.59)

† $R_{r.i.m.} = \sum_{hkl} [N/(N-1)]^{1/2} \sum_i |I_i(hkl) - \overline{I(hkl)}| / \sum_{hkl} \sum_i I_i(hkl)$, where *N* is the multiplicity.

exclusion chromatography indicates that BaNdk is hexameric in nature.

2.2. *in vitro* activity

The ability of purified Ndk to autophosphorylate was determined in an independent assay. The protein was incubated in a kinase buffer containing 50 mM Tris–HCl pH 7.4, 5 mM MgCl₂ in the presence of 0.5 μCi [γ -³²P]-ATP as a phosphate donor for 15 min at room temperature to assay autophosphorylation. The reactions were terminated by addition of SDS sample buffer, resolved on 15% SDS-PAGE and autoradiographed to determine the phosphorylation of Ndk.

Ndk activity was determined using a coupled-enzyme assay in which ATP was used as a donor and thymidine diphosphate (TDP) was used as a substrate. The decrease in absorbance owing to oxidation of NADH was measured at 340 nm at room temperature in a spectrophotometer (Perkin–Elmer, lambda25). Negative-control assays were performed in the absence of TDP and Ndk.

2.3. Crystallization

Crystallization experiments were set up at 295 K using the hanging-drop vapour-diffusion method and by exploring crystallization 'space' using sparse-matrix approaches (Mazeed *et al.*, 2003; Jancarik & Kim, 1991; Cudney *et al.*, 1994). Crystals were obtained in various screening solutions. Large single crystals (1.5 × 0.8 × 0.5 mm) were grown in 2–3 d from a hanging drop consisting of 2 μl 10 mg ml⁻¹ protein and 2 μl reservoir solution in 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 5 mM EDTA equilibrated against 500 μl of a reservoir solution consisting of 2.25 M ammonium formate and 0.1 M HEPES buffer pH 7.25 (Fig. 1).

2.4. Data collection

A single crystal was mounted in a capillary. Data were collected to 2.0 Å at room temperature using a MAR 345 area-detector system and a Rigaku RU-300 rotating-anode generator operating at 50 kV and 100 mA. The data were processed using the *MOSFLM/CCP4* suite of programs (Collaborative Computational Project, Number 4, 1994). The crystals were hexagonal, with unit-cell parameters *a* = 107.53, *b* = 107.53, *c* = 52.3 Å and space group *P*6₃22. The data-collection statistics are summarized in Table 1. The crystal mosaicity refined to around 0.6° and an overall data completeness of 100% was obtained. Calculation of the Matthews coefficient (Matthews, 1968) suggests that the asymmetric unit contains a single subunit. Assuming that the asymmetric unit contains a monomer, the calculated

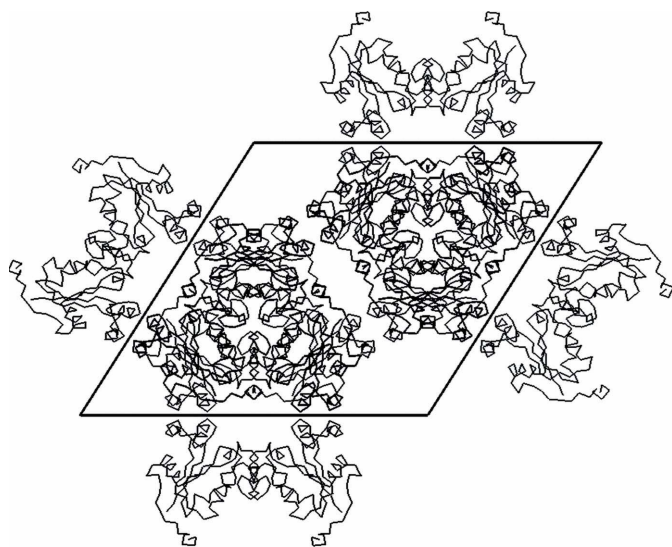


Figure 2
Packing of molecules in the crystal structure of *B. anthracis* Ndk. The association of the hexamer as a dimer of trimers is clearly visible.

Matthews coefficient is $2.1 \text{ \AA}^3 \text{ Da}^{-1}$, corresponding to a solvent content of about 36.9%.

2.5. Data processing and structure solution

A total of 79 277 measured reflections were merged into 12 490 unique reflections with an R_{merge} of 8.7%. A sequence comparison of BaNdk against the structures in the Protein Data Bank (<http://www.rcsb.org>) showed that BaNdk exhibits 56% identity to the human enzyme. Molecular-replacement calculations were carried out using *AMoRe* (Navaza, 1994). A clear solution with good packing was obtained with a correlation coefficient of 66.3 and an R factor of 42.4% using data between 20 and 2 \AA resolution. The packing of the molecules in the crystal was good (Fig. 2) and suggested that the hexamer observed in size-exclusion chromatography apparently arises from association as a dimer of trimers. Model building and refinement are presently in progress.

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