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Structure and analysis of nucleoside diphosphate kinase from *Borrelia burgdorferi* prepared in a transition-state complex with ADP and vanadate moieties

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Nucleoside diphosphate kinases (NDKs) are implicated in a wide variety of cellular functions owing to their enzymatic conversion of NDP to NTP. NDK from *Borrelia burgdorferi* (*Bb*NDK) was selected for functional and structural analysis to determine whether its activity is required for infection and to assess its potential for therapeutic inhibition. The Seattle Structural Genomics Center for Infectious Diseases (SSGCID) expressed recombinant *Bb*NDK protein. The protein was crystallized and structures were solved of both the apoenzyme and a liganded form with ADP and vanadate ligands. This provided two structures and allowed the elucidation of changes between the apo and ligand-bound enzymes. Infectivity studies with *ndk* transposon mutants demonstrated that NDK function was important for establishing a robust infection in mice, and provided a rationale for therapeutic targeting of *Bb*NDK. The protein structure was compared with other NDK structures found in the Protein Data Bank and was found to have similar primary, secondary, tertiary and quaternary structures, with conserved residues acting as the catalytic pocket, primarily using His132 as the phosphohistidine-transfer residue. Vanadate and ADP complexes model the transition state of this phosphoryl-transfer reaction, demonstrating that the pocket closes when bound to ADP, while allowing the addition or removal of a γ -phosphate. This analysis provides a framework for the design of potential therapeutics targeting *Bb*NDK inhibition.

1. Introduction

Lyme disease is the most common vector-borne illness in the USA (Centers for Disease Control and Prevention, 2015). It is estimated that over 300 000 people in the USA are affected by Lyme disease every year, placing the disease as the fifth most common nationally notifiable disease in 2014. In the USA, 96% of cases are concentrated in 14 states, predominantly in the northeast and the upper Midwest. Moreover, Lyme disease is found in over 80 countries globally, with the number of average cases per year on the rise (Centers for Disease Control and Prevention, 2015), and it continues to remain a difficult disease to diagnose, treat and eradicate.

The Lyme disease spirochete *Borrelia burgdorferi* is maintained in nature *via* transmission cycles between reservoir hosts and tick vectors. *B. burgdorferi* is transmitted to humans *via* the bite of infected ticks of the *Ixodes ricinus* complex, including *I. scapularis* in the northeastern, north central and middle Atlantic USA, *I. pacificus* in northern California, and

I. ricinus and *I. persulcatus* in Eurasia (Lane *et al.*, 1991). Lyme disease can vary dramatically in progression and presentation (Steere *et al.*, 2016). Eva Åsbrink and Anders Hovmark attempted to separate the untreated progression of the disease into three distinct phases (Åsbrink & Hovmark, 1988). Phase 1 (localized disease) was denoted by erythema migrans (EM), a radiating cutaneous manifestation with a characteristic ‘bullseye’ appearance at the bite site of the infected tick, along with flu-like symptoms. Phase 2 (disseminated disease) occurs within weeks after infection and is marked by early bacterial dissemination, potentially causing cardiac and joint abnormalities such as myocarditis, heart palpitations, muscle weakness, body aches and joint swelling. Phase 3 (late persistent disease) is noted by progressive systemic infection with potential neurological appearances such as meningitis, polyneuropathy, cognitive and psychiatric changes, debilitating fatigue and serious cardiac abnormalities. However, not all Lyme disease progresses according to this scheme, making it often difficult to diagnose Lyme disease and prescribe the appropriate treatment, which is essential as more life-threatening symptoms appear (Steere *et al.*, 2016).

B. burgdorferi was identified as the causative agent of Lyme disease in 1982 (Burgdorfer *et al.*, 1982). This spirochete is an obligate parasite in both ticks and mammals, and shares a twisted spiral morphology similar to other organisms in the *Spirochaete* phylum such as *Treponema pallidum* and *Leptospira interrogans* (Steere *et al.*, 2016). Other *Borrelia* species such as *B. mayonii* have recently been implicated in causing similar symptoms in humans (Pritt *et al.*, 2016). The Infectious Diseases Society of America recommends antibiotic treatment for Lyme disease, including doxycycline, amoxicillin or cefuroxime axetil, which usually completely clears the bacterial infection (Wormser *et al.*, 2006). In some cases, symptoms remain after antibiotic treatment (Berndtson, 2013). Persistent infection and ‘post-treatment Lyme disease syndrome’ (PTLDS) were observed in a small percentage of Lyme disease patients at the time they finished treatment. Patients still have lingering symptoms after treatment for Lyme disease with a recommended two- to four-week course of antibiotics. The mechanism of persistent symptoms is largely unknown and may not be owing to persistent infection in most cases, but novel therapies for Lyme disease would allow alternative treatments to be tested for a reduction in these symptoms after *B. burgdorferi* is cleared.

Nucleoside diphosphate kinases (NDKs; EC 2.7.4.6), which were discovered in 1973 (Fitz-Gibbon *et al.*, 2002), are an important class of enzymes that are responsible for maintaining cellular metabolism by providing pools of nucleotide triphosphates (NTPs) and deoxynucleotide triphosphates (dNTPs) for various energetic processes. NDKs produce NTPs and dNTPs from NDPs and dNDPs via a catalytic reversible phosphate-transfer mechanism (Ray & Mathews, 1992). After hydrolysis of the terminal phosphate on the substrate NTP (usually ATP, which is the most abundant within the cell), the γ -phosphate is transferred to the β -phosphate on the receiving NDP or dNDP via a ping-pong mechanism using a phosphorylated histidine intermediate. While Mg^{2+} is used as a

cofactor in catalysis and significantly increases the reaction rate of transfer in the second step, the reaction will proceed at a slower rate without the presence of Mg^{2+} (Williams *et al.*, 1993). The reversible two-step ping-pong mechanism is described as

NTP products are used for various metabolic functions such as nucleic acid synthesis (dNTPs and NTPs), lipid synthesis (CTP), protein elongation (GTP), signal transduction (GTP), microtubule polymerization (GTP), polysaccharide synthesis (UTP) and many other essential functions. Without the conversion of ATP to other essential NTPs and dNTPs, growth, communication and maintenance within the cell could be severely compromised. A wide pool of available NTPs is necessary for proper metabolism and function, supporting the evidence that NDK is an essential enzyme for the survival and adaptation of an organism.

NDKs have been implicated in a wide range of other important cellular functions. In *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* NDK has been found to play a significant role in regulating growth and surface polysaccharide synthesis in addition to replenishing NTPs (Chakrabarty, 1998). Surface polysaccharides often function as virulence factors or influence cell signaling, implying that NDKs could play an important role in bacterial pathogenicity. In eukaryotes, NDKs (also denoted Nm23) have been shown to influence cancer progression, with a reduction in the Nm23 ‘A chain’ gene correlating with more aggressive forms of melanoma, colon, breast and gastric tumors (Tee *et al.*, 2006). Conversely, elevated levels of Nm23 in the blood can promote hematologic malignancies such as acute myelogenous leukemia (AML; Okabe-Kado *et al.*, 2012). However, *B. burgdorferi* is extremely restricted by its metabolism; it cannot support the Krebs cycle, oxidative phosphorylation or *de novo* biosynthesis pathways for nutrients such as amino acids, carbohydrates or lipids (Corona & Schwartz, 2015). ATP production from glycolysis and nutrient acquisition from the host are the only sources of bioenergetics for metabolism, signaling and reproduction. As such, *B. burgdorferi* NDK is extremely important in retaining a diverse pool of NTPs and dNTPs for use in the bacterial cell. This creates a unique target to characterize for the development of new compounds against *B. burgdorferi*.

This article describes and analyzes two solved structures of *B. burgdorferi* nucleoside diphosphate kinase. We first show that *B. burgdorferi* NDK is important for robust infection in the mouse model and then present a detailed analysis of the structure of the enzyme. We solved two complex structures of the target protein: one structure with no bound substrate and another structure with ADP and vanadate bound to illustrate formations regarding the transition-state complex. Analysis of both the apoenzyme and the holoenzyme at high resolution (2.2 Å) offers clues to catalytic action, conformational

Table 1
Macromolecule-production information.

PDB code	4di6 (apo)	4dz6 (ADP + vanadate)
Source organism	<i>B. burgdorferi</i>	<i>B. burgdorferi</i>
DNA source	<i>B. burgdorferi</i> B31 genomic DNA (ATCC-35210D-5)	<i>B. burgdorferi</i> B31 genomic DNA (ATCC-35210D-5)
Forward primer	GGGTCCTGGTTCGATGTCAATGTTATTGCAAAAACTTTAT	GGGTCCTGGTTCGATGTCAATGTTATTGCAAAAACTTTAT
Reverse primer	CTTGTTTCGTGCTGTTTATTAACAATAATAATGCTCACATTCAT CATC	CTTGTTTCGTGCTGTTTATTAACAATAATAATGCTCACATTCAT CATC
Expression vector	pAVA0421	pAVA0421
Expression host	<i>E. coli</i> BL21(DE3)R3	<i>E. coli</i> BL21(DE3)R3
Complete amino-acid sequence of the construct produced	MAHHHHHHMGTLEAQTQGPGSMSMLLQKTLICIVKPDGVRRGLI GDVSRFRFVGLKMVAAMKMLIVDESLAKKHYLYDDIVFRHS EAVWNSLIKFISSNSPVFTFVVEGVESIEVVRKLCGATEPKL AIPGTIRGDFSYSFSFKYSNEKGFSIYNVIHASANE ADAMREIPIWFKDNEILNYKRDDCEHYCYC	MAHHHHHHMGTLEAQTQGPGSMSMLLQKTLICIVKPDGVRRGLI GDVSRFRFVGLKMVAAMKMLIVDESLAKKHYLYDDIVFRHS EAVWNSLIKFISSNSPVFTFVVEGVESIEVVRKLCGATEPKL AIPGTIRGDFSYSFSFKYSNEKGFSIYNVIHASANEADAMRE IPIWFKDNEILNYKRDDCEHYCYC

changes, active-site dynamics and the overall structure of the enzyme complex.

2. Materials and methods

2.1. Protein expression and purification

Cloning, expression and purification were conducted as part of the Seattle Structural Genomics Center for Infectious Disease (SSGIC; Myler *et al.*, 2009; Stacy *et al.*, 2011) following standard protocols described previously (Bryan *et al.*, 2011; Choi *et al.*, 2011; Serbzhinskiy *et al.*, 2015). The full-length gene (UniProt O51419) encoding amino acids 1–169 was PCR-amplified from *B. burgdorferi* B31 genomic DNA (ATCC-35210D-5). The gene was amplified using the following primer sequences: forward primer 5'-GGGTCC TGGTTCGATGTCAATGTTATTGCAAAAACTTTAT-3' and reverse primer 5'-CTTGTTTCGTGCTGTTTATTAACAATAATAATGCTCACATTCATCATC-3'. The gene was cloned into the ligation-independent cloning (LIC; Aslanadis & de Jong, 1990) expression vector pAVA0421 encoding a six-histidine fusion tag followed by the human rhinovirus 3C protease-cleavage sequence (MAHHHHHHMGTLEAQTQGP GS-ORF). The human rhinovirus 3C protease-cleavage site is between the Gln and Gly residues that are underlined; however, the tag was not cleaved during purification, giving 21 amino acids at the N-terminus of the 169-residue protein. Plasmid DNA was transformed into chemically competent *Escherichia coli* BL21(DE3)R3 Rosetta cells. The plasmid containing N-terminally six-His-tagged *B. burgdorferi* NDK (6-His-*Bb*NDK) was tested for expression and 2 l of culture was grown using auto-induction medium (Studier, 2005) in a LEX Bioreactor (Epiphyte Three) as described previously (Choi *et al.*, 2011).

6-His-*Bb*NDK was purified in a two-step protocol consisting of an Ni²⁺-affinity chromatography (IMAC) step and size-exclusion chromatography (SEC). All chromatography runs were performed on an ÄKTApurifier 10 (GE Healthcare) using automated IMAC and SEC programs according to previously described procedures (Bryan *et al.*, 2011; Serbzhinskiy *et al.*, 2015). Thawed bacterial pellets were lysed by sonication in 200 ml buffer consisting of 25 mM HEPES pH 7.0, 500 mM NaCl, 5% glycerol, 0.5% CHAPS,

30 mM imidazole, 10 mM MgCl₂, 1 mM TCEP, 250 µg ml⁻¹ AEBSF and 0.025% azide. After sonication, the crude lysate was clarified with 20 µl (25 units µl⁻¹) of Benzonase and incubated while mixing at room temperature for 45 min. The lysate was then clarified by centrifugation at 10 000 rev min⁻¹ for 1 h using a Sorvall centrifuge (Thermo Scientific). The clarified supernatant was then passed over an Ni-NTA His-Trap FF 5 ml column (GE Healthcare) which was pre-equilibrated with loading buffer composed of 25 mM HEPES pH 7.0, 500 mM NaCl, 5% glycerol, 30 mM imidazole, 1 mM TCEP, 0.025% sodium azide. The column was washed with 20 column volumes (CV) of loading buffer and was eluted with loading buffer plus 250 mM imidazole in a linear gradient over 7 CV. Peak fractions, as determined by UV absorbance at 280 nm, were pooled and concentrated to 5 ml. The pooled material was then passed over an SEC column (Superdex 75, GE Healthcare) equilibrated with running buffer composed of 300 mM NaCl, 20 mM HEPES, 5% glycerol, 2 mM DTT pH 7.0. The SEC peak fractions were collected and analyzed for the presence of the protein of interest using SDS-PAGE. The SEC peak fractions were then pooled and concentrated to 35 mg ml⁻¹ using an Amicon purification system (Millipore). Aliquots of 200 µl were flash-frozen in liquid nitrogen and stored at -80°C until use in crystallization. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

For the crystallization of apo *B. burgdorferi* NDK, purified protein was diluted to 17 mg ml⁻¹ in a buffer consisting of 25 mM HEPES pH 7.0, 500 mM sodium chloride, 2 mM DTT, 0.025% sodium azide, 5% glycerol. The diluted protein was crystallized by the sitting-drop vapor-diffusion method using 0.4 µl protein solution mixed with 0.4 µl crystallization solution composed of 20% PEG 3350, 200 mM sodium malonate over a reservoir containing 50 µl crystallization solution. The drops were incubated at 16°C and crystals appeared within two weeks. Crystals were prepared for data collection by transfer to a cryoprotectant solution consisting of the crystallization solution plus 25%(v/v) ethylene glycol and were then flash-cooled by plunging a crystallization mounting loop directly into liquid nitrogen. Crystals of ADP-vanadate-bound NDK protein were prepared in the same way, except that

Table 2
Crystallization.

PDB code	4di6 (apo)	4dz6 (ADP + vanadate)
Method	Vapor diffusion, sitting drop	Vapor diffusion, sitting drop
Plate type	Compact 300 (Rigaku Reagents)	Compact 300 (Rigaku Reagents)
Temperature (°C)	16	16
Protein concentration (mg ml ⁻¹)	17	17
Buffer composition of protein solution	25 mM HEPES pH 7.0, 500 mM sodium chloride, 2 mM DTT, 0.025% sodium azide, 5% glycerol	25 mM HEPES pH 7.0, 500 mM sodium chloride, 2 mM DTT, 0.025% sodium azide, 5% glycerol, 1 mM activated sodium vanadate, 2 mM ADP
Composition of reservoir solution	20% PEG 3350, 200 mM sodium malonate	20% PEG 3350, 200 mM sodium malonate
Volume and ratio of drop	0.8 µl; 50% buffer solution, 50% crystallization solution	0.8 µl; 50% buffer solution, 50% crystallization solution
Volume of reservoir (µl)	50	50

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

PDB code	4di6 (apo)	4dz6 (ADP + vanadate)
Diffraction source	Beamline 21-ID-G, APS	Beamline 21-ID-G, APS
Wavelength (Å)	0.97856	0.97856
Temperature (°C)	−173.0	−173.0
Detector	MAR Mosaic 300 mm CCD	MAR Mosaic 300 mm CCD
Crystal-to-detector distance (mm)	230	275
Rotation range per image (°)	0.5	1.0
Total rotation range (°)	150	150
Exposure time per image (s)	1.0	2.0
Space group	<i>P</i> 6 ₃	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	135.26, 135.26, 126.65	78.65, 81.00, 181.43
α , β , γ (°)	90, 90, 120	90, 90, 90
Resolution range (Å)	50.0–2.40 (2.46–2.40)	99.470–2.20 (2.26–2.20)
Total No. of reflections	295229	283371
No. of unique reflections	50359	87269
<i>R</i> _{meas}	0.109 (0.540)	0.053 (0.311)
Completeness (%)	97.9 (99.2)	99.6 (99.47)
Multiplicity	5.86 (5.79)	3.24 (3.22)
$\langle I/\sigma(I) \rangle$	15.3300 (4.24)	17.3200 (3.58)
<i>R</i> _{r.i.m.} (%)	11.96 (59.37)	7.94 (65.52)
Overall <i>B</i> factor from Wilson plot (Å ²)	32.59	40.87

2 mM ADP and 1 mM activated sodium orthovanadate (Gordon, 1991) were added to the protein solution immediately before the crystallization drops were set up. Crystallization information is summarized in Table 2.

2.3. Structure determination

X-ray diffraction data for both structures were collected on a MAR Mosaic 300 CCD detector on beamline 21-ID-G of the Advanced Photon Source (APS), Argonne, Illinois, USA. All data sets were reduced using *XDS* and scaled using *XSCALE* (Kabsch, 2010). The structure of the apo protein was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the CCP4 suite (Winn *et al.*, 2011) using PDB entry 2cwk (nucleotide diphosphate kinase from *Pyrococcus horikoshii*; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) as a search model. The model was refined by iterative manual rebuilding using *Coot* (Emsley *et al.*, 2010) and geometrically restrained refinement using *REFMAC*

Table 4
Structure solution and refinement.

Values in parentheses are for the outer shell.

PDB code	4di6 (apo)	4dz6 (ADP + vanadate)
Resolution range (Å)	46.22–2.40 (2.46–2.40)	99.470–2.20 (2.26–2.20)
Completeness (%)	99.20	99.73
σ Cutoff	$F > 0.000\sigma(F)$	$F > 0.000\sigma(F)$
No. of reflections, working set	50334 (3513)	59351 (3868)
No. of reflections, test set	2557 (197)	3003 (197)
Final <i>R</i> _{cryst}	0.175 (0.213)	0.183 (0.234)
Final <i>R</i> _{free}	0.228 (0.293)	0.223 (0.269)
No. of non-H atoms		
Protein	8048	8065
Ion	0	15
Ligand	0	101
Water	431	328
Total	8479	8509
R.m.s. deviations		
Bonds (Å)	0.009	0.009
Angles (°)	1.259	1.406
Average <i>B</i> factors (Å ²)		
Protein	26.3	36.7
Ion	—	46.1
Ligand	—	44.5
Water	24.7	34.4
Ramachandran plot		
Most favored (%)	97	97
Allowed (%)	3	3

(Murshudov *et al.*, 2011). X-ray data-collection and refinement statistics are summarized in Tables 3 and 4.

2.4. Construction of signature-tagged suicide *Himar1* delivery vectors

A signature-tagged suicide vector was created by inserting STM tags into a region between the ColE1 origin and inverted terminal repeat 2 (ITR2) in the pGKT *Himar1*-based transposon vector (Stewart & Rosa, 2008). The STM tags used in this study were designed based on previously published STM tags (Lehoux *et al.*, 1999); each contains a unique 7 bp sequence tag. pGKT was graciously provided by Drs Philip E. Stewart and Patricia Rosa (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Minnesota, USA). pGKT contains a gentamicin-resistance cassette gene driven by the *B. burgdorferi* *flgB* promoter (*flgB_P;**aacC1*) and a *colE1* origin of replication within the transposable element. The ‘backbone’ of the vector outside the transposable element

Table 5

Transposon mutants and parental strain utilized in this study.

Clone name	Missing plasmid†	Replicon‡	Insertion site	Gene	Insertion ratio§	Description	Common name
5A18NP1	lp28-4, lp56					Parental strain	5A18NP1
T01P01A11	lp5	lp25	15343	BBE22	0.44	Pyrazinamidase/nicotinamidase (<i>pncA</i>)	<i>pncA</i>
T05TC180	cp9, lp5	Chromosome	484917	BB0463	0.32	Nucleoside diphosphate kinase	<i>ndk</i>

† The parental strain 5A18NP1 and mutants are all missing the plasmids lp28-4 and lp56, but the additional plasmids lost in the transposon mutants are noted. None of these plasmids have been shown to affect infectivity in mice (Purser & Norris, 2000; Grimm *et al.*, 2004; Labandeira-Rey & Skare, 2001; Labandeira-Rey *et al.*, 2003; Revel *et al.*, 2005; Jewett *et al.*, 2007). ‡ Coordinate of the transposon insertion in the replicon. § Number of nucleotides (beginning of the open reading frame to the transposon insertion site)/number of nucleotides (open reading frame).

contains the highly active *Himar1* C9 transposase (Lampe *et al.*, 1999) and a constitutively expressed kanamycin-resistance cassette with a *B. burgdorferi* promoter (*flaB_{pr}::aph1*). Thus, gentamicin resistance is conferred by transposition of the transposable element, whereas kanamycin resistance and transposase activity are lost once transposition has completed. The modified regions containing the STM tags were amplified by PCR and sequenced for each pGKT-STM construct. This transposon-vector set could easily be expanded to include a larger number of STM tags (Lehoux *et al.*, 1999; Hunt *et al.*, 2004).

2.5. Generation of signature-tagged transposon mutants in BB0463

Transposon mutagenesis of the infectious *B. burgdorferi* B31 clone 5A18NP1 was performed by electroporation with 5 µg pGKT-STM plasmid using previously described methods (Lin *et al.*, 2012, 2014, 2018). Briefly, the cultures were incubated overnight in BSK-II medium without antibiotics, and transposon mutants were selected by subsurface plating on 0.7% solid BSK-II medium with 200 µg ml⁻¹ kanamycin and 40 µg ml⁻¹ gentamicin (Lin *et al.*, 2012). Colonies were selected and cultured in liquid BSK-II medium with the same antibiotics and stored at -70°C with the addition of 15% (v/v) glycerol. The transposon-insertion site was determined using *E. coli* rescue of circularized HindIII fragments (Lin *et al.*, 2012; Stewart *et al.*, 2004). The exact transposon-insertion site of each clone in the library was determined by dideoxynucleotide sequencing of the recovered plasmid using the clone sequencing primer. The replicon, insertion site, gene or intergenic region (IR) with insertion, transposon orientation and insertion ratio (number of nucleotides from gene start/total gene length) was obtained using *BLAST* analysis. Genome coordinates and annotation of *B. burgdorferi* B31-MI as reported by Fraser *et al.* (1997) were used, with the inclusion of more recent gene designations. As part of an overall goal to dissect the structure, function and mechanisms of pathogenesis of the *Borrelia* NDK protein, we selected transposon mutants in BB0463 (nucleoside diphosphate kinase, *ndk* gene) from a sequence-defined transposon library (Lin *et al.*, 2012) for further study. The effects of mutation of the *ndk* gene on the structure and infectivity were determined (Lin *et al.*, 2012). Four mutants in the *ndk* gene were obtained from our STM transposon mutant library and one *ndk* mutant, T05TC180, was selected for further mouse-infection study (see Table 5).

2.6. Mouse-infection studies

Prior studies indicated that genes present in the linear plasmids lp25, lp28-1, lp36, lp54 and cp26 in *B. burgdorferi* B31 are required for full infectivity in mice (Purser & Norris, 2000; Grimm *et al.*, 2004; Serbzhinskiy *et al.*, 2015; Labandeira-Rey *et al.*, 2003; Revel *et al.*, 2005; Jewett *et al.*, 2007). Thus, the plasmid content of each clone was determined using a PCR method as described previously (Lin *et al.*, 2009, 2015). None of the plasmids missing in the strains selected for study are reported to influence infectivity in mice. The insertion ratio (the distance in base pairs from the insertion site to the 5' end of the gene divided by the total gene length) was used to provide a convenient measure of the location of the transposon insertion within the gene. Transposon insertion in 5% of the 5' end and 10% of the 3' end of the gene might express functional protein (Lin *et al.*, 2012). Based on the transposon-insertion site and plasmid profile, we selected one representative *ndk* mutant, T05TC180 (the insertion site is 0.32), to perform the mouse-infection experiment. The infectivity of the transposon mutant in *ndk* (T05TC180) was determined by needle inoculation in groups of six four-week-old C3H/HeNHsd female mice. STM transposon mutants T01P01A11 with insertions in *bbe22* (pyrazinamidase/nicotinamidase, *pncA*) and the parental clone 5A18NP1 were utilized as negative and positive controls, respectively. The parental clone 5A18NP1, *ndk* mutants and the *bbe22* (*pncA*) mutant were cultured directly from frozen stocks in BSK-II medium to mid-log phase. The concentration of organisms was determined by dark-field microscopy. A group of six mice were inoculated with 1×10^5 organisms subcutaneously at the base of the tail as described previously (Lin *et al.*, 2009, 2015). Groups of three mice were sacrificed at days 14 and 28 post-inoculation, and skin, ear, tibiotarsal joint, heart and urinary bladder were harvested. The tissue specimens were cultured in 6 ml BSK-II medium containing kanamycin and gentamicin (Lin *et al.*, 2009, 2015).

3. Results and discussion

3.1. Infection experiment results

To determine whether *B. burgdorferi* NDK was important for bacterial infection *in vivo*, an infection experiment in a mouse model was performed with an *ndk* transposon mutant and compared with the wild type and a *pncA* transposon mutant known to be important in infection. The results demonstrated that the parental wild-type clone 5A18NP1 was fully infectious, in that all cultures from mice inoculated with

Table 6
Infectivity of *B. burgdorferi* NDK mutants in CH3/HeNHsd mice at days 14 and 28 post-inoculation.

Each of six mice per group was inoculated intradermally at the base of the tail with 10⁵ *B. burgdorferi* ndk mutant spirochetes.

Clone	Days post-inoculation	Positive sites/total				Inoculation site	All sites	No. of mice positive/total
		Bladder	Ear	Heart	Joint			
T05TC180†	14	1/3	0/3	1/3	2/3	0/3	4/15	2/3
	28	0/3	1/3	0/3	2/3	3/3	6/15	2/3
5A18NP1‡	14	3/3	3/3	3/3	3/3	3/3	15/15	3/3
	28	3/3	3/3	3/3	3/3	3/3	15/15	3/3
T01P01A11§	14	0/3	0/3	0/3	0/3	0/3	0/15	0/3
	28	0/3	0/3	0/3	0/3	0/3	0/15	0/3

† T05TC180 is a mutant in the *ndk* gene. ‡ 5A18NP1 is a parental strain. It exhibited full infectivity and was used as a positive control in this study. § T01P01A11 is a pyrazinamidase/nicotinamidase (*pncA*) mutant created in our STM library. This mutant showed a non-infectivity phenotype in single-clone inoculation and was used as a negative control in this study.

the parental clone 5A18NP1 were positive. The negative-control mutant T01P01A11, a transposon mutant in the *pncA* gene, demonstrated a non-infective phenotype in that all tissue cultures from mice inoculated with T01P01A11 were negative. However, the mutant in the *ndk* gene showed only partial infection at multiple sites, implying that a mutation in the *ndk* gene does not completely prevent infection, but rather that the strain shows a compromised ability to infect the host. In the *ndk* mutant both the 14 d data and the 28 d data showed a lower infectivity phenotype in multiple tissue sites, whereas the wild-type control showed that all tissue sites were infected at both time points (see Tables 5 and 6). Thus, inactivation of the *ndk* gene resulted in greatly reduced infectivity. These data support a requirement for NDK for robust infection and broad colonization by *B. burgdorferi*.

3.2. Comparison with other bacterial NDK structures

B. burgdorferi NDK consists of 11 α -helices and four β -pleated sheets, with the pattern alternating as $\beta\alpha\alpha\beta\alpha\alpha\alpha\beta\alpha\alpha\beta\alpha\alpha\alpha$ to form a single subunit. These moieties are bound together by both hydrophobic interactions and hydrogen bonds within the internal structure. Disulfide bonds do not seem to play a large role in the subunit structure: one internal bond is formed in each subunit between the terminal Cys169 and Cys164, which does not seem to interact with other subunits. Six identical subunits form the homohexameric enzyme complex. The enzyme complex forms a dihedral disk-like structure, with three subunits binding to form one side of the disk with its exposed active sites in a trigonal shape, and the other three subunits bound facing in the opposite orientation, as shown in Fig. 1. These structures are offset, possibly owing to electron repulsion or other steric interactions. The human homolog shares a similar structure, with the *A* and *B* chains of the trimers forming the homohexameric complex (Gilles *et al.*, 1991). This quaternary structure is highly conserved: almost all NDK structures deposited in the Protein Data Bank form a hexameric structure, including those from *Drosophila melanogaster* (Chiadmi *et al.*, 1993), *Mycobacterium tuberculosis* (Chen *et al.*, 2002) and *Oryza sativa* (Huang *et al.*, 2003). The only solved structure that does not conform to a hexameric conformation is the NDK from

Myxococcus xanthus (Williams *et al.*, 1993), which instead forms a tetrameric enzyme structure. The *M. xanthus* NDK subunit structures are offset similarly to the other NDK structures mentioned above. It is possible that this hexameric structure facilitates cooperativity between the subunits, but this will require further enzymatic study.

The 169-amino-acid primary sequence (190 residues including the His tag used in protein purification, 169 residues of the native protein sequence) forms the subunit in *B. burgdorferi* NDK. This primary sequence is present in all *Borrelia* species, with varying degrees of conservation depending on the species and its relation to *B. burgdorferi*. The highest degrees of sequence identity are found for *B. finlandensis* and *B. bissettii*, which show 97% identity, while *B. miyamotoi* and *B. hermsii* have 71% identity and the remaining *Borrelia* species fall within this range (71–97% identity). Other spirochetes and bacteria have varying identities below 50%, while human (*Homo sapiens*) NDK shares 35% sequence identity (Altschul *et al.*, 1997). Variance occurs in pockets within the sequence, indicating that while there may be variance among certain amino acids, there may also be specific residues that are highly conserved and important for catalysis.

While there is variance in amino-acid composition among the primary sequence, there are many highly conserved residues. Lys11, Arg92, Thr98, Arg109, Asn129 and His132 are highly conserved across multiple domains of life (Moréra *et al.*, 1995) and are present in the active pocket of the newly solved crystal structure. Lys11, Arg92, Thr98, Arg109 and Asn129 are residues that are implicated in binding ATP for the reaction. In *Drosophila* and *Dictyostelium* His132 is thought to be essential for phosphate-transfer activity via a phosphohistidine intermediate, acting as a protein histidine kinase (Moréra *et al.*, 1995; Attwood & Wieland, 2015), and is found in our solved structures. Fig. 2 shows a ClustalOmega comparison of the primary amino-acid sequence of *B. burgdorferi* NDK with those of another medically relevant spirochete (*T. pallidum*), *E. coli* and *H. sapiens* (Sievers *et al.*, 2011). ESPript was used to further illustrate the residue differences as well as the solved protein structures (Robert & Gouet, 2014).

A structural comparison was performed via the DaliLite software (Holm & Rosenström, 2010) to determine whether

any homologs in the Protein Data Bank (PDB) share similar three-dimensional structures. Numerous NDKs were represented ranging from 47 to 30% identity. *P. horikoshii* NDK (PDB entry 2dxl, Z-score 24.6; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) has 47% PDB identity, *Leishmania* spp. NDK (PDB entry 3ngt, Z-score 23.9; Souza *et al.*, 2011) has 40% identity and *H. sapiens* NDK (PDB entry 1nsk, Z-score 24.0; Webb *et al.*, 1995) has 38% structural identity. The human NM23-H2 transcription-factor complex (the A chain of human NDK) also shares 37% identity. Structurally, *B. burgdorferi* NDK shows marked differences from currently solved structures of other NDKs in specific areas when compared *via* structural alignment. At residues

5–6, 43–44, 77–78 and 86–87 *B. burgdorferi* NDK contains short amino-acid sequences to connect larger secondary topography instead of the helices that are mostly contained in the other structures. A larger divergence in the *B. burgdorferi* secondary structure compared with other similar NDKs occurs at residues 55–67, where there is an α -helix instead of an unstructured sequence, together with an unstructured sequence instead of an α -helix at residues 102–103 and a seven-residue helical insert at residues 118–125 that is not contained in any of the other constructs. This does not seem to affect the conserved key residues that interact with the active site, but the insert appears to be close to residues implicated in stabilizing the transition state.

Figure 1

Ribbon diagrams of the structure of apo *B. burgdorferi* NDK. The images on the left are viewed parallel to the threefold symmetry axis and the images on the right are viewed perpendicular to the threefold symmetry axis. The top views are colored by subunit with semi-transparent surfaces. The lower images show the secondary features of the protein with α -helices in cyan, strands in red and coils in magenta. Images were generated using PyMOL (Schrödinger).

3.3. Ligand and substrate binding

In addition to the solved apo structure, a solved structure with bound ADP and vanadate provided information about the active-site conformation. In the PDB, there are 103 structures of NDKs. Of these structures, roughly one third contain bound ligands such as (d)NTPs, (d)NDPs or other small molecules (Mg^{2+} , sulfate or vanadate). Some examples include *H. sapiens* NDK bound to ADP (Giraud *et al.*, 2006), *Thermus thermophilus* NDK complexed with GDP (PDB entry 1wkk; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) and even *Mimivirus* NDK bound to Mg^{2+} , phosphate, TDP and many other ligands (Jeudy *et al.*, 2009). We were primarily interested in showing the transition state during substrate turnover, and so we chose to attempt co-crystallization with both ADP and vanadate. Vanadate is used to mimic phosphate in phosphoryl-transfer reactions with a nontransferable moiety (Davies & Hol, 2004), preventing transfer and locking the enzyme into a transition-state structure. Fig. 3 shows the active pocket of our solved structure with bound ADP and vanadate. Hydrogen bonds and hydrophobic interactions of key residues are highlighted, indicating the forces that are responsible for stabilizing the reaction site. In this structure, vanadate is bound covalently to the β -phosphate of the ADP moiety, but it retains a trigonal bipyramidal conformation. The His134 residue responsible for the catalytic phosphohistidine mechanism has not completely dissociated (distance of 2.5 Å, with the β -phosphate bond at 2.0 Å), allowing us to visualize the transition state before the phosphohistidine offloads the phosphate group onto the

receiving ADP. A similar experiment using *D. discoideum* NDK bound to magnesium and ADP was visualized using aluminium fluoride. This yielded a similar transition-state trigonal pyramid configuration for this reaction, matching the results for our structure (Xu *et al.*, 1997). Magnesium is shown to interact with both phosphates of the ADP as well as the incoming moiety, suggesting greater stability and translating to a faster reaction rate. Although we did not utilize magnesium, it could provide similar stability in our complex.

3.4. Comparison of similar structures and complexes

Four other structures have been deposited in the PDB that are complexed with both ADP and vanadate. These include *Dengue virus* serotype 4 NS3 helicase (Luo *et al.*, 2008), MsbA from *Salmonella typhimurium* (Ward *et al.*, 2007), *D. discoideum* myosin including the Mg^{2+} cofactor (Smith & Rayment, 1996) and another NDK from *Staphylococcus aureus* (Srivastava *et al.*, 2011). Although both the *S. aureus* and *B. burgdorferi* NDK complexes are bound to the same ligands, there is an important difference in the locations and configurations of the vanadate moieties. In the *S. aureus* structure vanadate is not covalently bound to ADP, but seems to be bound to His115, the supposed histidine residue *via* which the reaction proceeds in this construct. This is supported by the tetrahedral shape of the vanadate molecule, implying that other interactions are too weak to change the electron configuration. In our structure, the vanadate is covalently linked to the β -phosphate on the bound ADP, but also interacts with the catalytic histidine. This was previously

Figure 2

A ClustalOmega analysis of amino-acid residues from *B. burgdorferi* NDK (sp|O51419|NDK_BORBU), *T. pallidum* NDK (sp|O83974|NDK_TREPA), *E. coli* NDK (sp|P0A763|NDK_ECOLI) and the A chain of *H. sapiens* NDK (sp|P15531|NDKA_HUMAN). Data were analysed with ESPrpt to include the structure of *B. burgdorferi* NDK over the residue alignment (Lehoux *et al.*, 1999). Identical residues are marked by red columns, while highly conserved are highlighted in red, with related residues in outlined boxes. Lys11, Arg92, Thr98, Arg109, Asn129 and His132 are all conserved across these four species and are used in the binding pocket and the transferase reaction (His132).

explained by the trigonal bipyramidal shape, implying contributing factors from both the ADP and the catalytic histidine residue. Fig. 4 shows these two vanadate moieties side by side to illustrate this important difference in shape and binding.

Although these structures are from different organisms, these images can be thought of as two separate reaction steps, further supporting the phosphohistidine-transfer theory. If we treat the vanadate similarly to the γ -phosphate that normally takes its place, we can illustrate how a charged interaction from the catalytic histidine is transferred to a covalent interaction on the β -phosphate of the ADP, as well as visualize the

bonds that are broken and formed using our transition-state mimic. Although neither of these structures have magnesium complexed as a cofactor, the architecture of the reaction is unaltered and provides visual evidence of the steps required to transfer the γ -phosphate in NDKs.

3.5. The active-pocket conformation changes owing to substrate binding

The structures of the apoenzyme and holoenzyme complex show conformational change on binding to the substrate. As mentioned previously, hydrogen bonds and hydrophobic interactions from surrounding residues stabilize the substrate

Figure 3

Binding-pocket image of the structure featuring bound ADP with covalently bonded vanadate. Atoms are colored according to atom type (green, carbon; red, oxygen; blue, nitrogen; orange, phosphorus; gray, vanadium). The two images depict the same structure viewed at roughly orthogonal angles. Red dashed lines indicate hydrogen bonds between ADP–vanadate and Tyr53, Arg61, His62, Arg94, Thr100 and Arg111. Hydrophobic and π -bonding interactions between the adenosine base and His62 and Phe119 provide further stabilization of the ligand. Contributions from both polarized hydrogen bonds and hydrophobic interactions seem to hold the substrate in place, with each side of the molecule providing a specific stabilizing interaction. Vanadate is covalently bound to the β -phosphate of ADP but is in a trigonal planar configuration, implying that there is still a small contribution from the catalytic histidine to the predicted tetrahedral bonding structure. This figure was generated using PyMOL (Schrödinger).

Figure 4

Side-by-side image of vanadate complexes for our solved *B. burgdorferi* NDK structure (left; the same color scheme is used as in Fig. 3) and the previously solved *S. aureus* NDK structure (PDB entry 3q8y; right; a similar color scheme is used but with C atoms in cyan; Srivastava *et al.*, 2011). Our structure shows vanadate in a trigonal planar conformation with three terminal O atoms. A covalent bond to the nearest O atom on the β -phosphate (2.0 Å) and the His134 interaction (2.5 Å) is shown *via* this transition-state interaction, as the moiety would normally be transferred fully to the receiving ADP. The *S. aureus* structure shows a vanadate that is unbound to ADP, with charged interactions from both His115 and ADP stabilizing the molecule. If the ADP was not bound, His115 could account for the vanadate stabilization. This figure was generated using PyMOL (Schrödinger).

Figure 5

Comparison of an empty active site and an occupied active site. The coloration for the ADP-vanadate-bound structure is the same as that in Fig. 3; the apo structure is colored with C atoms rendered in yellow. The pocket ‘closes’ around the substrate owing to hydrogen bonds and other hydrophobic interactions. This could allow the phosphohistidine bound to the γ -phosphate to pass sufficiently close to the β -phosphate to form a covalent bond. Once the bond has been formed, the repulsion could force the substrate out of the active site and relax the pocket again. Our transition-state mimic captures the possible transfer in action and how the electronic surface of the binding pocket is altered. This figure was generated using *PyMOL* (Schrödinger).

within the active pocket. Once the substrate has bound, there is very little shift in the binding pocket towards the phosphoryl-transfer site of the molecule, as much of the conformational shift is shown to relate to the nucleoside base of the ADP. Residues His52–Trp67 in an α -helix near the binding pocket move closer to the ADP substrate once bound. Conversely, other protein structures shift away from the bound molecule once it interacts with the binding pocket. Ala99–Asn131, a helix arm that starts close to the α -phosphate, turns and then travels underneath and upwards around the adenine base, while shifting in plane directly away from the ADP molecule. This information is visualized in Fig. 5, which shows the unbound active site, the active site bound with our ligands and a conformational overlay generated in *PyMOL* (Schrödinger). While this structural shift is

only shown here in ADP, other bases (CDP, GDP *etc.*) may have a similar alteration within the transition state owing to their carbon backbone. This shift could be owing to electron repulsion within the highly electronegative phosphate groups and the stabilizing nature of the carbon backbone that forms the nucleoside base. Ejecting the substrate NTP would then relax the pocket, returning the conformation of the enzyme to its original state for more catalytic production.

4. Conclusions

B. burgdorferi affects thousands of people each year in the US alone and an even greater number globally. The burden, persistence and suboptimal therapy of Lyme disease are ever-present issues that require new methods of intervention. NDK

has been identified as a unique target that is important in cellular growth and metabolism. In this article, we have shown that *B. burgdorferi* NDK is important for robust infection and colonization in a mouse model. We demonstrate the structure of the nucleoside diphosphate kinase at 2.2 Å resolution, providing structures of the apoenzyme and holoenzyme bound to ligands to visualize changes owing to binding and the phosphoryl-transfer mechanism. Analysis of the structure, function and biochemical properties are presented, identifying this NDK as an excellent enzyme for further enzymatic study and potential therapeutic inhibition.

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