Alonso A. López-Zavala,^a
Idania E. Quintero-Reyes,^b
Jesús S. Carrasco-Miranda,^a
Vivian Stojanoff,^c Andrzej
Weichsel,^d Enrique Rudiño-
Piñera^e and Rogerio R. Sotelo-
Mundo^{a*}

^aCentro de Investigación en Alimentación y Desarrollo, A.C. (CIAD), Carretera a Ejido La Victoria Km 0.6, Apartado Postal 1735, Hermosillo, 83304 Sonora, Mexico, ^bUniversidad de Sonora, Blvd Bordo Nuevo s/n, Ejido Providencia, 85039 Cd Obregón, Sonora, Mexico, ^cNational Synchrotron Light Source, Brookhaven National Laboratory, Upton, NY 11973, USA, ^dMacromolecular Crystallography Core, The University of Arizona, Biological Sciences West, 1041 East Lowell Street, Tucson, AZ 85721, USA, and ^eDepartamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología (IBT), Universidad Nacional Autónoma de México (UNAM), Avenida Universidad 2001, Colonia Chamilpa, Cuernavaca, 62210 Morelos, Mexico

Correspondence e-mail: rrs@ciad.mx

Received 9 June 2014
Accepted 3 July 2014

PDB references: LvNDK, complex with dCDP, 4uog; complex with ADP, 4uoh; complex with dADP, 4uof

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Structure of nucleoside diphosphate kinase from pacific shrimp (*Litopenaeus vannamei*) in binary complexes with purine and pyrimidine nucleoside diphosphates

Nucleoside diphosphate kinase (NDK; EC 2.7.4.6) is an enzyme that catalyzes the third phosphorylation of nucleoside diphosphates, leading to nucleoside triphosphates for DNA replication. Expression of the NDK from *Litopenaeus vannamei* (LvNDK) is known to be regulated under viral infection. Also, as determined by isothermal titration calorimetry, LvNDK binds both purine and pyrimidine deoxynucleoside diphosphates with high binding affinity for dGDP and dADP and with no heat of binding interaction for dCDP [Quintero-Reyes *et al.* (2012), *J. Bioenerg. Biomembr.* **44**, 325–331]. In order to investigate the differences in selectivity, LvNDK was crystallized as binary complexes with both acceptor (dADP and dCDP) and donor (ADP) phosphate-group nucleoside diphosphate substrates and their structures were determined. The three structures with purine or pyrimidine nucleotide ligands are all hexameric. Also, the binding of deoxy or ribonucleotides is similar, as in the former a water molecule replaces the hydrogen bond made by Lys11 to the 2'-hydroxyl group of the ribose moiety. This allows Lys11 to maintain a catalytically favourable conformation independently of the kind of sugar found in the nucleotide. Because of this, shrimp NDK may phosphorylate nucleotide analogues to inhibit the viral infections that attack this organism.

1. Introduction

Nucleoside diphosphates from either a *de novo* or a salvage pathway require a final phosphorylation stage that leads to substrates for DNA polymerase. Nucleoside diphosphate kinase (EC 2.7.4.6; NDK) is responsible for catalyzing the final phosphorylation reaction of nucleoside diphosphates. This reaction is a reversible phosphate transfer to both ribonucleoside and deoxyribonucleoside diphosphates using NTP as a phosphate donor to provide adequate nucleosides for RNA or DNA synthesis (Gonin *et al.*, 1999). The reaction catalyzed by NDK starts with the transfer of a phosphate from ATP to His117; ADP is then released and the acceptor nucleoside diphosphate is bound and phosphorylated. Nucleotide phosphates are coordinated by a network of basic residues, Arg94, Arg9 and His59, and the polar Thr98 (from the *Dictyostelium discoideum* amino-acid sequence; Cherfils *et al.*, 1994). All NDKs follow a ping-pong enzymatic mechanism involving a phosphorylated histidine residue in the active site (Morera *et al.*, 1995).

NDKs are known to have relaxed substrate specificity, since they phosphorylate both deoxyribonucleotides and ribonucleotides (Bilitou *et al.*, 2009; Deville-Bonne *et al.*, 2010). This enzyme has been characterized from several prokaryotic and eukaryotic sources, and has been described as an important housekeeping enzyme that maintains and controls the pool of intracellular nucleotides (Bernard *et al.*, 2000).

Crystallographic and biochemical studies have indicated that most NDKs are hexameric (Dumas *et al.*, 1992; Lasca *et al.*, 2000); exceptions are the dimeric NDK from *Halomonas* sp. 593 (Tokunaga *et al.*, 2008) and the tetrameric NDKs from *Escherichia coli* (Moynié *et al.*, 2007), *Aquifex aeolicus* (Boissier *et al.*, 2012) and *Myxococcus xanthus* (Williams *et al.*, 1993).

Table 1Data reduction and refinement statistics of *Lv*NDK binary-complex structures.

Values in parentheses are for the highest resolution shell.

Data set	<i>Lv</i> NDK-ADP (PDB entry 4uoh)	<i>Lv</i> NDK-dADP (PDB entry 4uof)	<i>Lv</i> NDK-dCDP (PDB entry 4uog)
X-ray source	NSLS X6A	NSLS X6A	NSLS X6A
Detector	Quantum 270 CCD	Quantum 270 CCD	Quantum 270 CCD
Wavelength (Å)	0.979	0.979	0.979
Space group	C222 ₁	C222 ₁	C222 ₁
Unit-cell parameters (Å)	<i>a</i> = 69.8, <i>b</i> = 120.8, <i>c</i> = 103.3	<i>a</i> = 70.1, <i>b</i> = 134.3, <i>c</i> = 104.7	<i>a</i> = 70.1, <i>b</i> = 135.3, <i>c</i> = 104.8
No. of residues	430	453	453
Monomers per asymmetric unit	3	3	3
Matthews coefficient (Å ³ Da ⁻¹)	2.1	2.4	2.4
Solvent content (%)	42.5	49.5	49.5
Resolution range (Å)	34.8–2.0 (2.1–2.0)	19.8–2.10 (2.2–2.1)	19.3–2.3 (2.4–2.3)
Total reflections	242640 (23658)	207177 (10259)	176768 (18322)
Unique reflections	29462 (2912)	26320 (2111)	21500 (2199)
<i>R</i> _{merge}	0.145 (0.735)	0.142 (0.621)	0.171 (0.702)
Completeness (%)	99.6 (96.4)	91.2 (95.1)	95.2 (97.7)
$\langle I/\sigma(I) \rangle$	16.7 (3.4)	15.4 (2.7)	13.4 (3.5)
Wilson plot <i>B</i> value (Å ²)	24.01	20.36	22.50
Multiplicity	8.2 (8.1)	7.8 (5.4)	8.2 (8.3)
<i>R</i> _{work} / <i>R</i> _{free} [†]	0.178 (0.241)/0.217 (0.298)	0.189 (0.243)/0.248 (0.299)	0.198 (0.296)/0.256 (0.368)
Content of the asymmetric unit			
Protein atoms	3371	3579	3579
Ligand atoms	56	81	75
Water molecules	422	481	221
R.m.s.d. from ideal			
Bond lengths (Å)	0.012	0.013	0.015
Bond angles (°)	1.27	1.87	1.55
Mean overall <i>B</i> value (Å ²)			
Protein	24.7	21.7	21.8
Ligand	27.2	25.7	33.9
Solvent	31.1	26.8	25.0
Ramachandran plot, residues in			
Most favoured regions	421 [98%]	439 [97.0%]	439 [97%]
Additionally allowed regions	6 [1.3%]	9 [2.1%]	10 [2.3%]
Outliers	3 [0.7%]	4 [0.9%]	3 [0.7%]

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ represent the intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements. R_{free} was calculated using 5% of the reflections.

Tetramers of NDKs are formed through a dimer of dimers, while the hexameric arrangement is achieved either by a trimer of dimeric units or dimer of trimeric units (Gonin *et al.*, 1999; Lascu *et al.*, 2000). Hexameric *D. discoideum* NDK is one of the most well characterized enzymes from this family. Like other NDKs, it shows a characteristic fold with a $\beta\alpha\beta\beta\alpha\beta$ motif, similar to the ferredoxin fold, where α -helices pack onto a β -sheet motif, creating a highly conserved hydrophobic central core (Janin *et al.*, 2000). A further two typical features that complete the folding of the NDK monomer are the Kpn loop (named after the *Drosophila* Killer of prune mutant) and the C-terminus. The Kpn loop is a small compact structure comprised of residues 96–113 in human NDK. The C-terminal fragment is composed of the last 20 residues in hexameric NDKs and this small domain interacts with neighbouring subunit residues. This interaction is associated with loss of enzymatic activity and quaternary stabilization of hexameric NDK. In the tetrameric *M. xanthus* NDK the C-terminus is shorter by ten residues and is not implicated in function (Williams *et al.*, 1993). In most of the NDK crystal structures in complex with a nucleoside diphosphate, the active site presents a phosphate moiety bound to the side of the β -sheet and the purine or pyrimidine base is located in a hydrophobic pocket formed by a conserved Phe and Ile/Val. The complex also indicates that the active site has a preformed rigid core with very little change in protein conformation after nucleotide binding and in the covalent phosphorylated histidine intermediate.

NDK from the marine shrimp (Crustacea, Decapoda) *Litopenaeus vannamei* (*Lv*NDK) contains 151 residues and shows high amino-acid identity (>75%) to the hexameric human and *D. discoideum* NDKs and only ~42% identity to the tetrameric NDKs from *M. xanthus*

(Williams *et al.*, 1993) and *E. coli* (Moynié *et al.*, 2007). In general, the sequence of the shrimp NDK is highly conserved among eukaryotic and bacterial NDKs (40 and 60% identity, respectively).

We have studied shrimp NDK in solution: it is a homotrimer of ~57 kDa and binds and phosphorylates nucleoside diphosphates (Quintero-Reyes *et al.*, 2012). The aim of this work is to experimentally characterize the structural interaction of an invertebrate NDK with either acceptor (dADP and dCDP) or donor (ADP) phosphate substrates in their binary complexes. Since this enzyme appears to be key in viral infection by *White spot syndrome virus* (Leu *et al.*, 2011), it may provide clues towards novel antiviral strategies.

2. Materials and methods

2.1. *Lv*NDK binary-complex co-crystallization with different nucleoside diphosphates

Recombinant nucleoside diphosphate kinase from *L. vannamei* (GenBank DQ907945) was obtained from a synthetic gene optimized for expression in *E. coli* and its purification has been described elsewhere (Quintero-Reyes *et al.*, 2012). A screening of crystallization conditions of *Lv*NDK was performed using Crystal Screen from Hampton Research (Aliso Viejo, California, USA) by the microbatch method in Greiner plates. Three different nucleotide complexes were obtained by incubating *Lv*NDK (20 mg ml⁻¹) with ADP, dCDP or dADP at 20 mM concentration in solution consisting of 100 mM NaCl, 40 mM MgCl₂, 4 mM DTT. Each nucleotide complex was incubated for 30 min at 277 K before the crystallization trials. The drops were set up by mixing 1 µl nucleotide–enzyme complex solu-

tion with 1 μ l Crystal Screen crystallization solution (50 solutions) and were covered with 10 μ l paraffin oil. The plates were incubated at 289 K and monitored every 3 d until crystals appeared.

After a week, crystals of all of the *Lv*NDK–nucleotide complexes could be grown using three different crystallization buffers. Specifically, *Lv*NDK binary-complex crystals were obtained using the following conditions: conditions 6 [0.2 M magnesium chloride hexahydrate, 0.1 M Tris–HCl pH 8.5, 30% (w/v) PEG 4000] and 22 [0.2 M sodium acetate trihydrate, 0.1 M Tris–HCl pH 8.5, 30% (w/v) PEG 4000] gave crystals for all nucleotides (*Lv*NDK–dADP, *Lv*NDK–dCDP and *Lv*NDK–ADP), while condition 19 [0.2 M ammonium acetate, 0.1 M Tris–HCl pH 8.5, 30% (v/v) 2-propanol] only gave crystals of the dCDP binary complex. Before cooling, the crystals were transferred into a cryocooling solution containing 30% (v/v) PEG 400 substituted for PEG 4000 in the mother liquor, except for condition 19 where 2-propanol acted as a cryoprotectant. Cryoprotectant solutions were supplemented with the appropriate ligands to give the same final concentration as in the crystallization drops. 24 single crystals, including all of the binary complexes, were mounted using CryoLoops according to their size, flash-cooled in liquid N₂ and stored at 100 K.

2.2. X-ray data collection and structure determination

*Lv*NDK crystals were transported to the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL), Upton, USA for data collection. Diffraction experiments were performed on beamline X6A using an ADSC Quantum 270 detector. A data set for each binary complex was collected from a single crystal at a wavelength of 0.979 Å (12 672 eV) at 100 K. 200 images were collected for each crystal with an oscillation range of 1.0° in all cases. The crystal-to-detector distance was 250, 290 and 230 mm for the *Lv*NDK–ADP, *Lv*NDK–dCDP and *Lv*NDK–dADP crystals, respectively. Data sets were reduced and scaled using *XDS* (Kabsch, 2010). Data-collection statistics are listed in Table 1. Initial analysis was performed with the *Lv*NDK–dADP data, and the phase problem was solved by molecular replacement using the NDK crystal structure

from bovine retina as a search model (PDB entry 1be4; Abdulaev *et al.*, 1998). The identity between the shrimp and bovine NDK amino-acid sequences was 78%. Phases were obtained by molecular replacement using *Phaser* in the *CCP4* suite (Winn *et al.*, 2011; McCoy *et al.*, 2005). The *Lv*NDK–dADP structure was refined and used as a starting model in the refinement of the other *Lv*NDK binary complexes. Several refinement cycles were performed using *PHENIX* (Adams *et al.*, 2010) and *Coot* for manual building (Emsley *et al.*, 2010). Ligands were added manually by visual inspection of the electron density at the active site using a $2F_o - F_c$ map at 1.5σ . All figures were produced using *CCP4mg* (McNicholas *et al.*, 2011).

3. Results and discussion

3.1. Overall *Lv*NDK binary-complex structures

To achieve the nucleotide phosphorylation reaction, the active site of *Lv*NDK has a nucleotide-binding site and a catalytic histidine nucleophile (His117), which is part of a phosphorelay that transfers a phosphoryl group obtained from ATP to the nucleoside diphosphate (Janin *et al.*, 2000). Enzymatic activity and isothermal titration calorimetry assays of *Lv*NDK have been reported (Quintero-Reyes *et al.*, 2012).

As we have previously shown, recombinant *Lv*NDK is active and tightly binds nucleoside diphosphates. The dissociation constant (K_d) was determined using ITC: the K_d for dADP was 14.6 μ M (Quintero-Reyes *et al.*, 2012) and the K_d for ADP was 17 μ M (unpublished data). The binding of dCDP is atypical, because although *Lv*NDK phosphorylated the nucleotide no heat of binding was observed by ITC (Quintero-Reyes *et al.*, 2012).

Crystallization plates were set up with *Lv*NDK pre-equilibrated with dADP, dCDP or the ribonucleotide ADP, in all cases including Mg²⁺. All crystals obtained belonged to space group *C*222₁ with similar unit-cell parameters and highest resolution limits in the range 1.8–2.3 Å. The Matthews coefficient indicates three monomers per asymmetric unit ($V_M = 2.4 \text{ Å}^3 \text{ Da}^{-1}$ and 49.5% solvent content for *Lv*NDK–dADP; Matthews, 1968; see Table 1 for details). This is consistent with our previous data showing that *Lv*NDK is a trimer in solution by size-exclusion chromatography studies (Quintero-Reyes *et al.*, 2012), as previously reported for *B. anthracis* trimeric NDK (Misra *et al.*, 2009). Crystal-packing analysis performed with *PISA* (Protein Interfaces, Surfaces and Assemblies (<http://pdbe.org/pisa/>; Krissinel & Henrick, 2007) suggested that a hexamer is actually the biologically significant molecule and therefore our experimental evidence suggests that a dimer of trimers is present instead of a trimer of dimers as observed elsewhere (Lascu *et al.*, 2000).

The overall structure of each *Lv*NDK monomer shows a typical α/β fold, including six α -helices with a central core formed by a four-stranded antiparallel β -sheet (see Fig. 1). This β -sheet, a small flexible loop (55–59 residues) and the Kpn loop participate in the nucleotide-binding site. Additionally, the Kpn loop located at the centre of the trimer plays an important role in stabilizing the quaternary structure (Lascu *et al.*, 2000). Likewise, the C-terminus of each monomer makes intersubunit contacts with neighbouring side chains, providing structural stability to the enzyme (Karlsson *et al.*, 1996).

The C α -atom root-mean-square deviation (r.m.s.d.) of each monomer within the complete structure is less than 0.6 Å. In this work, the nucleotide–Mg²⁺ complex occupies each active site. The catalytic histidine (His117) and other amino-acid residues that interact with the phosphate group (Arg87) and the sugar moiety (Lys11 and Asn114) and Phe59 and Val111 that stabilize the nitrogen base of the nucleoside diphosphate substrate are invariant and no

Figure 1
Ribbon representation of the asymmetric unit content of an *Lv*NDK structure with one substrate molecule bound to each monomer. Each subunit is shown in a different colour (yellow, blue and green) and dADP–Mg²⁺ is presented as spheres coloured by atom type.

conformational changes were observed upon ligand binding (r.m.s.d. of 0.22 Å).

One striking feature of NDK is the lack of specificity towards the nucleoside base. The positions of a purine (ADP) and a pyrimidine (dCDP) are superimposable and invariant, as are the phosphate-binding pocket and ribose contacts (Lys11 and Asn114; Fig. 2). The nitrogenous bases are contacted by a hydrophobic sandwich comprised of Phe59 and Val111, which are conserved among all known NDKs.

3.2. *Lv*NDK nucleotide-binding site: comparison of ribonucleoside and deoxyribonucleoside diphosphates

To understand the structural details of nucleotide binding, *Lv*NDK was co-crystallized with both ribonucleoside (*Lv*NDK–ADP) and

deoxyribonucleoside (*Lv*NDK–dADP and *Lv*NDK–dCDP) diphosphates.

Fig. 3 shows a superposition of the active site in the *Lv*NDK binary complexes with dADP and ADP. The positions of the amino-acid residues Arg87 and Lys11, Phe59, Val111 and His117 (not shown) are identical (r.m.s.d. < 0.22 Å). The hydrophobic ‘sandwich’ made by the side chains of Phe59 and Val111 with the purine ring is remarkable, so the electron density strongly supports the ligands modelled in two different structures.

The *Lv*NDK binary complex with ADP (Fig. 3, ligand in green) has two hydrogen bonds between Lys11 and the 2′-OH and 3′-OH ribose groups. These contacts are consistent with those reported by Cherfils *et al.* (1994) in the crystal structure of NDK–ADP from *D. discoideum* and also by other authors (Janin *et al.*, 2000; Souza *et al.*, 2011). When dADP is co-crystallized with *Lv*NDK (Fig. 3, ligand in magenta), the position of the ribose is identical apart from the lack of the 2′-OH group. Nonetheless, ‘replacing’ the ribose hydroxyl with a water interacting with Lys11 conserves the hydrogen-bonding pattern. The same water–Lys11 hydrogen bonding was also observed in the complex of *Lv*NDK with dCDP. Lys11 and Asn114 are well conserved among all NDK kinases, creating a preformed rigid pocket that includes an ordered water molecule as needed (Cherfils *et al.*, 1994). With regard to the lack of heat of dCDP binding as reported previously (Quintero-Reyes *et al.*, 2012), in the crystal structure we observed a larger *B* factor for dCDP (33.9 Å²) compared with 25.7 Å² for dADP or 27.2 Å² for ADP.

Although all of the structures reported here appear to be very similar, the main conformational change of NDKs occurs during the binding of nucleoside monophosphates to the ligand-free structures, as reported by Souza *et al.* (2011). We conclude that the broad substrate recognition of *Lv*NDK is achieved through a hydrophobic interaction between the Phe59 and Val111 residues and the heterocyclic base and that the binding of deoxynucleosides is assisted through a hydrogen bond to a water molecule. This suggests that invertebrate NDKs may phosphorylate nucleoside analogues that could act as chain terminators for DNA viruses such as *White spot syndrome virus* (Liu *et al.*, 2009).

AAL-Z, IEQ-R and JSC-M were supported by a PhD fellowship from CONACyT (Mexico’s National Science and Research Council). RS-M acknowledges financial support from CONACyT grants CB-2009-131859 and E0007-2011-01-179940 and grant 2011-050 from the Texas A&M–CONACyT Collaborative Grant Program. We thank Edwin Lazo for technical support at BNL NSLS X6A and Gerardo Reyna, Felipe Isac, Luis Leyva, Adalberto Murrieta, Jose Luis Aguilar and Martin Peralta for bibliographical and computational support from CIAD. The authors acknowledge the editorial reviewers for extensive corrections and suggestions.

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