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Shrimp oncoprotein nm23 is a functional nucleoside diphosphate kinase

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Abstract Biosynthesis of nucleoside triphosphates is critical for bioenergetics and nucleic acid replication, and this is achieved by nucleoside diphosphate kinase (NDK). As an emerging biological model and the global importance of shrimp culture, we have addressed the study of the Pacific whiteleg shrimp (*Litopenaeus vannamei*) NDK. We demonstrated its activity and affinity towards deoxynucleoside diphosphates. Also, the quaternary structure obtained by gel filtration chromatography showed that shrimp NDK is a trimer. Affinity was in the micro-molar range for dADP, dGDP, dTDP and except for dCDP, which presented no detectable interaction by isothermal titration calorimetry, as described previously for *Plasmodium falciparum* NDK. This information is particularly important, as this enzyme could be used to test nucleotide analogs that can block white spot syndrome virus (WSSV) viral replication and to study its bioenergetics role during hypoxia and fasting.

Keywords Nucleoside diphosphate kinase · DNA replication · WSSV · Shrimp · *Litopenaeus vannamei* · Deoxynucleotide

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Introduction

The biosynthesis of non-adenine nucleoside triphosphates is fundamental for bioenergetics, DNA replication, sugar and lipid biosynthesis, and signal transduction pathways (Lascu 2000). This function is done by the nucleoside diphosphate kinases, which catalyzes the ATP-dependent phosphorylation of ribo- and deoxyribonucleoside diphosphates. Maintenance of the deoxynucleotide triphosphate (dNTP's) pool is key for DNA replication to occur. In shrimp, there is a global menace to its farming in the form of a DNA-virus, the White Spot Syndrome virus (WSSV) (Leu et al. 2009).

From a transcriptome study in *Litopenaeus vannamei* (Clavero-Salas et al. 2007) a nucleoside diphosphate kinase cDNA (GenBank: DQ907945.1) was identified by its amino acid identity to the human oncoprotein nm 23, and it was demonstrated that it is up-regulated upon viral infection. This result was confirmed in another transcriptome study from WSSV-challenged organisms (Leu et al. 2011).

During viral DNA replication, a dNTP's pool is required and alteration of the nucleotide supply is an antiviral strategy used against human viral diseases. During herpes infection, the alteration of the dNTP's pool leads to an increase in mutation frequency and nucleoside kinases are involved in that mechanism (Pyles & Thompson 1994). The successive phosphorylation steps that lead to dNTP's are catalyzed by viral kinases

(WSSV thymidine kinase and thymidylate monophosphate kinase (Tzeng et al. 2002) or kinases from the host, unknown in shrimp to date.

Nucleoside diphosphate kinase (NDK, E.C. 2.7.4.6) catalyzes the transfer of the γ-phosphate of a donor (ATP or GTP) onto an acceptor (nucleoside or deoxynucleoside diphosphate). The mechanism of catalysis goes through a ping-pong mechanism, where the NDK implicates a covalent intermediate in which a catalytic histidine residue is transiently phosphorylated (Lascu et al. 2000; Garces & Cleland 1969; Deville-Bonne et al. 2010). NDK is considered the main source of the dNTP's pool for the DNA polymerase; and for CTP and UTP required by RNA polymerase for transcription (Bilitou et al. 2009; Deville-Bonne et al. 2010). This enzyme has been characterized from several prokaryotic and eukaryotic sources; although more detailed studies have been done in the human NDKs, where described as a housekeeping enzymes carrying out an important function in maintaining and controlling the intracellular nucleotides pool (Lacombe et al. 2000).

Several crystallographic and biochemical studies indicate that most NDP kinases are hexameric but tetramers and trimers also exist (Munch-Petersen et al. 1991; Lascu et al. 2000; Janin et al. 2000). NDK tetramers are formed by a dimer of dimers while the hexameric arrangement is achieved either by a trimer or dimeric units or dimer of trimeric units (Kumar et al. 2011). Three conserved residues from the active site of sNDK are histidine, glutamate and serine. However, the catalytic mechanism, involves only the histidine residue that coordinates a magnesium ion at the active site. The shrimp NDK (GenBank ABI93176.1) contains 151 residues, a predicted molecular mass of 16.9 kDa and a pI 7.8. The deduced amino acid sequence shares 72% and 77% identity with *B. mori* and *H. sapiens*, respectively. sNDK is overexpressed during WSSV infection (Clavero-Salas et al. 2007; Leu et al. 2011), but its biochemical activity has not been demonstrated yet.

Nucleoside kinases are antiviral targets of several pathogens, as they can process the activation of nucleotide or nucleoside analogues. Nowadays, nucleoside analogues as acyclovir, and ganciclovir, are pro-drugs directed to different virus kinases. However, there are no rational antiviral strategies against WSSV yet. Therefore, the aim of this study is to obtain recombinant *L. vannamei* shrimp NDK (NDK) to characterize its biochemical activity and binding to substrates which then could validate further structural studies as a *bona fide* antiviral target for drug design against WSSV.

Materials and methods

Production of recombinant *L. Vannamei* sNDK

The *L. vannamei* sNDK amino acid sequence was obtained from GenBank (Accession ABI93176.1) and its coding sequence was optimized and synthetically produced for optimal expression in *E. coli* using the pJexpress414 vector (DNA 2.0). The optimized synthetic gene construct contained a six-histidine sequence and a protease cleavage site. The construct was introduced in chemically competent *E. coli* BL21-SI (Novagen, USA) cells that had been previously transformed with a plasmid encoding bacterial chaperones (pG-KJE8, Takara, USA). The pJexpress414 vector contains an ampicillin resistance marker and a promoter inducible by isopropyl β-D-1-thiogalactopyranoside (IPTG). All reagents were research grade or better from Sigma-Aldrich, México.

The transformed bacteria was grown at 37 °C in LB media with no NaCl, in the presence of ampicillin and chloramphenicol at 50 μg/mL and 20 μg/mL, respectively. When the culture reached an optical density of 0.6 at 600 nm, it was induced with 1 mM of IPTG, 0.3 M of NaCl and L-arabinose (5 mg/mL) in order to induce expression of the heterologous protein and the chaperones. The induction was carried out for 5 h. The cells were harvested at 5,000 *x g* at 4 °C for 10 min and washed with 0.9% NaCl. The bacteria were busted by sonication in the presence of hen egg white lysozyme (500 μg/mL), and the soluble fraction obtained by centrifugation at 35,000 *x g* at 4 °C during 30 min. Evaluation of expression was done by SDS-PAGE of non induced and induced soluble fractions and stained with histidine-tag staining reagent (Pierce, USA).

Purification of *L. vannamei* sNDK by immobilized metal affinity chromatography (IMAC)

Bacterial soluble proteins were further clarified by addition of 10% streptomycin sulfate and centrifuged at 35,000 *x g* at 4 °C for 30 min. The supernatant was loaded onto a 5 mL His-Trap FF column (GE Healthcare, USA) previously equilibrated with 20 mM sodium phosphate buffer pH 7.4, 500 mM NaCl (10 column volumes). The sNDK protein was obtained with elution buffer (20 mM sodium phosphate buffer pH 7.4, NaCl 500 mM and 500 mM imidazole). Fractions of 3 mL were collected and analyzed by 15% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R-250.

L. vannamei sNDK kinase activity towards dTDP, dGDP, dCDP and dADP by a spectrophotometric-coupled assay

The sNDK activity was estimated measuring the oxidation of NADH to NAD^+ at 340 nm using a coupled assay as previously described (Kandeel & Kitade 2010). The total reaction volume was 1.5 mL. The mixture contained 50 mM Tris–HCl pH 7.5, 2 mM ATP, 5 ng of sNDK, 5 mM $MgCl_2$, 10 mM of KCl, 0.14 mM NADH, 2 mM phosphoenol pyruvate (PEP), 1.3 U pyruvate kinase (PK) and 2.3 U of lactate dehydrogenase (LDH). The reaction was started with the addition of

0.5 mM of the corresponding dinucleoside diphosphate (dTDP, dCDP, dGDP, dADP). One unit of enzyme is defined as the turnover of 1 μmol of substrate per second.

Determination of quaternary structure of sNDK by size exclusion chromatography

Determination of the oligomeric state of sNDK was done using size exclusion chromatography (Superdex 200 column; GE Healthcare); previously calibrated with molecular weight standards. The molecular weight markers (MWM) were prepared in 20 mM sodium phosphate buffer pH 7.4, 200 mM NaCl and 5% glycerol. The molecular weight standards were albumin (66 kDa), ovalbumin (44 kDa) and soybean trypsin inhibitor (21.5 kDa).

Isothermal titration calorimetry

To determine thermodynamic parameters of sNDK binding towards deoxynucleotides diphosphate, we used a VP-ITC (MicroCal Inc., USA). 1.4 mL of sNDK 20 μM was loaded into the sample cell and it was titrated with 1 mM of each deoxynucloside diphosphate. All the solutions were prepared with the final dialysis buffer of sNDK and thoroughly degassed. The experiments were performed at 30 °C with a stirring speed of 249 rpm. Each 10 μL-injection of nucleoside diphosphate was spaced 300 s to ensure proper equilibrium between each titration. To determine the heat of dilution two control experiments were made. First, each nucleoside was injected to the protein dialysis buffer (without sNDK). Second, the dialysis buffer was injected to the sNDK enzyme. The data were fitted to a single site model to yield thermodynamic parameters such as enthalpy of binding (ΔH), association constant (Ka) and stoichiometry of binding (n). The free energy of binding (ΔG) was calculated from the equation $\Delta G = RT \ln K$ and the entropy changes (ΔS) with $\Delta G = \Delta H - T\Delta S$.

In silico modeling and docking of sNDK

A theoretical molecular structure of sNDK was done based on its sequence identity to a human NDK deposited in the PDB (code: 2HVD) (Giraud et al. 2006). Homology modeling and docking were performed with MOE ver. 2010.10 (Chemical Computing Group, Canada). Molecular modeling was started with a multiple sequence alignment in order to improve the homology model. A total of 25 intermediate models were generated from the starting alignment under the CHARMM27 force field, which included ADP ligand coordinates from the template structure. The final model was further refined with the default parameters in MOE starting from the best of the intermediate models according to the protein geometry utility of the software.

Docking of deoxynucleosides diphosphate to the active site of sNDK was used to predict the binding mode and to correlate binding affinity of distinct dNDPs with structural issues. To dock, we first selected the closed conformation of the nucleoside-binding site of our molecular model of sNDK, without selecting those residues from the Mg^{+2} ion-binding site. Three independent stochastic runs of docking were performed in order to obtain convergence of the best positions of ligand-sNDK. The alpha-triangle placement method was used to search the orientations of nucleosides at the sNDK binding site. From 60,000 poses collected and evaluated for every nucleoside conformation, only 30 top-scoring positions were retained according to the default scoring function applied in MOE. Those positions were subjected to a refinement step to relax the poses at the binding site, where the 30 top-scoring poses were selected according to the affinity dG scoring function, which estimates the enthalpic contribution to the free energy of binding of the predicted interactions in the docked poses.

Results

Shrimp NDK was readily obtained as a soluble protein (Fig. 1) and it was purified in a single step using an IMAC His-trap FF column (Fig. 2). All NDKs are known to be oligomeric, and their activity is dependent on the quaternary structure. Eukaryotic NDKs are predominantly hexamers, although trimers are found in some species. Several crystallographic structures and biochemical studies indicated that most NDK's are hexameric, so the discovery of tetrameric *Myxococcus* NDK was novel (Williams et al. 1993). In this work, we were able to determine the sNDK quaternary structure, which was a 66 kDa trimer with no significant presence of other species (Fig. 3). The specific activity of this NDK was evaluated towards

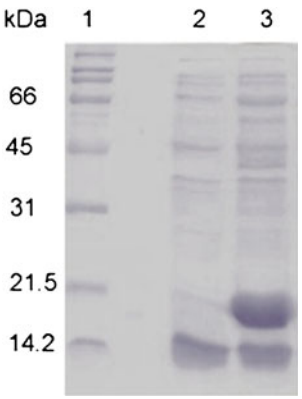

Fig. 1 Electrophoretic analysis of recombinant overexpression of sNDK. Lane 1: broad molecular weight marker, lane 2: supernatant before induction, lane 3: supernatant after induction with IPTG. The 14-kDa band that appears on lanes 1 and 2 is hen egg white lysozyme used to lyse bacteria. Gels were stained with Coomasie Blue R250

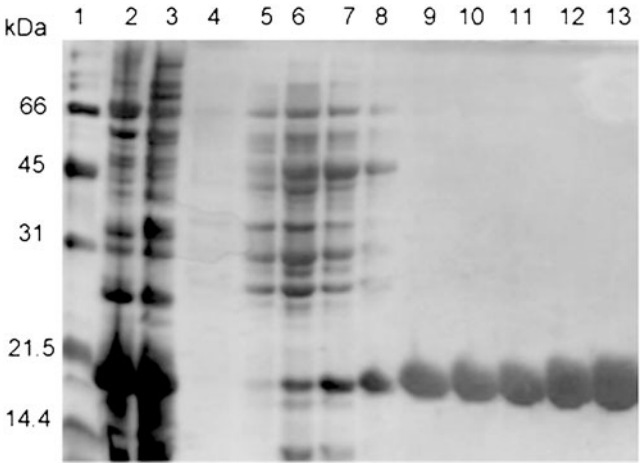

Fig. 2 Electrophoretic analysis of NDK by His-Tag chromatography. Lane 1: broad molecular weight marker, lane 2: crude extract of *E. coli* clarified, lane 3: flow through sample, lane 4–8: non-specific bound proteins, lane 9–13: pure recombinant sNDK from *L. vannamei*. Gels were silver stained

all four deoxynucleosides diphosphate (Table 1) using the PK-LDH enzyme coupled assay. We found that sNDK was active against all four deoxynucleosides diphosphate (dTDP 19,344 U/mg; dADP 14,626 U/mg; dCDP 9,648 U/mg; dGDP 5,505 U/mg). The assay detects the dNTP-dependent ATP degradation to ADP, and although the experiment has all proper controls to discount for non-specific ATPase activity, we pursued a more thorough characterization of dNDP's using isothermal titration calorimetry.

Among the studied nucleotides, dGDP (Fig. 4) had greater affinity (K_d=7.6 μM) for sNDK, and the experimental thermodynamic parameters indicate that binding is enthalpy-driven (Table 1). The amine group and the additional carbonyl in the guanine ring are critical for active site binding and this

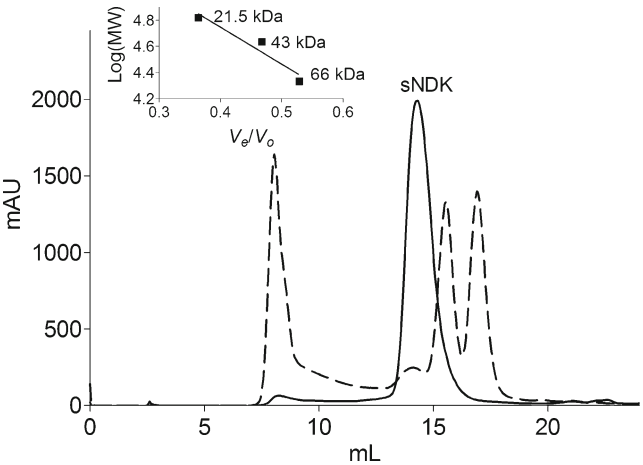

Fig. 3 sNDK quaternary structure. The native molecular weight was 66 kDa from calibration with molecular weight standards (see inset). This corresponds to a trimer

Table 1 Thermodynamic parameters and binding constants of sNDK towards deoxynucleoside diphosphates obtained by isothermal titration calorimetry (ITC)

Ligand	ΔH	$T\Delta S$	ΔG	K_d (μM)	n
dGDP	−4.4	2.7	−7.1	7.6	1.2
dADP	−2.7	4.0	−6.7	14.6	1.4
dTDP	−2.2	4.6	−6.8	13.7	2.3
dCDP	No detectable heat of interaction				

Values of ΔH, TΔS and ΔG are in kcal mol^{-1}

indicates that hydrogen bonds are present in the complex. dADP has a similar dissociation constant compared to dGDP but binding of the complex is entropy driven. dTDP shows a similar thermodynamic behavior as dADP. Interstingly, the purine nucleotides occupy only one of the subunits while dTDP bind two molecules per sNDK trimer (Fig. 4).

No heat was produced upon dCDP injection (Fig. 4). Since enzymatic activity was detected in a coupled-enzyme assay, it is possible that the enthalpy is so small for dCDP interaction that it cannot be detected by the equipment, and the stability of the complex is entropy-driven, by enthalpy-entropy compensation. Furthermore, strong enthalpy-entropy compensation indicates a higher magnitude of conformational changes, which is required for correct placement of active site residues facing the ligand. Thus, we expect that the binding of pyrimidyne nucleosides is accompanied by reduced mobility of the ligand accompanied by increased hydrophobic forces, lower conformational changes and less favorable enthalpy due to lower hydrogen bond formation (Kandeel & Kitade 2010).

The molecular model (Fig. 5) was predicted as a trimer according to the high identity (76%) of the amino acid sequence between sNDK and the human homolog oncoprotein nm23 (Chen et al. 2003), and experimentally it was confirmed by gel filtration chromatography (Fig. 3). Each sNDK monomer had a α/β domain, with two connecting α-helices at one side of an antiparallel β-sheet stabilized by hydrophobic interactions. The residues for binding and catalysis are conserved in sNDK (Chen et al. 2003). The position of the active site His117 residue for phosphoryl transfer is conserved by the presence of Ser119 and Glu128 (see Fig. 5, numbers from the sNDK amino acid sequence). The presence of other residues important for ligand interactions was also noted, such as Phe59 for nucleobase rings stacking, Lys11 and Asn114 for ribose 3′-OH H-bonding, and several basic residues that line the phosphate binding site (Fig. 5b). It is interesting to note that NDK was proposed to fit almost all planar nucleobase rings stacked at the active site (Janin et al. 2000), but it is a common feature in most NDKs that deoxycytidine nucleotides have a poor binding affinity (Kandeel & Kitade 2010).

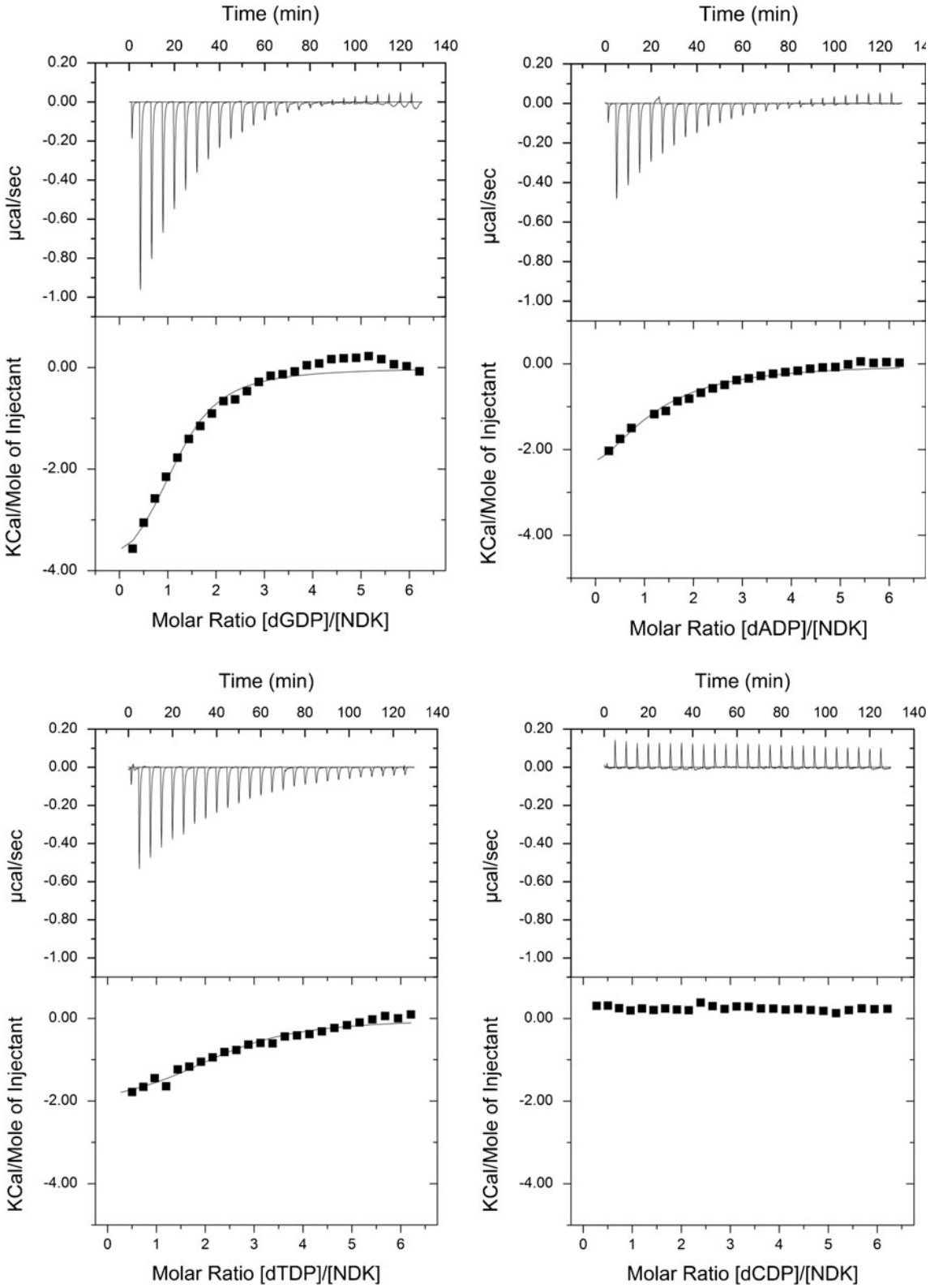

Fig. 4 Thermograms and ITC binding isotherms for the binding of deoxyribonucleosides diphosphate with *L. vannamei* NDK. The observed binding isotherm is normalized as kcal per mole of injectant and plotted against the molar ratio of ligand to macromolecule. The top panels show the raw data heat changes produced by successive injections of nucleotides into *L. vannamei* NDK. The lower panel shows the integrated binding isotherms as a function of the molar ratio of ligand to enzyme

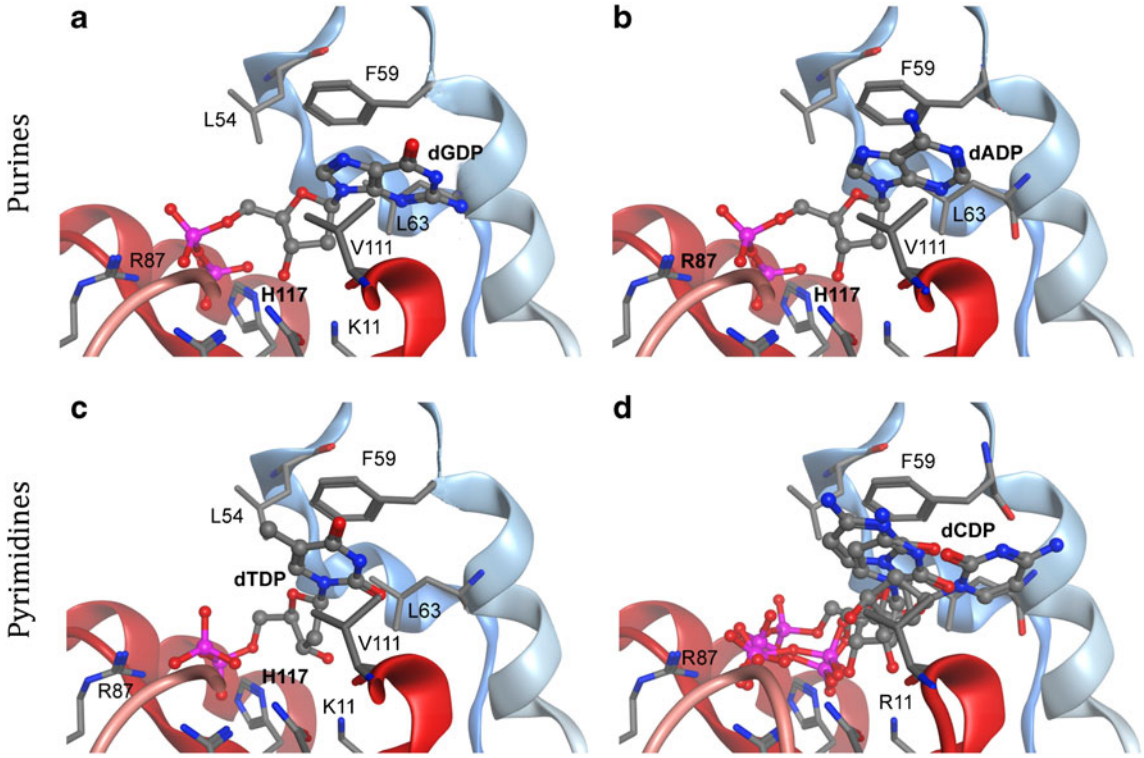

Fig. 5 Theoretical molecular models for nucleotide docking into the sNDK active site. **a** Binding of dGDP into the sNDK active site. A favorable interaction between the purine ring and F59, L63 and V111 is observed. R87 interacts with the phosphate groups. **b** For dADP the same interactions are found in the docked model. **c** The stacking interaction is comprosed by F59 and V111. K11 appears closer to the ribose ring compared to other models. **d** For dCDP several conformers of the nucleotide were found in the docking experiment. This is consistent with the lack of detectable interaction in ITC but enzymatic activity in the coupled phosphorylation assay

In hexameric NDK's the amino acids at the extreme C-terminus interact with the neighboring dimer, contributing to the hexamer stability. The residues involved in specific interactions within the dimer are conserved in all NDK's. Dimer assembly is due essentially to the formation of a continuous ß-strand between the two subunits and the interactions of two antiparallel alpha$_1$ helixes. In *Myxococcus* NDK the corresponding C-terminal segment is shorter and interacts with the neighboring subunit (Williams et al. 1993). In these structures, glutamate 29 makes two hydrogen bonds with main chain amide residues Val21 and Gly22 from the neighboring subunit. Lys31 makes hydrogen bonds with three main chain carbonyls of the neighboring subunit. Pro101 from three subunits makes a network of hydrogen bonds with three water molecules on the top and bottom of the hexamer. Finally, the C terminus makes contact with the neighboring subunit. None of these interactions occur in the *Myxococcus* tetrameric NDP kinase (Williams et al. 1993). In this structure, the C-terminus is shorter and reinforces the interaction within the dimer. These NDP kinases have shorter C-termini and do not have well conserved Pro101 and Lys31, which are essential for hexamer assembly. Other residues, totally conserved in eukaryotic kinases, are not conserved in the shrimp NDK, like His55. Interestingly, the side chains involved in the assembly of dimers into tetramers in *Myxococcus* NDP are not conserved in this group. A lack of conservation of interactions has already been reported for other bacterial proteins (Lascu et al. 2000).

Discussion

sNDK has no specific activity differences between purine and pyrimidyne deoxynucleoside diphosphate. A recombinant cytosolic NDK from *Pisum sativum* had a specific activity of 2,700 U/mg when measured using dCDP as a phosphate acceptor. This corresponds well to the reported activity 2,038 U/mg of purified pea mitochondrial NDK by the same assay (Struglics & Hakansson 1999) and sNDK is in the same order of magnitude (9,648 U/mg). Mutants containing the H117D mutation, lacked significant enzymatic activity, even thought dCDP is the least preferred acceptor for NDKs (Lascu & Gonin 2000).

NDK from eukaryotes have been found to be hexameric in the crystal state, like human NDK, *Drosophila melanogaster*, and *Dictyostelum discoideum*, but have been reported to be tetrameric or trimeric in solution. In both kinds of quaternary

structure, the assembly of dimers from monomers is identical. An example of *Dictyostelium* and *Myxococcus* NDK's dimers may overlap perfectly. Two trimers may associate to generate a hexamer, whereas, two dimers may associate to generate the tetramer. The main difference between the hexameric and tetrameric NDK's is at the C-terminal part of the molecule.

NDK is a key enzyme in the control of cell energy and nucleotide metabolism; furthermore, it shares in the activation of various chemotherapeutic pro-drugs. Currently, little is known about the enzyme substrate specificity and enzyme drug recognition. Furthermore, the design of new substrates and chemotherapeutics targeting of viral enzymes requires accurate knowledge of the binding mode of such compounds interaction with the enzyme and the forces underlying this process. Structural and calorimetric studies with antiviral compounds such as acyclovir diphosphate or ribonucleosides involved in cell bioenergetics are proposed to further understand the diverse roles of NDK in crustaceans.

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