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ORIGINAL PAPER

The nucleoside diphosphate kinase from mimivirus: a peculiar affinity for deoxypyrimidine nucleotides

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Abstract The first viral Nucleoside Diphosphate Kinase was recently identified in the giant double-stranded DNA virus *Acanthamoeba polypha*ga Mimivirus (ApM). Here we report its expression and detailed biochemical characterization. NDK_{apm} exhibits unique features such as a shorter Kpn-loop, a structural motif previously reported to be part of the active site and involved in oligomer formation. Enzymatic activity measurements on the recombinant NDK_{apm} revealed its preferential affinity for deoxypyrimidine nucleotides. This property might represent an adaptation of NDK_{apm} to the production of the limiting TTP deoxynucleotide required for the replication of the large A+T rich (72%) viral genome. The NDK_{apm} might also assume a role in dUTP detoxification to compensate for the surprising absence of Mimivirus dUTPase (deoxyuridine triphosphate pyrophosphatase) an important enzyme conserved in most viruses. Although the phylogenetic analysis of NDK sequences sampled through organisms from the three domains of life is only partially informative, it favors an ancestral origin for NDK_{apm} over a recent acquisition from a eukaryotic organism by horizontal gene transfer.

Keywords Nucleoside diphosphate kinase · *Acanthamoeba polyphaga* mimivirus · Nucleocytoplasmic large DNA virus · Enzymatic activity

Introduction

Nucleoside triphosphate (NTP) and 2′-deoxynucleoside triphosphate (dNTP) are essential metabolites to all living organisms. There are both used as precursor for the synthesis of nucleic acids and as energy suppliers in a large variety of metabolic pathways, including protein, lipid and oligosaccharide synthesis. Nucleoside diphosphate kinases (NDK) catalyse the last step of NTP and dNTP synthesis (Lascu and Gonin, 2000) and as such are ubiquitous in all autonomous organisms. NDK genes encode short 150 amino acids long protein sequences highly conserved across evolutionary distant organisms. For instance, *Escherichia coli* and human NDKs share more than 40% identical residues through their entire lengths, despite having diverged from their common ancestor 3 billion years ago (Almaula et al., 1995; Lascu et al., 2000). The NDK are multimeric enzymes, most often hexamers, with few instances of bacterial tetrameric structures (Almaula et al., 1995; Lascu et al., 2000).

At the biochemical level these enzymes catalyse the phosphate transfer reaction between a nucleoside triphosphate and a nucleotide diphosphate (NDP). This reaction is Mg^{++} dependant and the phosphate donor is usually ATP due to its abundance in the cell relative to ADP and other NTPs (Roisin and Kepes, 1978). NDK have been described as non specific enzymes, able to use purine and pyrimidine nucleotides as well as ribo- and desoxyribo- nucleotide as substrates (Lascu and Gonin, 2000). NDK thus produces NTP for nucleic acid synthesis, CTP for lipid synthesis, UTP for polysaccharide synthesis and GTP for protein synthesis elongation, signal transduction or microtubules polymerization.

A renewed interest for these enzymes arose ten years ago, when they started to be suspected of assuming additional cellular roles beyond their housekeeping function, such as metabolism regulation with potential biomedical application (Munoz-Dorado et al., 1993; MacDonald et al., 1996; Hartsough and Steeg, 2000; Timmons and Shearn, 2000). For example, they have been involved in cell growth and differentiation in vertebrates (nm23 gene) through their tumor

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metastasis suppressor activity (Lacombe et al., 2000). NDK is also responsible for the last step of AZT conversion to AZT-TP in AIDS treatments. NDK are now actively studied for their potential implication in many aspects of cell regulation.

The recently discovered double-stranded DNA virus, amoeba infecting, *Acanthamoeba polyphaga* Mimivirus (La scola et al., 2003), is by far the largest known virus, with an icosahedrical particle of 600 nm in diameter (Xiao et al., 2005). Its highly complex 1.2 Mb genome (Raoult et al., 2004) exhibits 911 protein coding genes, including all genes characteristic of previously defined nucleocytoplasmic large DNA viruses (NCLDV) families such as Iridoviridae, Poxviridae, Phycodnaviridae, and Asfarviridae. However, Mimivirus exhibits a large additional complement of genes encoding enzymatic activities never yet encountered in any other viruses such as four amino-acyl tRNA synthetases and other components of the translation apparatus, a full set of DNA repair enzymes, and many other enzymatic proteins suggesting that Mimivirus life cycle requires specific metabolic activities not provided by its host (Raoult et al., 2004). Following the identification of the first viral NDK homologue in *Acanthamoeba polyphaga* mimivirus, its detailed biochemical characterization was given high priority, for various reasons. First, to demonstrate its activity at the molecular level, second to gain some insight into its role in the virus life cycle, and third to elucidate the consequences of sequence variations specific to NDK_{apm}, in particular a much shorter "Kpn-loop." This structure, highly conserved among known NDKs is involved in substrate binding and oligomeric state of the enzyme (Janin et al., 2000). The determination of the crystal structure of the NDK_{apm} molecule is pursued in parallel (Jeudy et al., 2005).

Following tyrosyl-tRNA synthetase (Abergel et al., 2005) and topoisomerase IB (Benarroch et al., 2006), NDK_{apm} is the third Mimivirus gene product for which a detailed functional characterization is presented.

Materials and methods

Expression of the *Acanthamoeba polyphaga* mimivirus NDK gene product (NDK_{apm})

The gene encoding the *Acanthamoeba polyphaga mimivirus* Nucleoside Diphosphate Kinase was amplified from mimivirus genomic DNA and directional cloning was performed using the Gateway system (Invitrogen). The PCR product was inserted by homologous recombination in the pDIGS02 expression plasmid in phase with a N-terminal His_6-tag, under the control of a T7 promoter. The pDIGS02 is an in-house vector engineered to selectively co-express the GroEL-GroES chaperon complex together with the gene of interest, following tetracyclin induction, in order to improve protein folding. After transformation into DH10B cells (Invitrogen), the purified plasmids were used for the over-expression of the recombinant proteins using a specific expression screening protocol (Abergel et al., 2003; Jeudy et al., 2005).

The best results were obtained when transforming E.coli Rosetta (DE3) pLysS at 298 K. Since the decrease in temperature was visibly a positive factor for soluble expression, we optimized our results by further decreasing the growth temperature. After initial growth at 310 K in 2YT media containing ampicillin and chloramphenicol and induction with 0.5 mM IPTG when A600 reached 0.6–0.8, the temperature was set to 290 K. Chaperon co-expression had no significant effect on the protein solubility and was not used in the final protocol. Pellet was resuspended in a 50 mM sodium phosphate, 300 mM NaCl buffer, pH 9.0 (buffer A), containing 0.1% Triton X-100 and 5% glycerol and total proteins were extracted by sonication.

Purification and biochemical characterization of the NDK_{apm}

The recombinant NDK_{apm} protein was purified as described earlier (Jeudy et al., 2005). Purification was performed using Ni^{2+} column for affinity purification. The NDK protein studied here thus corresponds to the Mimivirus protein in which the N-terminus methionine is replaced by an extended His-tag inherent to the use of the GATEWAY system (21 residue-long tag: SYYHHHHHHLESTSLYKKAGL). Interestingly, 90% of the NDK recombinant protein has been transformed in a truncated form after two weeks. N-terminal sequencing revealed a N-terminal cleavage of the 15 first tag residues. After 2 months of storage at 297 K the NDK protein was fully degraded. The NDK_{apm} oligomeric state was measured by gel filtration on an analytical S200 column. The purified NDK_{apm} was loaded on the column at a 17 mg/ml concentration in Tris buffer 100 mM pH 9.0, 300 mM NaCl. Five molecular weight standards were initially run onto the column using the same buffer to calibrate the column.

Enzymatic activity measurements

Two different coupled systems were used to assay the NDK_{apm} activity both on NTP and on NDP. The NDK activity was measured spectrophotometrically by following the absorbance at 340 nm. The commercially available *Saccharomyces cerevisiae* NDK enzyme (NDK_{sc}) was used as a reference.

1. The first coupled assay involved pyruvate kinase and lactate dehydrogenase and the NDK activity is

measured through NADH oxidation (Li et al., 1996). The reaction was performed at various (d)NDP concentrations in a 1-ml volume containing 5 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate potassium salt (SIGMA, Ref 79415), 0.2 mM NADH, 1 mM ATP, 1 active unit of pyruvate kinase/lactate dehydrogenase, 0.65 μg NDK (from mimivirus or yeast), in 100 mM Tris-HCl buffer pH 7.5. This system allows the NDK activity to be assayed for all (d)NDP except ADP as phosphate acceptor, using ATP as phosphate donor.

2. The second coupled assay involved hexokinase and glucose-6-phosphate dehydrogenase and the NDK activity is measured through NADP reduction (Ogawa et al., 2001). The reaction was performed at various (d)NDP concentrations in a 1-ml volume containing 5 mM $MgCl_2$, 10 mM KCl, 1 mM Glucose, 0.2 mM NADP, 15 μM ADP, 2,5 active units of hexokinase/glucose-6-phosphate dehydrogenase and 0.65μg of NDK (mimivirus or yeast) in 100 mM Tris-HCl buffer pH 7.5. This system allows the NDK activity to be assayed on the various (d)NTP except for ATP. This system allows the activity on ADP to be measured using GTP as phosphate donor at 1 mM concentration.

The kinetic constants for NDK_{apm} and the reference NDK_{sc} were determined by fitting the Michaelis-Menten equation on the experimental data. Activitymeasurements were performed for each substrate concentration, retaining duplicate results differing by less than 5%.

Results and discussion

Oligomeric state of NDK_{apm}

After affinity purification, the NDK_{apm} protein oligomerization state was measured on a gel filtration column (Fig.1). The elution pick corresponds to a 111.92 kDa molecular weight according to the linear regression calibration curve thus corresponding to the hexameric form. This was not totally expected given the NDK_{apm} C-terminal sequence size, intermediate in between what is observed for the known hexameric and tetrameric NDK enzymes. The uniquely shorter NDK_{apm} Kpn-loop, a structure that has been involved in the oligomerization process (Lascu et al., 2000), also could have resulted in a different monomer assembly. However, an analysis of a sample of NDK sequences through a multiple alignment combining structural and sequence information (Poirot et al., 2004; http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) highlights the conservation of a proline (P95) and a glycine (G96) residue in the Kpn-loop known to be located at the oligomer interface (Fig. 2). These residues are also conserved in the NDK_{apm} sequence and may explain the maintenance of the hexameric state.

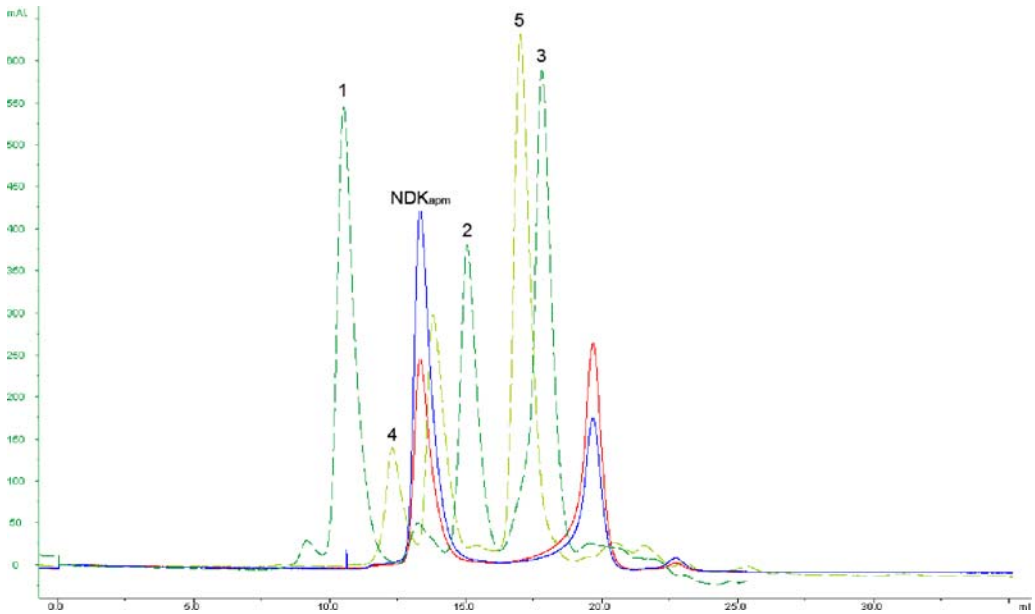

Fig. 1 Oligomerization state of NDK_{apm}. Molecular weight of the NDK_{apm} oligomer determined through size exclusion chromatography. Dotted line peaks correspond to molecular weight markers used to calibrate the Superdex S200 column (First calibration, dark green peaks 1: Ferritine 440 KDa, 2: Ovalbumine 43 KDa, 3: Ribonucléase 13.7 KDa. Second calibration, light green peaks 4: Catalase 232 KDa, 5: Chymotrypsinogène 25 KDa). The solid line peaks correspond to the NDK_{apm} elution (blue: $OD_{280\ nm}$ red: $OD_{260\ nm}$)

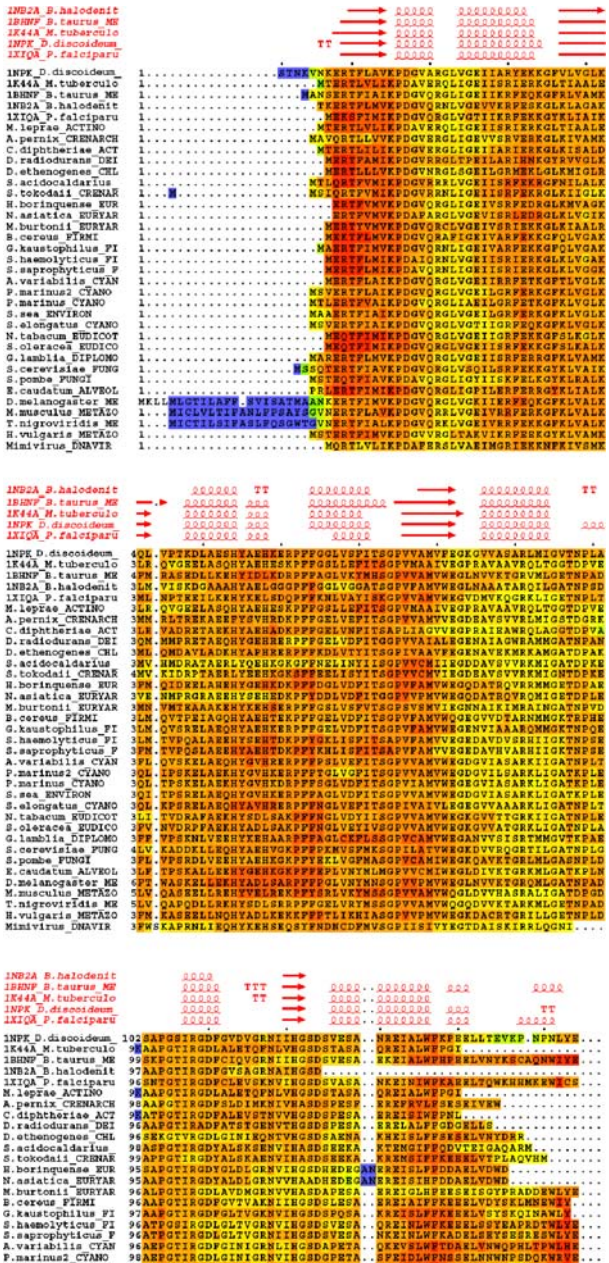

Fig. 2 Structural alignment of NDK sequences using the 3D-coffee server (reference, http://www.igs.cnrs-mrs.fr). The Core index (consistancy index) goes from red (high Core index) to blue (low Core index). Secondary structure elements are reported on top of the multiple alignment. The NDK structures used in the multiple alignment are identified through their PDB ID (1K44: *Mycobacterium tuberculosis*; 1NPK: *Dictyostelium discoideum*; 1XIQ: *Plasmodium falciparum*; 1W7W: mitocondrie de *Pisum sativum*; 1BHN: BOVINE RETINA; 1EHW: Human; 1ND2: *Bacillus Halodenitrificans*; 1S57: *Arabidopsis thaliana*; 1NDL: *Drosophila melanogaster*). While NDK sequences are identified through the name of the organism they originate from

Enzymatic activity of NDK_{apm} on purine and deoxypurine nucleotides

As the Kpn-loop has also been involved in the formation of the NDK active site (Janin et al., 2000), the shorter homologous loop exhibited by NDK_{apm} could be expected to have significant consequences on its enzymatic properties. This was explored by systematically assaying the activity and specificity of the NDK_{apm} enzyme on the various types of nucleotides. In all cases the commercially available *Saccharomyces cerevisiae* enzyme NDK_{sc} was used as a reference to help interpreting the experimental results obtained through a protocol in which the NDK activity competes for the same substrates also used by enzymes of the coupling systems (pyruvate kinase and hexokinase, see Materials and Methods).

Using purine and deoxypurine nucleotides first allowed us to establish that the unique viral NDK_{apm} exhibited the activity predicted from its sequence similarity with cellular NDKs, with kinetic constants of the same order of magnitude then the ones measured on NDK_{sc} (Table 1).

Enzymatic activity of NDK_{apm} on pyrimidine and deoxypyrimidine nucleotides

Given the known absence of specificity of cellular NDK enzymes, similar results were expected when using pyrimidine and deoxypyrimidine nucleotide substrates. To our surprise, this proven not to be the case. We first compared the NDK_{apm} activity on CDP (0.2 mM), dCDP (0.5 mM) and dTDP (0.05 mM) nucleotides using ATP (1 mM) as the phosphate donor. While we could measure both NDK activities on CDP (Fig. 3a), the behavior of the two enzymes were found to be markedly different with dCDP and dTDP. At the above concentration the NDK_{apm} activity was barely detectable (Fig. 3b, c) compared to the one of NDK_{sc}. Table 2 presents the kinetic constants of the two NDK with pyrimidine ribonucleotide substrates.

We then compared the NDK_{apm} activity on CTP (0.5 mM), dCTP (0.5 mM) and dTTP (0.1 mM) using the second

Table 1 Kinetic constants of the NDK_{apm} (red) and NDK_{sc} (black) on purine and deoxypurine nucleotides

	NDK_{mimi}		NDK_{sc}	
Substrate	Km (mM)		Vm (mM/min)	
dGDP	0.14	0.22	0.099	0.132
dGTP	0.041	0.022	0.027	0.038
ADP	0.005	0.025	0.05	0.13
ATP	0.77	0.22	0.14	0.17
GTP	0.83	0.15	0.45	0.35

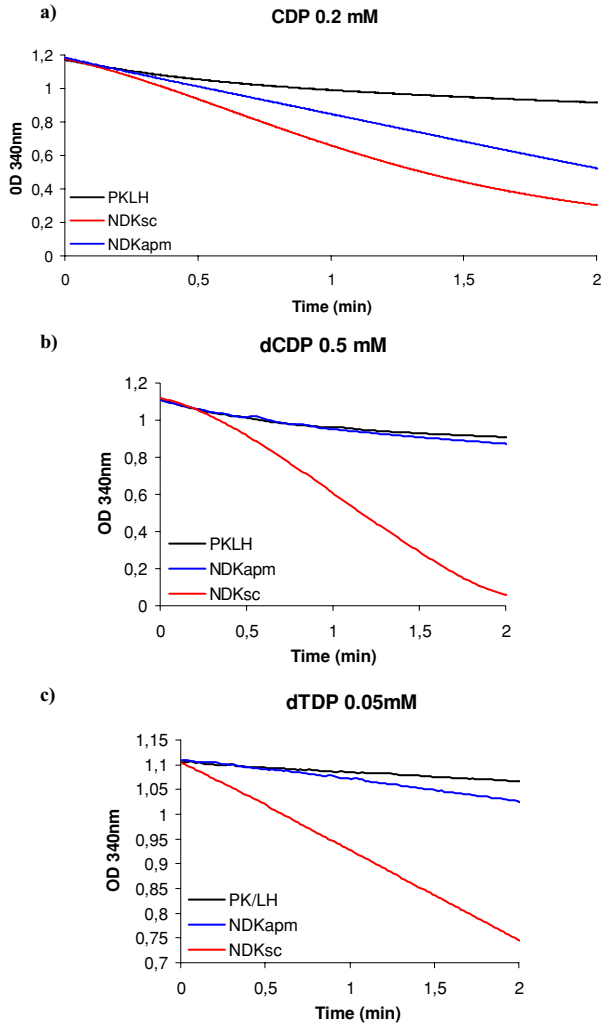

Fig. 3 NDK activities using the first coupling system on (a) CDP 0.2 mM, (b) dCDP 0.5 mM and (c) dTDP 0.05 mM substrates. The background (black curve) corresponds to the pyruvate kinase activity. NDK_{sc} activity corresponds to the red curve and NDK_{apm} to the blue curve

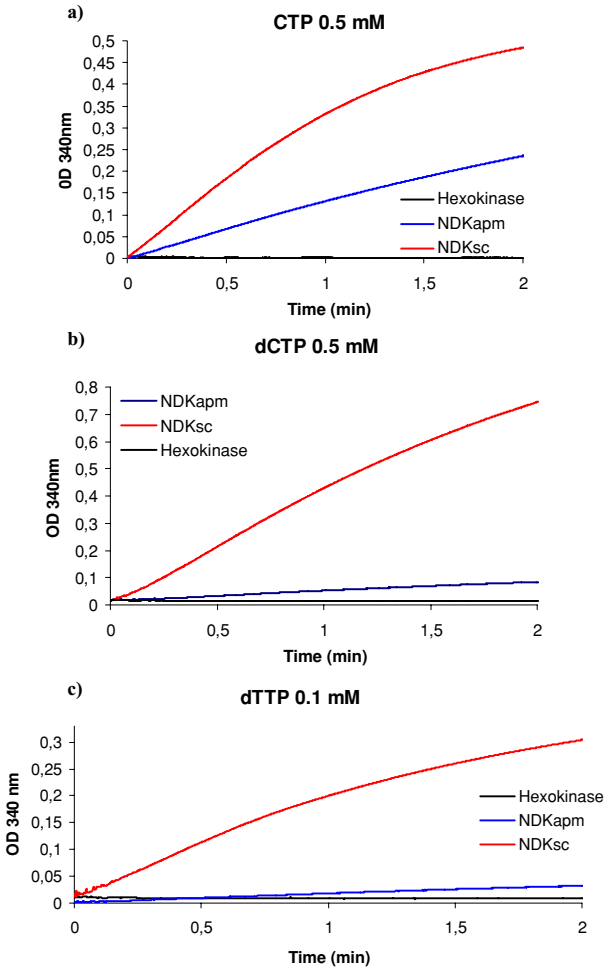

Fig. 4 NDK activities using the second coupling system on (a) CTP 0.5 mM, (b) dCTP 0.5 mM and (c) dTTP 0.1 mM substrates. The background (black curve) corresponds to the hexokinase activity. NDK_{sc} activity corresponds to the red curve and NDK_{apm} to the blue curve

coupling system (see materials and Methods). The NDK_{apm} and NDK_{sc} activities were again found comparable on CTP (Fig. 4a), but NDK_{apm} exhibited a barely detectable activity on dCTP and dTTP as if it could not metabolize these substrates (Fig. 4b,c).

Since the NDK_{sc} activity on pyrimidine and deoxypyrimidine was in the same range than the one measured on purine and deoxypurine nucleotides we hypothetized that the NDK_{apm} activity on deoxypyrimidine could be inhibited by excess of substrate. To test this hypothesis, we conducted inhibition experiments on NDK_{apm} and NDK_{sc} using 0.15 mM dGDP as substrate in absence or presence of 0.15 mM dTDP (Fig. 5a).

As expected, the addition of dTDP produced an increase in the NDK_{sc} activity while it resulted in an inhibition for NDK_{apm} (Fig. 5). The minimal inhibitory substrate concentration was then estimated by performing a series of experiment with decreasing concentrations of dTDP and dCDP. The minimal inhibitory concentration was estimated at 1 μM for dTDP and at 10 to 50 μM for dCDP (Fig. 6a, b). Table 3 summarized the kinetic constants of NDK_{sc} on deoxypyrimidine nucleotides to be compared with the ones estimated for NDK_{apm}.

Table 2 Kinetic constants of the NDK_{apm} (red) and NDK_{sc} (black) on pyrimidine nucleotides

	NDK_{mimi}		NDK_{sc}	
Substrate	Km (mM)		Vm (mM/min)	
UDP	0.1	0.69	0.11	0.4
UTP	0.11	0.14	0.041	0.075
CTP	0.04	0.1	0.025	0.083
CDP	0.05	0.3	0.07	0.22

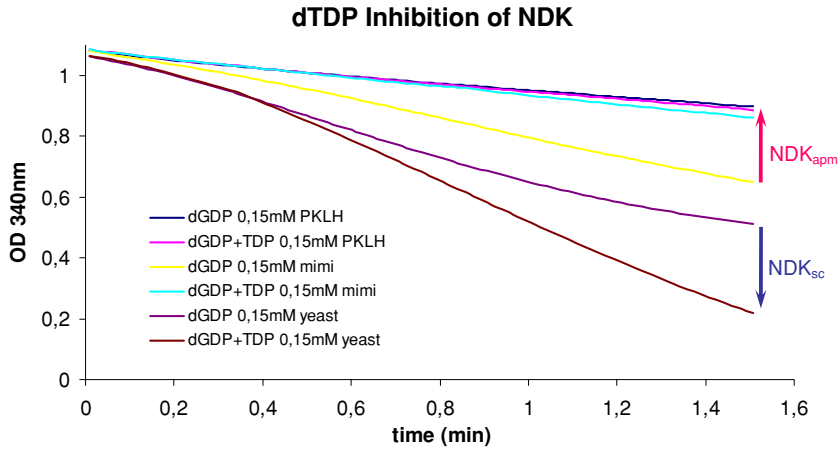

Fig. 5 Inhibition of the NDK_{apm} activity and activity of NDK_{sc}: on 150 μM dGDP substrate in the presence of 150 μM dTDP (first coupling)

The discovery of a NDK in a viral genome raised a number of questions. The first enzymatic assays (on dGDP) demonstrated that NDK_{apm} was indeed active. We thus proceeded to a thorough characterization of this enzyme to better understand its role in the replicative cycle of the mimivirus. Two main features appear to distinguish the viral NDK_{apm} enzyme from its cellular counterparts. At the sequence level, the NDK_{apm} Kpn-loop is uniquely shorter than the homologous loops exhibited by cellular NDKs. At the enzymatic level, NDK_{apm} exhibits a much higher affinity for deoxypyrimidine nucleotides. It is thus tempting to postulate that these two differences might be related. We hope that the

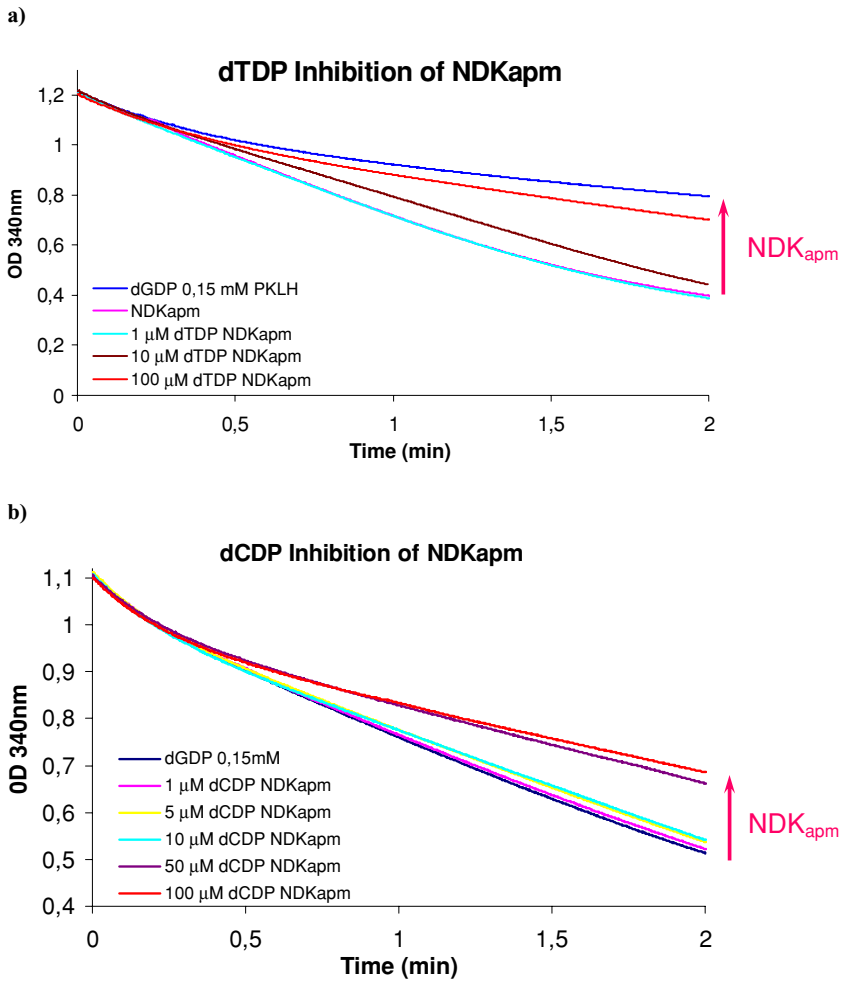

Fig. 6 Inhibition of the NDK_{apm} activity: on 150 μM dGDP substrate by increasing concentration of a) dCDP and b) dTDP (first coupling)

Table 3 Kinetic constants of NDK_{sc} (black) and estimated Km values of NDK_{apm} on deoxypyrimidine nucleotides

	NDK_{mimi}		NDK_{sc}	
Substrate	Km (mM)		Vm (mM/min)	
dCDP	<0.01	0.4	NA	0.38
dCTP	<0.02	0.17	NA	0.11
dTDP	>0.001	0.37	NA	0.33
dTTP	0.030	0.11	NA	0.105
dUDP	<0.01	>0.05	NA	NA
dUTP	0.025	0.13	NA	0.09

structure determination of the NDK_{apm} enzyme, now in progress (Jeudy et al., 2005), might help understanding the nature of this relationship. In the meantime, we also found that despite its shorter Kpn-loop, NDK_{apm} retained the hexameric form typical of most cellular NDKs. Here again the 3D structure of the NDK_{apm} protein might be precious to elucidate the impact of the Kpn-loop on the oligomerization status (Janin et al., 2000) and the oligomer stability (Lascu et al., 2000).

Finally, what might then be the reasons for the much increased affinity of the NDK_{apm} enzyme for deoxypyrimidine nucleotides ? It might first be linked to high A+T content (72%) of Mimivirus genome. At replication time, dTTP is probably the most limiting nucleotide to be incorporated in the newly synthetized viral DNA. It thus makes some sense that the NDK_{apm} might have evolved in order to take a better advantage of the small concentration of dTDP in the amoeba host cell. In support of this hypothesis, Mimivirus genome encodes additional enzymes involved in dTTP biosynthesis such as a thymidine kinase (responsible for dT/dU transformation into dTMP/dUMP) and a thimidylate synthase (producing dTMP from dUMP). Mimivirus also possesses its own deoxyribonucleotide monophosphate kinase able to transform any dNMP into dNDP and thus able to produce dTDP out of dTMP. Finally, Mimivirus also possesses a ribonucleoside reductase responsible for the conversion of ribonucleosides diphosphate into deoxyribonucleosides diphosphate (e.g. dADP, dGDP and dCDP). It thus appears that throughout its evolution, Mimivirus maintained the capacity to actively participated to its own DNA synthesis. On the other hand, Mimivirus genome is lacking a gene encoding for the dUTP pyrophosphatase (dUTPase), while this gene is an essential feature of all cellular organisms and is present in most viruses (Raoult et al., 2004). dUTPase is a key enzyme to prevent the incorporation of dUTP in DNA. As our results indicate that NDK_{apm} exhibits a very high affinity for dUDP and dUTP nucleotides (Table 3), it is tempting to postulate that this enzyme might assume a detoxifying role, by converting an eventual excess of dUTP into the harmless dUDP. Mimivirus encoded uracil-DNA glycosylase, together with its comprehensive DNA repair pathways (Raoult et al., 2004) might then remove the residual misincorporated uracyl.

A phylogenetic analysis of a large sample of various NDK sequences from the 3 domains of life was used to investigate the origin of Mimivirus NDK_{apm}. A high quality multiple alignment was obtained by combining 3D-structure and sequence information using the 3D-Coffee software (Poirot et al., 2004). NDK sequences remain highly similar (over 40% identical residues) across evolutionary distant organisms (e.g. bacteria *vs*. vertebrates). However, despite its quality, this alignment was found to contain little phylogenetic information, leading to a "star-like" consensus tree associated with many low bootstrap values (Data not shown). Such a result is typical of sequences mixing strongly conserved residues and highly variable positions that have been saturated with mutations (i.e. corresponding to residues that have been changed many times over). In this tree NDK_{apm} lies on an independent branch far from, and basically equidistant from all cellular organisms. In particular, NDK_{apm} failed to exhibit an affinity with protist NDKs (e.g. from *Entamoeba histolitica*, *Plasmodium falciparum*, *Giardia lambia* or *Dictyostelium discoideum)* that would suggest an horizontal gene transfer from an amoebal host. NDK_{apm} thus appears of ancestral origin, as most of Mimivirus genes that have been analysed so far (Raoult et al., 2004 ; Ogata et al., 2005). This is consistent with the hypothesis that large DNA viruses such as *Acanthamoeba polyphaga* Mimivirus might result from the reductive evolution of an ancestral parasitic cellular organism rather than from the random acquisition of laterally transferred host components (Raoult et al., 2004 ; Ogata et al., 2005 ; Suhre et al., 2005).

It is our hope that the determination of its 3D structure, together with the characterization of other Mimivirus enzymes involved in DNA synthesis will lead to a complete understanding of the role of NDK_{apm} in the virus life cycle.

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