The in vitro DNA binding properties of NDP kinase are related to its oligomeric state

Sébastien Mesnildrey, Fabrice Agou, Michel Véron*

Unité de Régulation Enzymatique des Activités Cellulaires, Institut Pasteur, CNRS UMR 321, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France

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Abstract Genetic and biochemical evidences suggest that the enzymatic activity of NDP kinase is necessary but not sufficient for its biological function. While the human NDPK-B binds specifically single-strand polypyrimidines sequences, the hexameric enzyme from *Dictyostelium* does not. We demonstrated by electrophoretic mobility shift assay and filter binding assay that a dimeric mutant from *Dictyostelium* binds to an oligodesoxynucleotide while the wild-type does not. These data suggest that the differences in the DNA binding properties of several eucaryotic NDP kinases might be correlated to the differences in the stability of their hexameric structure.

Key words: Nucleoside diphosphate kinase; DNA binding protein; Dimeric mutant; Filter binding assay; Development

1. Introduction

Nucleoside diphosphate kinase (NDP kinase) is an ubiquitous enzyme which catalyses the transfer of a phosphate from a nucleoside triphosphate to a nucleoside diphosphate [1]. Thus, this enzyme contributes to maintain the cellular pool of nucleoside triphosphates. The genes encoding NDP kinase have been cloned from many organisms, both eukaryotes and prokaryotes. They all encode a small 17–20 kDa polypeptide. Moreover, crystal structure of several NDP kinases has also been solved at high resolution including the enzyme from the eukaryotes *Dictyostelium discoideum* [2], *Drosophila melanogaster* [3] and human [4,5] and from the prokaryote *Myxococcus xanthus* [6]. As expected from its sequence, the topology of folding is highly conserved, built around a βαββαβ motif, also present in other DNA binding proteins [4]. However the oligomeric structure of NDP kinases varies. While eukaryotic NDP kinases are hexamers made of two trimers or three dimers, the enzymes from *Myxococcus* and *E. coli* are tetramers with two 2-fold axes [7]. Interestingly however, the association areas allowing the formation of dimers within the oligomers are totally conserved.

There are several indications that NDP kinase is involved in cellular processes which are not directly correlated with its catalytic activity. In *Drosophila*, a null mutation in the NDP kinase gene (awd) leads to the abnormal wing disc phenotype [8]. In human, three genes that encode cytoplasmic NDP kinases, *nm23-H1*, *nm23-H2* and *DR-nm23* [9–11] have been described. One of these, *nm23-H1*, was isolated as a potential metastasis suppressor gene. NDPK-B, encoded by *nm23-H2*, was shown to bind to the NHE element in the promoter of the *c-myc* proto-oncogene and to activate its transcription in vitro [12,13]. A direct interaction of NDPK-B with chromatin was reported, indicating its nuclear localization [14]. This DNA binding activity is maintained with a catalytically inactive mutant, indicating that the two functions are distinct [26]. We also demonstrated that NDPK-B preferentially binds to single-strand polypyrimidine rich DNA sequence including a (CT)n dinucleotide repeat, and to polypyrimidine rich RNAs [15]. Whereas the human NDPK-B is able to bind to the DNA, the *Dictyostelium* enzyme does not [15].

We have recently reported the characterization of two mutant NDP kinases from *Dictyostelium*, P100G and P100S-N150stop which have altered interactions in the association areas leading to the formation of trimers. While the P100G mutant protein was very unstable as compared to the native enzyme, the P100S-N150stop mutant protein was shown to be synthesized as a catalytically inactive dimer in recombinant bacteria [16].

Here we report on the DNA binding activity of these mutant NDP kinases. We show that the dimeric mutant protein binds to an oligodesoxynucleotide while the hexameric wild-type *Dictyostelium* enzyme does not. The significance of this preferential binding to dimeric form is discussed in relation to possible functions in different cellular processes.

2. Material and methods

2.1. Purification of Dictyostelium recombinant proteins

Wild-type NDP kinase was purified as described [17]. Briefly, 500 ml cultures of *E. coli* XL1-Blue were grown at 37°C overnight in LB medium containing 200 μg/ml Ampicillin and 50 μg/ml tetracyclin. The cells were harvested and resuspended in buffer A (50 mM Tris-HCl pH 8.4 containing 5 mM $MgCl_2$) and broken in a French Press at 4000 psi. After centrifugation (50 000×*g*) for 30 min at 4°C, the supernatant was applied to a Q-sepharose column equilibrated in buffer A (Pharmacia, 5×8 cm). The NDP kinase, recovered in the flow-through fraction, was approximately 70% pure as judged by SDS-PAGE. It was completely separated from *E. coli* NDP kinase. The pH was adjusted to 7.4 and NDP kinase was further purified by affinity chromatography on Blue Sepharose (Pharmacia), eluted at 250 mM by a 0–2 M NaCl gradient. Fractions containing NDP kinase activity were pooled and concentrated with a stirred ultrafiltration cell (Amicon). The excess salt was removed after multiple step of dilution and concentration of protein sample using an ultrafiltration cell. NDP kinase activity was assayed by a coupled assay using 0.2 mM dTDP as the phosphate acceptor in the presence of 1 mM ATP as described [16].

The mutant recombinant NDP kinases P100G and P100S-N150stop from *D. discoideum* were generated as described previously [16]. For the P100S-N150stop NDP kinase, the first step of purification was identical to that of the wild-type protein except that the Blue sepharose column was replaced by an Orange column (Amicon) which had a better affinity for the mutant protein. The protein was eluted at 500 mM by a 0–1 M NaCl gradient and the fractions were concentrated as above. The proteins were pure as determined by SDS-PAGE [18]. The

*Corresponding author. Fax: +33 1-45-68-83-99.
E-mail: mveron@pasteur.fr

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protein concentrations were determined using absorption coefficients of 0.55 A_2 units$\times mg^{-1} \times cm^2$.

2.2. Probe radiolabelling and EMSA experiments

Three different synthetic probes were purchased from Genset, France and were used for EMSA. A 9 CT repeat: $(CT)_9$, a 17 CT repeat: $(CT)_{17}$, and a 27 CT repeat: $(CT)_{27}$ have been previously shown to bind to the human B isoform of NDP kinase. 200 ng of each probe have been radiolabelled with [γ-^{32}P]ATP and T_4PN kinase. After a 10 min inactivation at 65°C and a phenol/chloroform extraction, the DNA was precipitated in ethanol and resuspended in a final concentration of about 20 nM. The oligonucleotide concentrations were determined by absorbance at 260 nm taking values of absorption coefficients given by Genset.

For EMSA experiments, we used a constant concentration of labelled oligonucleotide (1 nM). About 0.1 ng of oligonucleotide was incubated in 20 μl in the presence of various NDP kinase samples. Each sample was incubated 20 min on ice in a buffer 12.5 mM HEPES pH 7.9, containing 10 mM $MgCl_2$, 50 mM KCl, 0.05% NP40, 0.5 mM DTT and 12.5% glycerol and in final volume of 20 μl. We used various amounts of enzyme, from 100 ng to 10 μg, corresponding to a final monomeric concentration of 300 nM to 30 μM. We used 100 ng of single-strand dIdC (SsdIdC) as unspecific competitor for each assay. SsdIdC has been previously prepared beforehand by boiling for 10 min and rapidly freezing in dry-ice. Samples were separated on 4% polyacrylamide native gel under low ionic strength conditions in the presence of 0.25×TBE buffer. After a prerun at 200 V of 15 min, the separation was performed for ≈30 min at 170 V.

2.3. Filter binding assays

For filter binding experiments, the oligonucleotides labelled were purified by using denaturing polyacrylamide gel electrophoresis according to standard procedures [19]. Nitrocellulose filter binding reactions (30 μl) were set on ice in a binding buffer 20 mM Mes pH 6.0 containing 5 mM $MgCl_2$, 10 mM KCl and 20% glycerol. After binding was complete (1 h), 5 μl of each reaction mix was counted to provide an accurate measure of the total amounts of DNA in the reaction. The remaining 25 μl was then filtered under reduced pressure in a cold room through prewashed nitrocellulose filters (0.45 μM pore size, Millipore HA filters). Filters were washed once with twice 500 μl of binding buffer containing 150 mM KCl or not. The filters were then dried and counted by liquid scintillation.

To determine the dissociation constant, we used a conventional model in which the oligonucleotide has a single class of identical, independent binding sites [20]. The data were fit to the model with the Levenberg-Marquardt algorithm in the commercially available graphics software package KaleidaGraph (Abelbeck Software).

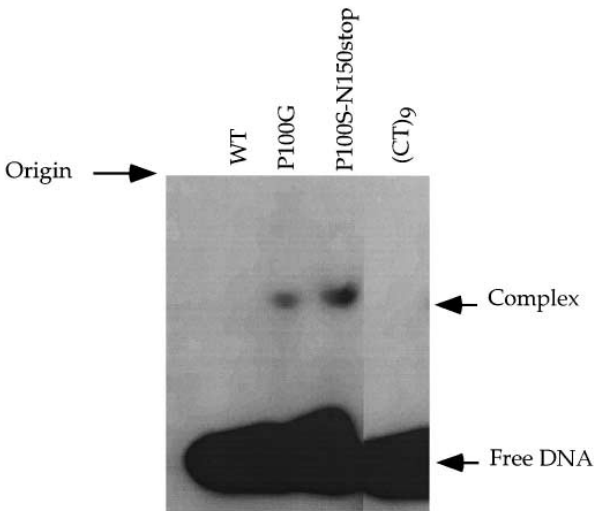

Fig. 1. Gel mobility shift assay of different forms of NDP kinase with oligonucleotide [^{32}P](CT)$_9$. Radiolabelled oligonucleotide [^{32}P](CT)$_9$ (1 nM) was incubated at 4°C with the mutants P100G, P100S-N150stop or the native NDP kinase (1 μM). Electrophoretic mobility was conducted as described in Section 2. The electrophoretic mobility of the [^{32}P](CT)$_9$ free or bound are indicated.

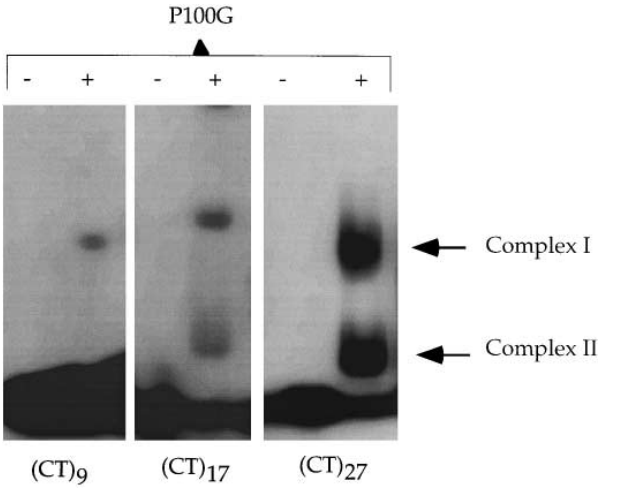

Fig. 2. Effect of the oligonucleotide length on DNA binding properties of the mutant P100G NDP kinase. Radiolabelled oligonucleotides [^{32}P](CT)$_9$, [^{32}P](CT)$_{17}$ or [^{32}P](CT)$_{27}$ (1 nM) were incubated at 4°C with the mutant P100G NDP kinase (1 μM). Two types of DNA:protein complexes are observed, most likely corresponding to stoichiometries of 1:1, 1:2.

3. Results

Wild-type and mutant recombinant NDP kinases showed a similar level of expression in *E. coli*. However, P100G and P100S-N150stop mutant NDP kinases were unexpectedly contaminated by nucleic acids in spite of their passage on DEAE Sepharose which usually retains most of the cellular polyanions. In contrast, the wild-type enzyme does not show any association with nucleic acids upon purification. This constituted a first indication that the mutant proteins bind preferentially to nucleic acids. In order to separate the nucleic acids from the enzymatic preparations of mutants, we used another affinity matrix (Orange, Amicon) which required a higher salt concentration for the elution of protein. Under such conditions, the nucleic acids were totally separated from the enzymatic fraction as shown by SDS-PAGE analysis as well as by the shape of the absorption spectra.

3.1. Detection of the mutant NDP kinases·(CT)$_9$ complexes by mobility shift assay using native gel electrophoresis

To examine the DNA/NDP kinase interactions, we used a single-strand oligodesoxynucleotide including nine CT repeats, $(CT)_9$. The binding of the mutant and of the wild-type NDP kinases were detected by a DNA shift assay. In this assay, 5′ labelled $(CT)_9$ (≈1 nM) was incubated at a constant protein concentration in the presence of a 100-fold molar excess of single-strand dIdC to prevent non-specific interactions. As shown in Fig. 1, a distinct band shift is formed only with the P100G and P100S-N150stop mutant NDP kinases, indicating that the mutant proteins bind to $(CT)_9$ whereas wild-type does not.

3.2. Effects of polypyrimidine strand length on the formation of DNA·NDP kinase complexes

It was possible that the low affinity of the mutants for $(CT)_9$ could be due to the short length of the oligonucleotide.

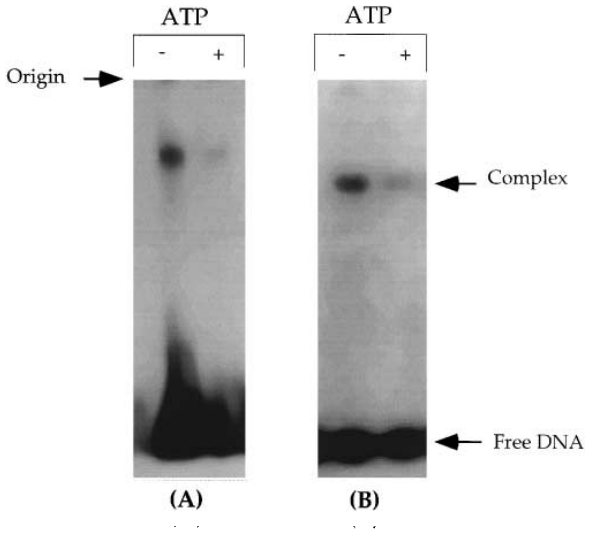

Fig. 3. Inhibition by ATP of the binding of $[^{32}P](CT)_9$ to the mutant P100G and P100S NDP kinases. $[^{32}P](CT)_9$ was incubated with the mutant (1 μM) P100G (A) or P100S-N150stop NDP kinase (B) in the absence (−) or in the presence (+) of ATP (2 mM).

To evaluate the approximate number of nucleotides involved in the interaction with the mutant proteins, we tested the ability of two other oligonucleotides, varying only in length, to bind to the P100G mutant.

Fig. 2 shows the effect of the length of the oligonucleotide on the formation of DNA/NDP kinase complex. In the presence of $(CT)_9$, only one retarded band was obtained whereas two bands were observed with 34-mer $(CT)_{17}$ or 54-mer $(CT)_{27}$. Similar results were obtained with the mutant P100S-N150stop (data not shown). These results indicate the presence of two DNA-protein complexes most probably with the stoichiometry 1:2 (complex I) or 1:1 (complex II) (see Fig. 2). Similar results were observed in EMSA experiments using human NDP kinase B [15]. For these reasons, further studies to analyse the DNA binding properties of mutant NDP kinases were performed with the $(CT)_9$ oligonucleotide.

3.3. Competition mobility assays with ATP

We examined the competition effect of $(CT)_9$ binding with NDP kinase substrates. For this, we performed a mobility shift assay with the two mutants in the presence or absence of ATP (Fig. 3). As shown in Fig. 3, the addition of ATP (2 mM) prevents the formation of oligo/protein complex. Similar results were also observed in the presence of AMP or ADP (data not shown). Molar bases equivalent of single-strand dIdC does not inhibit the $(CT)_9$/mutant complex formation (the retarded band remained in the presence of a 1000-fold excess of dIdC). Considering that NDP kinase binds preferentially to pyrimidine bases, we conclude that the mononucleotide ATP, ADP or AMP acts as competitor according to a mechanism not only based on the straightforward electrostatic competition. In that way, this inhibition could reflect an indirect effect of ATP.

3.4. Interaction studies by filter binding assay

Nitrocellulose filter binding has been used extensively to measure equilibrium binding constants for sequence specific protein interactions [21,22]. Fig. 4 shows an experiment in which we have examined the capacity of the oligonucleotides $(CT)_9$ and $(CT)_{27}$ to bind to the dimeric mutant P100S-N150stop by filter binding assay. The results again indicate the formation of a DNA/protein complex for the dimeric mutant whereas no complex is formed with the hexameric wild-type protein. Using a variable protein concentration, ranging from 10 nM to 70 μM, and a constant $(CT)_9$ concentration of 42 nM, we have measured a dissociation constant equivalent to 2.5 ± 0.5 μM. When a similar experiment was performed

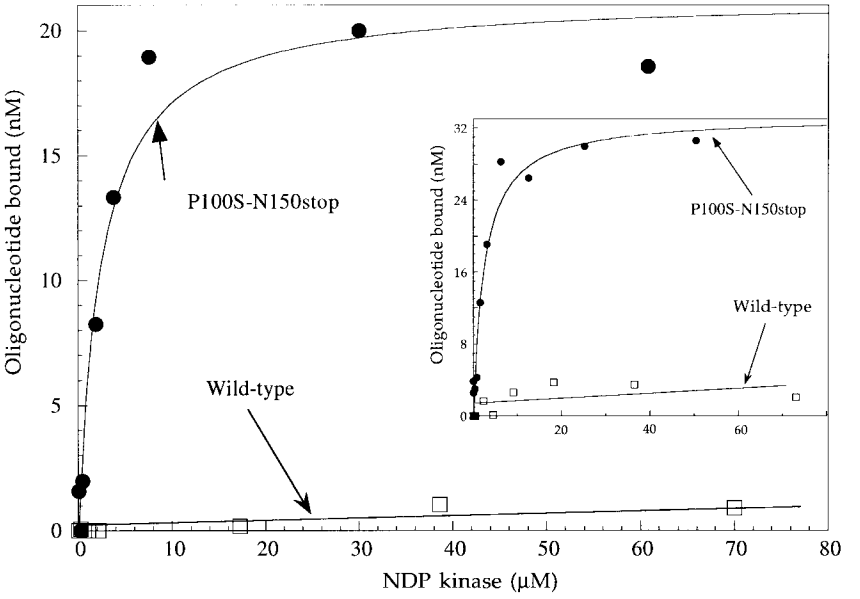

Fig. 4. Determinations of interaction constants of $(CT)_n$ for the P100S-N150stop and wild-type NDP kinases by nitrocellulose filtration assay. Equilibrium isotherms were determined by nitrocellulose filter binding assay at constant $[^{32}P](CT)_9$ concentration (42 nM) and increasing wild-type (□) or P100S-N150stop (●) NDP kinase concentrations under conditions described in Section 2. Solid curves represent simulated theoretical binding isotherm. Inset: binding isotherm with the 54-mer $[^{32}P](CT)_{27}$ (52 nM) determined by increasing wild-type (□) or P100S-N150stop (●) NDP kinase concentrations.

with $(CT)_{27}$ (Fig. 4, inset) an identical dissociation constant was obtained, indicating that the 18-mer is optimal to interact with the dimeric protein. This dissociation constant is consistent with the amount of DNA that was band-shifted in the EMSA (see Fig. 1). Considering that the retention efficiency in the titration is 55%, the stoichiometry of the complex $(CT)_9$/mutant monomer given by the intersection point of the two linear portions of the curve, is approximately 0.5 ± 0.3 units. A similar value was measured with the $(CT)_{27}$ oligonucleotide indicating that a single protein/complex was isolated by these methods. It is well known that the strength of the filter binding assay is its sensitivity to the first protein molecule bound; its weakness is its relative insensitivity to the binding of the second. So, it is not so surprising to detect only one DNA/protein complex by filter binding assay.

The binding of $(CT)_9$ to the mutant protein was dependent upon salt concentration or pH. Thus, using 150 mM KCl in the washing buffer, a 10-fold increase of K_D for $(CT)_9$ was observed (data not shown). The efficiency of the assay drops rapidly with increasing pH and temperature. At pH 8.0, 20°C and in the absence of glycerol in the binding buffer, only 10% of complex is retained on the filter with a K_D of about 90 μM indicating the formation of a very labile complex (data not shown).

4. Discussion

We have demonstrated by mobility shift assay and nitrocellulose filter binding assay that the oligomeric state of the NDP kinase has crucial effects on DNA binding.

The relatively low affinity of the synthetic oligodesoxynucleotides $(CT)_9$ and $(CT)_{27}$ for the dimeric mutant may result from several causes. (i) The CT repeats are likely not to be the most appropriate base sequences. (ii) The nucleic acid binding properties of NDP kinase are not consistent with those of a conventional transcriptional factor binding strongly DNA. In fact, the properties of NDP kinases are somewhat similar to those of the aminoacyl-tRNA synthetases [23]. These ubiquitous enzymes have to recognize specifically one particular tRNA among about 60 species present in the cell, and then to form specific, but nevertheless very labile, RNA/protein complexes to allow the aminoacylation reaction to occur. This suggests that another putative cellular function of NDP kinase in relation with its DNA binding properties might be considered to be an enzyme acting on DNA or RNA molecules, rather than a typical transcriptional factor. In addition, the dissociation constant of the polynucleotide for the dimeric enzyme is at least two orders of magnitude above the Michaelis constant of several mononucleotide substrates measured for the wild-type enzyme [24]. Compared to the natural substrates for the NDP kinase, the affinity of the oligodesoxynucleotide is not so low. It can be noted that the conditions (pH 8.0) used for optimal NDPK activity in the formation of the nucleoside triphosphate seem to be the worst for the interactions with an oligonucleotide.

The fact that a dimeric NDP kinase can bind to a polypyrimidine rich sequence and displays no catalytic activity contrary to the hexameric form [25] leads us to propose a plausible regulation mechanism implicating the NDP kinase in different cellular processes. As shown in Fig. 3, we observed an inhibition effect of ATP in the formation of the complex oligo/protein. Since the hexameric form does not bind to DNA, and dimeric form binds, such an effect may result from a change of the oligomeric state from the dimeric to hexameric form upon addition of ATP.

In light of these results, a possible explanation concerning the DNA binding properties of the NDP kinase from several organisms can be proposed. As we previously reported [15], neither NDP kinase from *Drosophila* nor NDP kinase from *Dictyostelium* show DNA binding properties in spite of their very basic isoelectric point which is very similar to human NDPK-B. In fact, the hexameric state of these NDP kinases might be more stable compared to the human isoform B, preventing, in that way, the formation of the competent form for DNA binding. The fact that a saturated retardation signal was never observed with NDPK-B even if excess of recombinant enzyme was applied [12,15], also supports this hypothesis.

The functional duality of the *nm23* gene product which is involved in the control of tumor metastasis, might be correlated to two opposite effects: in some cases metastatic potential has been correlated with reduced *nm23* expression, and in other tumor types, overexpression of *nm23* correlates with metastatic spread. Genetic and biochemical approaches are in progress in our laboratory in order to shed further light into the hypothetic cellular function of the NDP kinase related to its DNA or RNA binding properties.

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