Communication

Cellular Phosphorylation of Anti-HIV Nucleosides

ROLE OF NUCLEOSIDE DIPHOSPHATE KINASE*

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Nucleotide analogs are widely used in antiviral therapy and particularly against AIDS. Delivered to the cell as nucleosides, they are phosphorylated into their corresponding nucleotides, especially against adenine nucleotides (15, 16), for the host. The last step in this series of phosphorylations is catalyzed by nucleoside diphosphate (NDP) kinase, an enzyme that can catalyze the non-adenine nucleoside diphosphates into triphosphates.

Using pure recombinant human NDP kinase type B (product of the gene nm23-H2), we have characterized the kinetic parameters of several nucleotide analogs for this enzyme. Contrary to what is generally assumed, diphospho- and triphospho- derivatives of azidothymidine as well as of dideoxyadenosine and dideoxythymidine are very poor substrates for NDP kinase. The rate of phosphorylation of these analogs varies between 0.05% and 0.5%, as compared to the corresponding natural nucleotides. The reactions leading to mono- and diphosphates of the nucleosides are catalyzed by base-specific enzymes, i.e. the phosphorylation of purines and pyrimidines in the cell is catalyzed by distinct nucleoside kinases and nucleoside monophosphate kinase. In contrast, the step leading from the nucleoside diphosphate to the triphosphate is catalyzed by a single enzyme, nucleoside diphosphate (NDP) kinase, independent of the nature of the base and of the sugar (EC 2.7.4.6) (3).

The main function of NDP kinase in the cell is to phosphorylate the non-adenine nucleoside diphosphates into triphosphates. The reaction has a ping-pong mechanism, with a phospho-histidine intermediate according to the following reactions (Reactions 1 and 2):

\[
\begin{align*}
N_1TP + E & \rightleftharpoons N_1DP + E \sim P \\
E \sim P + N_2DP & \rightleftharpoons E + N_2TP
\end{align*}
\]

ATP is believed to be the main phosphate donor in the cell. Renewed interest in this enzyme resulted recently from its cloning from several species including the prokaryote Myxococcus xanthus (4), the primitive eukaryote Dictyostelium discoideum (5), and higher eukaryotes including mammals. Two highly homologous NDP kinases, NDPK-A and NDPK-B, have been isolated in human erythrocytes and sequenced (6), and these proteins were identified to be essential to the products of the genes nm23-H1 and nm23-H2, respectively (6, 7). nm23-H1 has been shown to be involved in tumor metastasis (8, 9). All NDP kinases are made of identical 17-kDa subunits. Eukaryotic NDP kinases are hexamers, whereas some bacterial enzymes are tetramers. The high resolution structure of the NDP kinases from Dictyostelium (10, 11), M. xanthus (12), Drosophila (13), and human (14, 15) show that the subunit fold and active site of NDP kinases are highly conserved throughout evolution. This fold is original for a phosphotransferase, showing no similarities with the usual nucleotide binding fold of nucleotide-binding proteins. High resolution data are also available for Dictyostelium and Myxococcus NDP kinase complexed with ADP, a purine nucleotide (15, 16), for Dictyostelium complexed with TDP, a pyrimidine deoxynucleotide (17), and for human NDPK-B complexed with GDP (14). These data, along with the study of several mutant proteins modified in active site residues by in vitro mutagenesis (18), provide a comprehensive description of the active site.

Nucleoside analogs are thought to be phosphorylated by the same enzymes as the natural nucleotides. For example, thymidine kinase and thymidylate kinase catalyze the first and second steps in the phosphorylation of AZT. However, AZT-TP is a poor substrate for thymidylate kinase and accumulates in the cell (19), which may be responsible for a major part of its activity. AZT-DP, 3-azidothymidine 5'-diphosphate; AZT-TP, 3-azidothymidine 5'-triphosphate.
cytotoxic effects (20). In contrast to the numerous studies performed on AZT phosphorylation to AZT-MP and AZT-DP by thymidine kinase and thymidylate kinase, no study is available on the last step in the phosphorylation cascade, i.e., the phosphorylation of AZT-DP in AZT-TP. This may be due to the lack of specificity of NDP kinase toward the nucleobase of natural nucleotides, which has led to the general assumption that this enzyme would also easily phosphorylate diphosphates of nucleotides, which has led to the general assumption that this enzyme would also easily phosphorylate diphosphates of nucleoside analogs and in particular AZT-DP and dADP. However, the cellular concentration of AZT-TP is even lower than that of AZT-DP, unlike ATP which is much more abundant than ADP (19). This suggested to us that AZT-DP may be a poor substrate for NDP kinase and that the reaction catalyzed may be a second limiting step in the phosphorylation pathway.

In this paper we have investigated the ability of antiviral diphospho- and triphosphonucleotides to be used as substrates by human NDP kinase. The results are discussed in the context of the crystal structure of NDP kinase and in particular of the role played by the 3'-OH of the ribose moiety in substrate binding and in catalysis.

**MATERIALS AND METHODS**

Purification of Recombinant Human NDPK-B—Human NDP kinase-B was expressed in Escherichia coli as described (21) and purified according to (14) with the following modifications. Cells were resuspended in 50 ml of Tris-Cl buffer (pH 8.4) containing 5 mM MgCl₂, 1 mM dithiothreitol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine (Buffer A). They were lysed in a French press, the lysate was spun at 20,000 rpm for 30 min and the supernatant was loaded on a DEAE-Sephal column equilibrated with buffer A. Under these conditions, the endogenous E. coli NDP kinase bound to the resin and was separated from recombinant NDPK-B which was recovered in the flow-through fractions. The latter were loaded on a Blue-Sepharose column (5 ml) equilibrated in buffer A at pH 7.4. The column was washed with 2 x 1 M NaCl and NDPK-B was eluted with a linear gradient of NaCl (2 M to 5 M). The high salt concentration, which was necessary for elution from the column, was immediately lowered by dialysis of the fractions against 50 ml Tris-Cl buffer (pH 7.4) containing 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM bovine serum albumin. NDPK-B was purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. It was stored at -20°C in the same buffer containing 20% glycerol.

Nucleotides—The concentration of the commercially available nucleotides (Pharmacia Biotech Inc.) was determined by absorption spectrophotometry. [γ-32P]GTP (5000 Ci/mmol) was from Amersham Corp. and [14C]ADP (58 mCi/mmol) was from DuPont NEN. Phosphoribosyltransferase was from Sigma.

To synthesize phosphoderivatives of AZT, the free 5'-OH of AZT was phosphorylated by condensation with β-cyanoethyl dihydrogen phosphate (22) in the presence of DCC in anhydrous pyridine to give the phosphoester, followed by treatment with 0.4 LIOH for 1 h. AZT-DP and AZT-TP were obtained one-pot from AZT-MP via the phosphoribosyltransferase which is prepared from the prokaryote and 1-hydridolimidazole (23). The di- and triphosphate were isolated by chromatography on a DEAE-Sephadex A-25 column (HCl form) eluted with a linear gradient of triethylammonium hydrogen carbonate buffer (pH 7–8; 0.05–0.5 M).

ddADP was enzymatically synthesized from ddATP in presence of 3-fold excess fructose 6-phosphate and phosphofructokinase in 50 mM Tris-HCl buffer (pH 7.4) 5 mM MgCl₂ for 3 h at 20°C. It was purified by reversed-phase chromatography on a C18-column eluted with acetonitrile-water (0–25%).

AZT-DP, AZT-TP, and ddADP were repurified by reversed-phase high performance liquid chromatography (Nucleosil 100, 5 μm, 250 mm × 10 mm; A = 0.1 M TEAA, B = MeCN from 0–20% in 20 min, flow rate 5 ml/min), and their purity was checked by HPLC, 1H, and 31P NMR and by mass spectrometry (fast atom bombardment).

**Kinetic Measurements—**

When the ability of NDP kinase to use the analog as a phosphate donor was studied, we measured the formation of [14C]ATP from [14C]ADP (0.1 mM), at various concentrations of nucleoside triphosphates. When the analog was tested as a phosphate acceptor, 1.0 mM [γ-32P]GTP was used as a phosphate donor and the amount of [γ-32P]NTP formed was measured. [γ-32P]GTP was not used in this study because of high background. It should be noted that the GDP formed during the reaction competes with the analog phosphosphate studied, leading to nonlinear kinetics. In order to avoid this difficulty, repurification of GDP was achieved by adding pyruvate kinase (0.05 mg/ml at 600 units/mg) and phosphoenolpyruvate (1 mM) along with 50 mM KCl in the assay mix. We checked that the analog nucleo-side diphosphates were not substrates for pyruvate kinase.

The assays were started by adding 3 μl of enzyme to a reaction mixture (10 μl) containing 50 mM Tris-Cl (pH 7.4), 5 mM MgCl₂, and the substrates at 37°C. The amount of NDP kinase added per assay varied from 10 pg for natural nucleotides to 5 ng with poor analogs. When the nucleotide triphosphates were assayed, the reaction was stopped by adding 3-μl aliquots to a 2-ml stop solution consisting of 0.7 M formic acid and 10 mM each of ADP and ATP. When assaying nucleotides diphosphates with [γ-32P]GTP, the reaction was stopped by placing a 3-μl aliquot of the reaction mixture at 85°C for 2 min. After cooling on ice, 2 μl of a 10 mM solution of cold nucleotide was added. The nucleotides were separated on TLC plates with UV indicator (Macherey-Nagel, Germany) which were developed with 400 ml NH₄HCO₃ (1 M) formic acid and 1.5 mM LiCl when [γ-32P]GTP or [14C]ATP were used, respectively. The products formed were quantified with the WIN-Q program (Molecular Dynamics) using a PhosphorImager. Linear readings of the radioactivity were obtained in a range covering 5 orders of magnitude in nucleotide concentration. Kinetic parameters were calculated by nonlinear fitting using Kaleidograph software.

**TABLE I**

| Acceptor pyrimidine | dTDP | AZT-DP |
|---------------------|-----|-------|
| kcat (s⁻¹)          | 800 ± 100 | 1.3 ± 0.1 |
| Kₐₚ (mM)            | (100%) | (0.17%) |
| kₐₚ/Kₐₚ (m⁻¹ · s⁻¹) | 0.06 ± 0.02 | 6.0 ± 0.6 |
| kₐₚ/Kₐₚ (m⁻¹ · s⁻¹) | 10¹⁻ | 2 × 10² |

![Image](http://www.jbc.org/Downloadedfrom)
RESULTS AND DISCUSSION

We have investigated the ability of NDP kinase to use the diphosphate and triphosphate forms of AZT, ddA and ddT, as phosphate acceptor and phosphate donor, respectively. Since the two human isozymes of NDP kinase, NDPK-A and NDPK-B, do not differ in their enzymatic properties (6), we have used only the isozyme NDPK-B encoded by the gene Nm23-H2 (9), to perform the experiments reported in this paper. Preliminary experiments using NDPK-A gave similar results (data not shown).

Fig. 1 shows a typical kinetic experiment. The rate of product accumulation was constant for at least 6 min, allowing determination of initial velocities (Fig. 1, inset). It should be noted that the \( K_m \) and \( V_{max} \) values derived from these experiments are apparent kinetic parameters measured by varying the concentration of one substrate only. Due to competition between the nucleoside di- and triphosphates, inhibition by excess of substrate makes a more complete study difficult. However, for an enzyme with a ping-pong mechanism, the ratio of the apparent \( k_{cat} / K_m \) is equal to the true value of \( k_{cat} / K_m \); therefore, it is a useful parameter when comparing the natural substrates to the analogs.

### Table II

| Donor purine | dTTP | ATP | 2’-dATP | 3’-dATP | 2’,3’-d-dATP |
|--------------|------|-----|---------|---------|-------------|
| \( k_{cat} (s^{-1}) \) | 1300 ± 100 | 700 ± 0.05 | 150 ± 0.05 | 30 ± 0.05 | 0.02 ± 0.001 |
| \( K_m (mM) \) | 1.2 ± 0.2 | 2.0 ± 0.5 | 5 ± 2 | 10^1 | 10^3 |
| \( k_{cat} / K_m (s^{-1} \cdot M^{-1}) \) | 10^3 | 3.5 × 10^3 | 3 × 10^4 | 3 × 10^6 |

As shown in Tables I and II, AZT nucleotides are very poor substrates for the NDP kinase reaction. When used in the diphosphate form as an acceptor, the apparent \( k_{cat} \) is 0.17% of that of TDP (Table I), while it is 0.05% of that of TTP when used in the triphosphate form as the phosphate donor (Table II). The ratio \( k_{cat} / K_m \) is high with natural nucleotides, actually close to the value predicted for diffusion-controlled reactions. It drops by several orders of magnitude for all analogs with a modified 3’-OH position on the ribose moiety. This is true, for instance, for analogs in which the 3’-OH is missing, such as 3’-dATP (which yields only 0.4% of the activity with ATP) or the 6-deoxy analogs (Tables I and II). Very low \( k_{cat} \) are measured when ddTTP or ddATP is used as donor (0.01% and 0.04% of TTP and ATP, respectively), or when ddADP is used as the acceptor (0.4% of ADP). These results point to the importance of the 3’-OH group as opposed to the 2’-OH. It is interesting to note that similar results were obtained with 3’-dATP and AZT-TP, suggesting that steric hindrance by the bulky azido group in AZT nucleotides is not the reason for their poor performance as substrates of NDP kinase. In contrast, preliminary measurements showed that arabinofuranose (where the sugar moiety is the epimer of ribose in the 2’ position) is a good substrate for NDP kinase (data not shown).

We also performed experiments where the analogs were tested as competitors in the reaction of phosphorylation of [\(^{14}\text{C}\)]ADP by TTP. ATP, ADP, and TTP were both inhibitors (data not shown), with \( I_{50} \) values approximately equal to their respective \( K_m \) (see Tables I and II), indicating that a lack of binding to the enzyme is not the reason of the poor activity described above. Under the conditions used, no transfer of \( \gamma \)-phosphate from either analog to ADP could be detected.

The X-ray structures of several NDP kinases in complex with nucleotides explain the lack of specificity of the enzyme for the nucleoside-base. Unlike most nucleotide-binding proteins, NDP kinase does not form specific hydrogen bonds with the base (Fig. 2). In contrast, there is extensive bonding to the 3’-OH of the sugar, which accepts hydrogen bonds from the Lys-16 and Asn-119 side chains (numbers correspond to the Dictyostelium NDP kinase sequence). The role of these amino acids has been confirmed by site-directed mutagenesis (18). Moreover, the 3’-OH donates a hydrogen bond to one of the \( \beta \)-phosphate oxygens (16, 17). This internal bond maintains the nucleotide in a folded conformation, which is probably needed to position...
the γ-phosphate correctly for in-line attack by the N4 nitrogen of the catalytic histidine. Its presence also suggests that the 3'-OH plays a role in catalysis by donating its proton to the leaving group and helping release of the nucleoside diphosphate product. Our data on the study of nucleoside analogs support this suggestion.

**CONCLUSION**

We have shown that the di- and triphosphate forms of AZT, ddA, and ddT, are poor substrates for NDP kinase and that the absence of a 3'-OH on the sugar is largely responsible for their lack of activity. These results are in agreement with previous studies showing some in vivo accumulation of the AZT-DP (19) and dideoxynucleotides in MT-4 cells (24). Although they suggest the possibility that these and other nucleoside analogs lacking a 3'-OH group such as the acyclic nucleosides, may not be phosphorylated by NDP kinase in vivo, it should be kept in mind that the turn over of NDP kinases is unusually high (more than 1000 s⁻¹), and therefore that even poor substrates may be phosphorylated in the cell. Our results may help understanding the pharmacokinetics of nucleoside analogs. They may provide a rational basis for the drug design of new active molecules, with the hope that analogs more efficiently phosphorylated by NDP kinase can be used at a lower dose and elicit less toxic and secondary effects.

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