Pre-steady State of Reaction of Nucleoside Diphosphate Kinase with Anti-HIV Nucleotides*

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The pre-steady-state reaction of Dictyostelium nucleoside diphosphate (NDP) kinase with dideoxynucleotide triphosphates (ddNTP) and AZT triphosphate was studied by quenching of protein fluorescence after manual mixing or by stopped flow. The fluorescence signal, which is correlated with the phosphorylation state of the catalytic histidine in the enzyme active site, decreases upon ddNTP addition according to a monoequilibrium time course. The pseudo-first order rate constant was determined for different concentrations of the various ddNTPs and was found to be saturable. The data are compatible with a two-step reaction scheme, where fast association of the enzyme with the dideoxynucleotide is followed by a rate-limiting phosphorylation step. The rate constants and dissociation equilibrium constants determined for each dideoxynucleotide were correlated with the steady-state kinetic parameters measured in the enzymatic assay in the presence of the two substrates. It is shown that ddNTPs and AZT triphosphate are poor substrates for NDP kinase with a rate of phosphate transfer of 0.02 to 3.5 s⁻¹ and a Kₜ of 1–5 mM. The equilibrium dissociation constants for ADP, GDP, ddADP, and ddGDP were also determined by fluorescence titration of a mutant F64W NDP kinase, where the introduction of a tryptophan at the nucleotide binding site provides a direct spectroscopic probe. The lack of the 3'-OH in ddNTP causes a 10-fold increase in Kₜ. Contrary to "natural" NTPs, NDP kinase discriminates between various ddNTPs, with ddGTP the more efficient and ddCTP the least efficient substrate within a range of 100 in kₐₑₛₜ values.

Nucleoside analogues like 3'-deoxy-3'-azidothymidine (AZT) and dideoxynucleosides (ddN) are widely used as antiviral drugs, particularly in the multitherapy protocols now defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

In recent years, genes encoding NDP kinase have been cloned from a number of prokaryotic and eukaryotic organisms (4, 5). NDP kinases are made up from 17-kDa subunits with highly conserved sequences. The x-ray structures of NDP kinases from several species have also been determined at high resolution (5–8), and these studies have shown that both the subunit fold and the active site are remarkably conserved.

Solving the NDP kinase structure in the presence of dTDP (9), ADP (10), and GDP (11) was an important step toward an understanding of the phosphatase transfer mechanism. The nucleotides in the active site are different from other known nucleotide-binding proteins, with the base stacking on a phenylalanine near the protein surface without polar interactions with the protein side chains. The ribose and phosphate moieties are located deeper inside the active site, forming numerous bonds with a Mg²⁺ ion and protein side chains. The nucleotide conformation is original, with a hydrogen bond between the 3'-OH of the sugar and the β-phosphate. In addition, the 3'-OH accepts H-bonds from Lys¹⁸ and Asn¹¹⁹ (in this report, we use the numbering of Dictyostelium NDP kinase). The catalytic His²²² points its N6 toward the phosphate, a well defined water molecule bridging it to the β-phosphate oxygen in the ADP complex, at the presumed position of ATP γ-phosphate.

A precise model for the reaction product and the transition state has been proposed, based on the crystal structures of the enzyme phosphorylated by phosphoramidate (12) and of the ternary complex with ADP and AlF₃ (13). This model is also supported by the results of a large series of substitutions of con-

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² The abbreviations used are: AZT, 3'-deoxy-3'-azidothymidine; ddNDP, 2',3'-dideoxynucleoside diphosphate; ddNTP, 2',3'-dideoxynucleoside triphosphate; HIV, human immunodeficiency virus.

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served residues of the active site (14). NDP kinase has a very high turnover with $k_{\text{cat}}$ around 1000 s$^{-1}$ for “natural” ribo- or deoxyribonucleotides. Comparing the structures of free enzyme and enzyme complexed with nucleotide diphosphates demonstrates that the change of conformation upon nucleotide binding is minimal. The movement is limited to the $\alpha_i/\alpha_2$ helices hairpin, which forms one side of the nucleotide binding site (8–10). Dictyostelium NDP kinase has a single tryptophan (Trp$^{137}$), located at the proximity of the active site. We have shown previously that its fluorescence could be used as a probe for the phosphorylation state of the catalytic histidine (15).

We have recently reported that the diphospho-derivatives of some nucleotide analogues used as antiviral drugs are poor substrates for NDP kinase, as measured by the global enzymatic assay (16). Thus, when the diphospho- and triphospho-derivatives of azidothymidine, deoxyadenosine, or deoxythymidine are used as substrates, the rate of phosphate transfer is $10^2$ to $10^4$ times less than for natural nucleotides.

In this study, we present pre-steady-state kinetic experiments to investigate the phosphorylation of the enzyme by antiviral ddNTPs. Both stopped-flow and conventional techniques were used to measure fluorescence changes on a time scale ranging from milliseconds to several minutes. Using a mutant NDP kinase (F64W) in which an additional tryptophan mutant NDP kinase (F64W) in which an additional tryptophan was placed near the nucleotide binding site, we also measured the affinity constants of nucleotides and nucleosides.

**EXPERIMENTAL PROCEDURES**

**Materials—**ATP, ADP, CDP, GDP, dTDP, lactate dehydrogenase, and pyruvate kinase were from Boehringer Mannheim, and dideoxyribonucleotides from Amersham Pharmacia Biotech or from Boehringer Mannheim. [14C]ADP (57 mCi/mmol) was from NEN Life Science Products. The synthesis of phosphodervatives of AZT and of ddNDP has been described previously (16).

**Site-directed Mutagenesis—**The mutation F64W in Dictyostelium NDP kinase was made by site-directed mutagenesis according to Runkel (17), using the oligonucleotide 5'-GAAAGACCATGGTTCCGGTTGTTT-3'. Altered bases as compared with the wild type sequence are bold and underlined. The mutation was verified by DNA sequencing.

**Enzyme Purification—**Dictyostelium NDP kinase was overexpressed in Escherichia coli (XL1-Blue) using plasmid pNDK as described (4) with small modifications. The cell extract was loaded at pH 8.4 onto DEAE-Sepharose which retained only E. coli NDP kinase (14) and the flow-through was adsorbed on Blue-Sepharose (Amersham Pharmacia Biotech) at pH 7.5. After washing with Tris buffer, the enzyme was eluted by a NaCl gradient (0–1.5 M) in 50 mM Tris-HCl, pH 7.5. After dialysis, the protein was concentrated using an Amicon ultrafiltration cell, equilibrated in 50 mM Tris-HCl, pH 7.5, and stored frozen at $-20^\circ$C. Protein concentration was determined using an absorbance coefficient of $\Delta A_{280}$ = 0.85 for a 1 mg/ml solution according to Gill equation for a 1 mg/ml solution. Mutant F64W NDP kinase was purified according to the same procedure. The absorption coefficient of F64Wmutant NDP kinase was estimated to $\Delta A_{280}$ = 0.85 for a 1 mg/ml solution according to Gill equation for a folded protein in water (18). All proteins were purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis. Enzyme concentration was expressed as concentration of 17-kDa subunits.

The phosphorylated enzyme was prepared as described previously (15), the enzyme that had been preincubated in T buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM MgCl$_2$ and 75 mM KCl) with a saturating amount of ATP was made free of nucleotides by gel filtration on Sephadex G-25. The concentration of the phosphorylated enzyme as well as the absence of nucleotides were checked from the absorbance spectrum of the protein. The stoichiometry of phosphorylation was determined in a parallel experiment using [\gamma-$\text{P}$]ATP, as described in Ref. 15. The phosphorylated enzyme was then kept at 4°C and used within 3 h after synthesis.

**Activity Assays—**Two different assays were used to measure NDP kinase activity. In the first assay, activity of NDP kinase was measured at 20°C with ATP and dTDP as substrates using coupled enzymes (lactate dehydrogenase and pyruvate kinase) as described previously (16). The second assay was used when small reaction volumes (a few microliters) were needed, in particular with radioactive substrates (16). When ddNTPs were tested as phosphate donors, [14C]ADP was used as an acceptor. The initial rate of the reaction was determined at 20°C in the presence of a constant amount of [14C]ADP (0.1 nm) during 2, 4, and 6 min. After separation of the radioactive nucleotides by thin layer chromatography on PEI-cellulose (Macherey-Nagel, Germany), their radioactivity was quantified using a PhosphorImager (Molecular Dynamics).

The ratio of apparent $k_{\text{cat}}$/apparent $K_m$ measured at a constant concentration of the other substrate is equal to the true value of $k_{\text{cat}}$/apparent $K_m$ for a ping-pong enzyme. It is a useful parameter when comparing natural substrates to nucleotides analogs. The nonlinear least-squares fit of the data was performed using Kaleidagraph (Abelbeck Software). Unless otherwise indicated, this software was also used for all of the fittings described below.

**Binding Studies—**The affinity of NDP kinase for NDP kinase was measured by following the variation of intrinsic fluorescence of the mutant F64W enzyme upon nucleotide binding. All fluorescence measurements were performed at 20°C in T buffer on a Photon Technology International (PTI) spectrofluorometer (Quantamaster$^\text{TM}$). Successive aliquots of the nucleotide were added to the enzyme solution (2 µM), and the fluorescence was measured at 340 nm with excitation at 295 nm for ADP and ddADP (2-nm excitation slit and 2-nm emission slit), or at 304 nm in the case of the other nucleotides (emission slit was then 4 nm). Exponential tailing curves were fitted to a hyperbolic ligand-protein curve after correction for dilution. The inner filter effect was found to be negligible.

**Slow Kinetics Experiments—**Slow kinetics experiments were performed at 20°C on a PTI spectrofluorometer (Quantamaster$^\text{TM}$), sampled with continuous stirring. The reaction of wild type NDP kinase with NTP analogues was initiated by addition of the nucleotide (less than 25 µM) to an initial enzyme solution ratio of 1:100 (19). The fitted curves were found to correspond to a single exponential progress, either decreasing for phosphate transfer to the enzyme or increasing in the case of phosphate transfer to the NDP analogues. The pseudo first-order rate constants ($k_{\text{cat}}$) were determined as a function of substrate concentration ([S]), with [S] in excess to [E], the enzyme concentration.

**Stopped-flow Kinetic Experiments—**Stopped-flow kinetic experi-
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RESULTS

Changes in Fluorescence Associated with Phosphorylation of NDP Kinase by Antiviral Nucleotide Analogues—We have shown previously that the fluorescence of the single tryptophan in Dictostelium NDP kinase is quenched upon phosphorylation of the enzyme by ATP (15). To test whether the interaction with ddNTP also results in a change of fluorescence, steady-state emission spectra ($\lambda_{exc} = 295$ nm) were collected in presence of ddATP or ddADP at saturating concentrations, and compared with the spectrum of the native enzyme. A strong decrease (20%) in fluorescence intensity near 320–340 nm was observed in the presence of ddATP. In contrast, the fluorescence was insensitive (less than 3% decrease) to ddADP binding, as reported previously for ADP. Quenching (at least 10%) was also observed with other natural NTPs or ddNTPs ($\lambda_{exc} = 304$ nm) and was likely due to the phosphorylation of the catalytic histidine. Conversely, the fluorescence signal of the phosphorylated enzyme was enhanced upon ddADP addition as the phosphate was transferred from the protein back to the nucleotide. This provided a convenient method to monitor histidine phosphorylation at different ddNTP/ddNDP ratios and to derive the equilibrium constants. As shown in Fig. 1, the equilibrium constant was the same for the ddTTP/ddTDP pair ($K_{eq} = 0.13 \pm 0.02$) and for the AZTTP/AZTDP pair. It did not differ significantly from natural nucleotides. A value of $K_{eq} = 0.19 \pm 0.02$ was obtained previously for the ATP/ADP pair by fluorescence titration (15). Applying the Haldane relationship to steady-state kinetic parameters yielded the same value. We conclude that the absence of 3′-OH or its replacement with an azido group in the analogue has no significant effect on the phosphorylation equilibrium between the enzyme and the nucleotides.

Steady-state Kinetic Parameters of NDP Kinase with ddNTP—Steady-state kinetic parameters were first measured in the phosphate exchange reaction between ddNTP and [3H]ADP (reactions A and B, shown in Scheme 1). Fig. 2 shows initial rate data for the native enzyme for ddGTP at various concentrations and the inset shows the comparison of the four ddNTPs at a given concentration. A constant concentration of ADP (0.1 mM), corresponding to twice the $K_m$ (20), was used. The data were adjusted with the Michaelis equation. Table I summarizes the kinetic constants (apparent $K_m$ and apparent $V_{max}$) determined for ddATP, ddGTP, ddTTP, and ddCTP used as phosphate donors.

The lack of the 3′-OH in ddNTP dramatically affected the catalytic activity. An increase by a factor of 10 in apparent $K_m$ and a decrease of 500–10,000 in apparent $k_{cat}$ were observed when comparing kinetic parameters of ddNTPs with those of ATP (20). Under the conditions used, the kinetic parameters of natural nucleotides were very similar (results not shown). In contrast, the kinetic constants of dideoxynucleotides varied strongly with the ddNTP used. In particular, ddCTP showed a very low value of $k_{cat}/K_m$, with a drop of more than 10^4 as compared with CTP.

Pre-steady-state Kinetics of NDP Kinase Phosphorylation by ddNTPs—The fluorescence signal allowed us to monitor the time course of the phosphate transfer reactions between NDP kinase and nucleotides. However, the time-dependent change of the fluorescence was too fast to be observed when 1 μM enzyme was reacted with 100 μM ATP. This is due to the fact that the time response of the stopped flow is slow compared with the $k_{cat}$ of the enzyme (−600 s−1 at 20 °C). In contrast, when the enzyme was reacted with ddATP under the same conditions, a time-dependent quenching of the enzyme fluorescenc...
cence was observed in the second-to-minute time scale (Fig. 3A), with no observable lag. For all concentrations of ddATP tested, a single exponential decay was observed, characterized by a pseudo-first order rate constant $k_{obs}$. The signal amplitude was constant, corresponding to a complete phosphorylation of the enzyme. The pseudo-first order rate constant of the phosphorylation reaction is shown in Fig. 4A as a function of [ddATP] in the 0.1–3 mM range. The observed rate constant ($k_{obs}$) increased linearly for [ddATP], before reaching a plateau. These data were best adjusted to the equation of a saturation curve with a maximum rate constant of 1 s$^{-1}$ at saturating ddATP and an apparent equilibrium dissociation constant $K_S$ of 0.75 mM (Fig. 4A and Table I).

Similar monoexponential decays were found when reacting the enzyme with ddGTP, ddTTP, and ddCTP. The pseudo-first order rate constants varied with [ddNTP]. The corresponding parameters (Table I) showed noticeable differences between nucleotides; thus, ddGTP appears to be the best substrate, ddATP and ddTTP being slightly less efficient. ddCTP is a very poor substrate for NDP kinase, which was nearly inactive with ddCTP in the 0.1–3 mM range. Therefore, the kinetic parameters for ddCTP could not be determined precisely.

Fig. 4B shows a similar dependence of the pseudo-first order rate constant when the reaction was initiated by hand mixing. In this case, only the linear part of the previous saturation curves were measured. The slope had the dimension of a bimolecular association rate constant and was sufficient to characterize the interaction of NDP kinase with low concentrations of NTP analogues. The values for these slopes are given as $k_{obs} = k_1/K_S$ in Table I.

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Pre-steady-state Kinetics of NDP Kinase Dephosphorylation with ddNDP—The dephosphorylation of the phosphorylated enzyme ($E_P$) by ddTDP and by AZTDP (reaction B, Scheme I) also displayed a monoexponential time course in the minute range (Fig. 5). The apparent time constant (apparent $k_{depho}$)
of ADP. The values of the dissociation constant binding curve, where the fluorescence decreases as a function of kinase under equilibrium conditions. Fig. 6 shows a typical curve to determine the affinity of nucleotide diphosphates for NDP kinase was recently resolved (21). It shows that the analogue AZT-DP complexed with a point mutant of $\text{NDP}^\text{Dictyostelium}$ shows essentially identical kinetic parameters to the wild type enzyme (Table II) and was stable up to 4.5M urea. NDP kinase showed essentially identical kinetic parameters to its two tryptophans in position 64 and 137. The fluorescence of Trp137 is the signal which enzyme catalyzing the transfer of 1 mol of phosphate/min. Each value is the mean of three independent determinations for wild type and 2 for the mutant NDP kinase.

**TABLE II**

| Specific activity (units/mg) | Wild type protein | F64W mutant protein |
|-----------------------------|------------------|---------------------|
| $k_{\text{cat}}$ (s$^{-1}$)  | 570              | 2000                |
| $K_{\text{ATP}}$ (mM)       | 0.44             | 0.28                |
| $K_{\text{dTTP}}$ (mM)      | 0.11             | 0.12                |
| $k_{\text{cat}}/K_{\text{ATP}}$ (M$^{-1}$ s$^{-1}$) | $1.3 \times 10^6$ | $2.0 \times 10^6$ |
| $k_{\text{cat}}/K_{\text{m}}$ (M$^{-1}$ s$^{-1}$)      | $5.1 \times 10^5$ | $4.7 \times 10^5$ |

**DISCUSSION**

In this work, we have conducted steady-state and pre-steady-state experiments to study the interaction of NDP kinase with antiviral dideoxynucleotide triphosphates as substrates. The enzyme from the slime mold Dictyostelium discoideum is a 102-kDa hexamer. It is highly homologous to both isofoms of the human enzyme (57% sequence identity), and has a very similar three-dimensional structure, especially at the active site (8–10) (Fig. 7). Conclusions drawn from this study should therefore be applicable to the human enzymes. The structure of AZT-DP complexed with a point mutant of Dictyostelium NDP kinase was recently resolved (21). It shows that the analogue binds at the same site and in the same orientation as the natural substrate dTDP. As could be expected, the phosphorylation equilibrium between the enzyme and its substrates is unaffected by the absence of the 3'-OH in dideoxynucleotides or its substitution in AZT derivatives. However, the analogues are very poor substrates (16), and we have found in the present study that the rate of phosphate transfer was strongly reduced in pre-steady-state experiments when either the histidine phosphorylation or the dephosphorylation step was followed as a function of time and substrate concentration. In Dictyostelium NDP kinase, the fluorescence of Trp137 is the signal which we use to monitor the phosphate transfer step in the kinetics.
as it is sensitive to the state of histidine phosphorylation, but not to substrate binding.

Our kinetic data are compatible with the following reaction.

\[ E + ddNTP \xrightarrow{k_{+1}} E \cdot ddNTP \xrightarrow{k_{+2}} E \cdot ddNTP \xrightarrow{k'_{-1}} E \cdot ddNTP \xrightarrow{k'_{+1}} E + ddNTP \]

**REACTION 1**

When studying the forward reaction, the concentration of the ddNDP product remains very low and the product binding reaction can be neglected. Under these circumstances, the \([E-P \cdot ddNTP]/[E-P]\) ratio must be less than \(k_{-2}/k'_{-1}\) and therefore much smaller than 1, given the very low rate of phosphorylation observed with the analogues. Then, the mechanism of phosphorylation by ddNTP simplifies to Reaction 2.

\[ E + ddNTP \xrightarrow{k_{+1}} E \cdot ddNTP \xrightarrow{k_{+2}} E \cdot ddNTP \xrightarrow{k'_{-2}} E + ddNTP \]

**REACTION 2**

When studying dephosphorylation by ddNDP in the absence of ddNTP, the same argument leads to writing the reaction mechanism as shown in Reaction 3.

\[ k_{obs} = \frac{k_{+2} \cdot [ddNTP]}{(k_{+1} + K_{d})} + [ddNTP] \]  

(Eq. 1)

The observed rate of phosphorylation should increase with [ddNTP] to reach a plateau value equal to \(k_{+2}\), the true rate of histidine phosphorylation, with half-saturation occurring for [ddNTP] = \(k_{+1}/K_{d}\) = \(K_{o}\), the equilibrium dissociation constant of the nucleotide substrate. At low [ddNTP], \(k_{obs}\) is expected to increase linearly with [ddNTP] with an apparent bimolecular rate constant that is \(k_{+2}/K_{d}\).

Equation 1 was used to fit the curves in Fig. 4 (A and B), yielding rate and dissociation constants listed in Table I. Whereas the two purine dideoxynucleotides have similar dissociation constants \(K_{d}\) \(\approx\) 1 mM, the rate of phosphorylation is larger for ddGTP \((k_{+2} = 3.5 \text{ s}^{-1})\) than for ddATP \((1 \text{ s}^{-1})\). On the other hand, pyrimidine dideoxynucleotides are relatively poorer substrates for NDP kinase phosphorylation. ddTDP has \(k_{+2} \approx 2.5 \text{ s}^{-1}\) and \(K_{d} \approx 5 \text{ mM}\), too large a value for saturation to be reached under our experimental concentrations. With ddCTP, the catalytic efficiency is so poor that neither \(K_{d}\) nor the rate of phosphate transfer could safely be determined in stopped-flow experiments at nucleotide concentrations up to 3 mM.

Because the affinity of NDP kinase for its natural nucleotide substrates could not be determined in the same way, we resorted to designing a mutant where a tryptophan replaces Phe\(^{64}\) at the base binding site (Fig. 7). The substitution provides a spectroscopic signal that monitors ligand binding. The stability and steady-state catalytic properties of the F64W NDP kinase given in Table II are very similar to those of the wild type enzyme. Equilibrium dissociation constants for E-NDP dead-end complexes were determined by fluorescence measurement of F64W NDP kinase for selected nucleoside diphosphates

### TABLE III

| Nucleoside diphosphate | Equilibrium dissociation constants \((K_{d})\) for F64W NDP kinase |
|------------------------|---------------------------------------------------------------|
| ADP                    | \(25 \pm 5\) (3)                                              |
| ddADP                  | \(220 \pm 50\) (3)                                            |
| GDP                    | \(14 \pm 5\) (2)                                              |
| ddGDP                  | \(120 \pm 30\) (2)                                            |
| dTDP                   | \(100 \pm 50\) (2)                                            |
| AZTDP                  | \(80 \pm 20\) (2)                                             |
| CDP                    | \(200 \pm 100\) (3)                                           |

FIG. 7. Nucleotide binding site. Figure is a stereoview of the NDP kinase active site with bound dTDP (adapted from Ref. 9). The thymine base is stacked between Phe\(^{64}\) and Val\(^{116}\) at the entrance of the active site and points down toward outside the protein. The phosphates carry a Mg\(^{2+}\) ion and point toward the active His\(^{122}\) on top. The single Trp\(^{137}\) is located near the catalytic His\(^{122}\). The 3’-OH of the natural nucleotide makes hydrogen bonds with residues Lys\(^{105}\) and Asn\(^{110}\) and with the β phosphate.
titration for the natural nucleotides and their analogues. Values listed in Table III show that the absence of the 3'-OH in the dideoxy analogues raises the dissociation constant by a factor of about 10 in purine nucleotides. This same ratio is also seen in steady-state parameters, $K_{eq}$ values being $\sim$10 times larger for ddNTP than for NTP substrates (16). Differences between natural nucleotides are apparent in Table III, with ADP and GDP having similar affinities that are significantly better than for ddNTP and especially CDP. The trends are the same for $K_S$ values obtained for the ddNTPs from the analysis of pre-steady-state kinetics above.

Although the dephosphorylation reaction was not studied in the same details, our data suggest that it obeys similar rules. Phosphate transfer from the phospho-enzyme to a dideoxy-nucleoside diphosphate substrate is slow and rate-limiting compared with substrate binding. Because saturation of the observed rate of dephosphorylation was not achieved at substrate concentrations used for manual mixing experiments (Fig. 5), only the apparent bimolecular rate constant $k_{cat}/K_S$ can be derived from these data. For ddTTP, $k_{cat}/K_S = 4500 \text{ M}^{-1} \text{ s}^{-1}$, which exceeds by a ratio of $\sim$5 the corresponding value of $k_{cat}/K_S$ values found for ddTTP in Table I. According to the Haldane equation, this ratio should be equal to the equilibrium constant $K_{eq}$ for phosphorylation, which we find to be 1/0.13 $\approx 7$ by direct measurement (Fig. 1). The two determinations of $K_{eq}$ are completely independent, and their agreement strongly supports our interpretation of the kinetic data.

An early study by Wälinder et al., in 1969, investigated the phosphorylation of bovine NDP kinase by [γ-$\text{32P}$]ATP using a rapid mixing technique (24). The pseudo-first order rate constants for phosphorylation by ATP and for dephosphorylation by dGDP exceeded the turnover number of the overall reaction, indicating that the phosphoenzyme could be an intermediate in the NDP kinase reaction. With these substrates, both steps are fast and, in the case of the Dictyostelium enzyme where $k_{cat}$ is on the order of 1000 s$^{-1}$, they are completed in less than a millisecond, too fast for stopped-flow studies. With less efficient substrates such as the antiviral analogues studied here, the turnover rate constant drops to 1–2 s$^{-1}$. Phosphorylation by ddNTP in one direction and dephosphorylation by ddNTP in the other direction are slow and rate-limiting in the overall reaction. Accordingly, the rates of phosphorylation $k_{cat}$ derived from the analysis of pre-steady-state data are in very good agreement with steady-state $k_{cat}$ values measured with the same nucleotide analogues as substrates (Table I).

Our results indicate that the absence of the 3'-OH in the analogues results in a 10-fold increase in the dissociation constant and in a 300–5000 decrease in the rate of phosphate transfer, resulting in a factor $3 \times 10^3$ to $5 \times 10^4$ in catalytic efficiency. The 3'-OH of the nucleotide sugar is involved in a hydrogen bond network with Asn$^{119}$, Lys$^{16}$ on the protein, and also with the oxygen that bridges the $\beta$- and $\gamma$-phosphates of the nucleotide itself (Fig. 7). Removing the Asn$^{119}$ or Lys$^{16}$ side chains results in mutant NDP kinases that display a much less dramatic decrease in catalytic efficiency than when the 3'-OH is removed; $k_{cat}/K_{eq}$ drops by a factor of 10 in the N119A mutant (21) and by a factor of 200 in the K16A mutant. The loss of the internal hydrogen bond between the 3'-OH and the bridging phosphate oxygen in ddNTP is likely to be the major reason for the low activity of the enzyme on dideoxy- or AZT derivative substrates. Additional differences are observed between the nucleotide analogues themselves, with ddCTP being the poorest substrate of all, but these differences have no obvious interpretation at present.

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