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Role of Nucleoside Diphosphate Kinase in the Activation of Anti-HIV Nucleoside Analogs

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Nucleoside analogs are currently used in antiretrovirus therapies. The best known example is AZT one of the first drug to be used for the treatment of AIDS. However, only the triphosphate derivatives of these compounds act as substrates of the viral reverse transcriptase. Since they do not enter cells, nucleoside analogs are administered and phosphorylated by cellular kinases. The last step in this phosphorylation pathway is catalyzed by nucleoside diphosphate (NDP) kinase. The incorporation of the nucleoside triphosphates into nascent viral DNA chain results in termination of the elongation process. We have performed kinetics studies of the phosphorylation reaction by NDP kinase of dideoxynucleoside diphosphates such as 2′,3′-dideoxy-3′-azidothymidine diphosphate (AZT-DP) and 2′,3′-dideoxy-2′,3′-didehydrothymidine diphosphate (d4T-DP). We show that the catalytic efficiency is strongly decreased and, therefore, that the reaction step catalyzed by NDP kinase constitutes a bottleneck in the processing pathway of anti-HIV compounds. In addition, the affinity of the analogs in the absence of catalysis was determined using a catalytically inactive NDP kinase mutant, showing a reduction of affinity by a factor of 2 to 30, depending on the analog. The structure of NDP kinase provides a structural explanation for these results. Indeed, all nucleoside analogs acting as chain terminators must lack a 3′-OH in the nucleotide deoxyribose. Unfortunately, this same substitution is detrimental for their capacity to be phosphorylated by NDP kinase. This defines the framework for the design of new nucleoside analogs with increased efficiency in antiretroviral therapies.

KEY WORDS: HIV; nucleoside analogs; dideoxynucleoside; AZT; d4T; phosphorylation; NDP kinase.

INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTI) are antiviral drugs widely used in anti-HIV therapy, generally in combination with non nucleoside reverse transcriptase inhibitors or HIV proteases inhibitors. All NRTI lack the 3′-OH in their ribose moiety. The most commonly used of nucleoside analogs are 2′, 3′-dideoxynucleosides, such as dideoxyinosine (ddI) or dideoxycytidine (ddC), or 2′, 3′-dideoxynucleosides substituted on the 3′-OH position of the sugar, such as AZT and d4T (Fig. 1). Their incorporation into a nascent DNA chain by HIV reverse transcriptase (RT) terminates elongation and blocks viral DNA synthesis. However, to exert their activity, NRTI must be activated into triphosphate derivatives by cellular kinases (Balzarini, 1999; Larder, 1992; Mitsuya *et al.*, 1985). It is generally considered that antiviral analogs are phosphorylated by the same cellular kinases as the natural nucleotides. The activation of nucleosides into their mono- and diphosphate forms is catalyzed by nucleoside kinases and nucleoside monophosphate kinases with various degrees of specificity (Johansson *et al.*, 1999). In humans, deoxygua-

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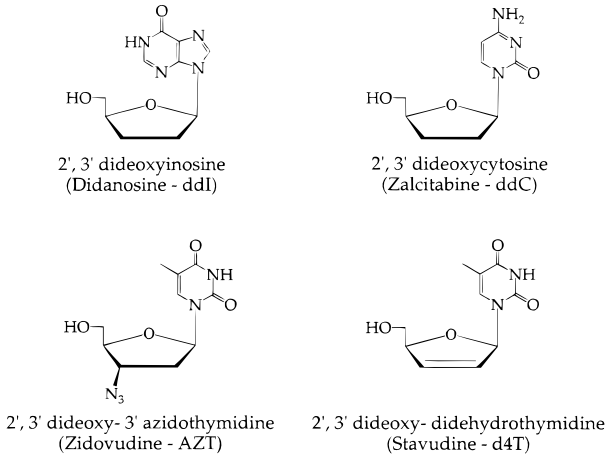

Fig. 1. Chemical formula of some nucleoside analogs currently used in anti-HIV therapies.

nosine kinase (dGK) is specific for purine nucleosides, while thymidine kinases cytosolic (TK1) and mitochondrial (TK2), as well as thymidylate kinase (TMP kinase), accept only pyrimidine nucleosides and nucleotides. Although deoxycytidine kinase (dCK) shows some specificity for 2′-deoxycytidine, it also phosphorylates 2′-deoxyadenosine and 2′-deoxyguanosine. The antiviral analogs, which are processed by three different kinases, each adding the α-, β-, or γ-phosphate, may be poor substrates of a kinase at a given step. This results in the accumulation of intermediates along the phosphorylation pathway. The level of the monophosphate (NMP), the diphosphate (NDP), or the triphosphate (NTP) forms of several nucleotide analogs in human peripheral blood mononuclear cells of patients infected by HIV (Solas *et al.*, 1998) or in lymphocytes in culture (Furman *et al.*, 1986), shows accumulation of NMP or NDP. AZT-MP is known to accumulate in cells and to cause toxic effects at the mitochondria level (Lewis and Dalakas, 1995). The accumulation of an intermediate allows us to identify the rate-limiting steps that are bottlenecks in the activation of an analog. For example, Lavie *et al.* (1997a) have characterized, in detail, the reduced activity of TMP kinase with AZT-MP.

In contrast to the nucleoside kinases involved in the first two steps of the nucleotides phosphorylation pathway, NDP kinase is nonspecific for the nucleobase or for the 2′ position of the sugar. It accepts purines and pyrimidines and both ribo- and deoxyribonucleoside diphosphates as substrates. For this reason, it has long been considered that NDP kinase was unlikely to constitute a limiting step in the phosphorylation pathway of NRTI. In this paper, we review our recent data showing the poor activity of NDP kinase with nucleotide analogs modified on the ribose moiety. We show that this last phosphorylation step is also a bottleneck in the activation pathway of several nucleoside analogs that are currently in clinical use for the treatment of AIDS.

PHOSPHORYLATION OF ANTI-HIV ANALOGS AT THE STEADY-STATE LEVEL

The X-ray structure of NDP kinases from several species has been determined (Chiadmi *et al.*, 1993; Dumas *et al.*, 1992; Webb *et al.*, 1995) showing that both the subunit fold and the structure of the active site are highly conserved (Janin *et al.*, 2000). All eukaryotic NDP kinases are hexameric, composed of 17-kDa monomers. The catalytic reaction of NDP kinase involves the transient phosphorylation of a histidine at the active site (Garces and Cleland, 1969; Lecroisey *et al.*, 1995; Morera *et al.*, 1995a). The nucleotide binding site does not involve a P-loop motif as observed in other ATP-binding sites. The base makes no hydrogen bonds with the protein and stacks a conserved phenylalanine (Phe60 in human NDP kinase B numbering). The ribose and phosphates moieties of the nucleotide are located deeper inside the active site, forming several H-bonds with protein side chains. In particular, the 3′-OH of the sugar is involved in a hydrogen bond network with the conserved residues Asn115 and Lys12. An internal H-bond within the nucleotide is also created between the 3′-OH and the O_7 bridging the β- and γ-phosphates. A single Mg^{2+} ion is coordinated by oxygens from the phosphate chain and is involved in phosphotransfer (Cherfils *et al.*, 1994; Morera *et al.*, 1994, 1995b; Xu *et al.*, 1997b).

NDP kinase A and NDP kinase B, the two main isoforms of human NDP kinase, are 88% identical (Gilles *et al.*, 1991). They show similar kinetic parameters for natural substrates (Schaertl *et al.*, 1998), as well as for a series of thymidine diphosphate analogs (Gonin *et al.*, 1999). In this study, we used both NDP kinase A and the NDP kinase from the lower eukaryote *Dictyostelium discoideum* (Dd-NDP kinase), which shares 63% sequence identity with human enzymes. Moreover, Dd-NDP kinase, the first crystal structure solved for a eukaryotic NDP kinase (Dumas *et al.*, 1992), has proved to be an excellent model for both

structural and kinetic studies (Karlsson *et al.*, 1996; Lascu *et al.*, 1993; Tepper *et al.*, 1994).

Anti-HIV drugs, such as AZT or d4T, were chemically phosphorylated, as described in Bourdais *et al.* (1996). The nucleoside analogs di- and triphosphates were purified by chromatography on a DEAE-Sephadex A-25 column, eluted with a linear gradient of triethylammonium hydrogen carbonate. ddNDP were enzymically synthesized from the triphosphate form in presence of an excess of fructose 6-phosphate and phosphofructokinase and purified by reverse phase chromatography on a C-18 column eluted with an acetonitrile gradient. The purity of each phosphorylated compound was assessed by [^{1}H], [^{13}C], and [^{31}P] NMR and by mass spectrometry.

The catalytic parameters of NDP kinase with natural and antiviral drugs were measured under the steady state conditions with either the classical spectrophotometric assay (Lascu and Gonin, 2000) or an isotopic miniaturized assay (microliters volumes) using [γ-^{32}P]GTP or [^{14}C]ADP. The radioactive assay is less consuming in phospho-derivative analogs, which are not easy to synthesize chemically. In this assay, the reaction products are separated on TLC plates and quantified by a PhosphoImager (Bourdais *et al.*, 1996). Saturation curves are obtained for a relatively wide range of nucleotide concentrations leading to the measure of k_{cat} and K_m values (not shown). Although the saturation plateau could not be reached when the analog was available in small amounts, the catalytic efficiency could nevertheless be calculated, since for an enzyme with a ping-pong mechanism, the slope of the initial rate v_i versus [NDP] at any NTP concentration is equal to the true ratio k_{cat}/K_m (Bourdais *et al.*, 1996).

The fluorescence emission spectrum of *Dictyostelium* NDP kinase upon excitation at 295 nm shows a 20% decrease in presence of saturating amounts of ATP (Fig. 2, inset; and Deville-Bonne *et al.*, 1996). A similar quenching also occurs with human NDP kinase A and B (Deville-Bonne *et al.*, 1998). No quenching is observed in the presence of ADP or AMP-PCP, a nonhydrolyzable analog of ATP. Conversely, the fluorescence signal of the phosphorylated enzyme is enhanced upon ADP or other NDP addition as the phosphoryl residue is transferred from the protein to the nucleotide. We have demonstrated that this variation in protein fluorescence is due to phosphorylation of the catalytic histidine (Deville-Bonne *et al.*, 1996).

The equilibrium constant of the reaction K_{eq}:

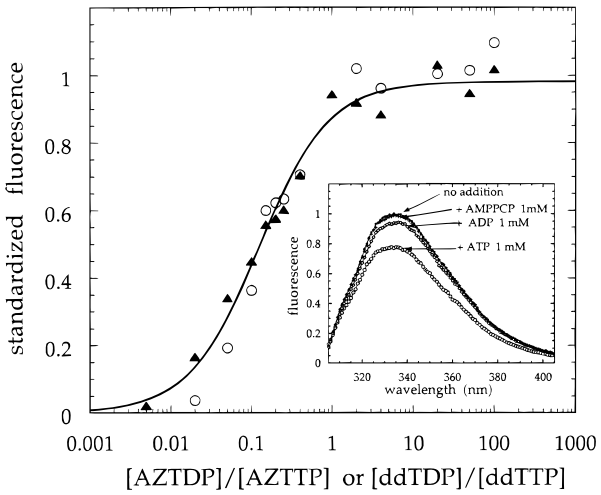

Fig. 2. Intrinsic fluorescence of NDP kinase as a function of ddTTP/ddTDP and AZT-DP/AZT-TP. The equilibrium constant of the reaction with ddTTP/ddTDP or AZT-DP/AZT-TP was measured using protein fluorescence. The enzyme (1 μM) in buffer T (Tris-HCl 50 mM, pH 7.5, $MgCl_2$ 5 mM, KCl 75 mM) was incubated at the equilibrium with NTP and NDP (total amount of nucleotides = 25 μM, in varying [NDP]/[NTP] ratio): ddTDP/ddTTP (○); AZT-DP/AZT-TP (▲). Data were fitted with the equation K_{eq} = [E∼P] · [NDP]/[E] · [NTP] modified to the following formulation: $\Delta F = (\Delta F_{max} \cdot r)/(r + K_{eq})$, where r = [NDP]/[NTP], where ΔF_{max} is the maximum variation in fluorescence, and ΔF the variation observed at a given r value. Fitting is shown as a solid line for AZT-DP/AZT-TP only. The value for K_{eq} is 0.19 ± 0.02 in both cases. Inset: NDP kinase fluorescence in the presence of: ATP 0.5 m*M* (○); AMP-PCP (+); ADP (Δ); no addition (▲).

$$K_{eq} = [E \sim P] \cdot [NDP] / [E] \cdot [NTP]$$
$$= [E \sim P] \cdot r / [E]$$

can be calculated from fluorescence measurements at different ratios r = [NDP] / [NTP], where NDP is a natural nucleoside diphosphate or an analog and NTP its triphosphate counterpart. The fluorescence intensity varies according an hyperbolic saturation curve (Fig. 2). When r is low ([NTP] > [NDP]), the enzyme is mainly under the phosphorylated form and the fluorescence is quenched while, at high r values ([NTP] < [NDP]), the enzyme is dephosphorylated and the fluorescence is maximum. A K_{eq} value of 0.19 ± 0.02 was found for natural nucleotides. Similar K_{eq} values were found for the couples ddGDP/ddGTP, ddTDP/ddTTP or AZT-DP/AZT-TP, indicating that the absence of a free 3′-OH does not affect the equilibrium of the phosphorylation reaction (Deville-Bonne *et al.*, 1996; Schneider *et al.*, 1998).

PRESTEADY-STATE STUDIES OF REACTION OF ANTI-HIV ANALOGS BY NDP KINASE

The intrinsic fluorescence quenching upon phosphorylation of the catalytic histidine provides a convenient method to monitor the phosphoryl transfer, either from the phosphorylated enzyme (E ~ P) to a NDP acceptor or from a NTP donor to the enzyme. We have used this signal in stopped-flow experiments to compare the kinetics of phosphorylation and dephosphorylation of several nucleoside analogs. Figure 3A shows a typical experiment where the fluorescence change is monitored upon d4T-TP addition. The time course always follows a monoexponential progress curve without lag, burst, or biphasicity in a time scale up to 100 s. Each fitted curve is characterized by a pseudo first-order rate

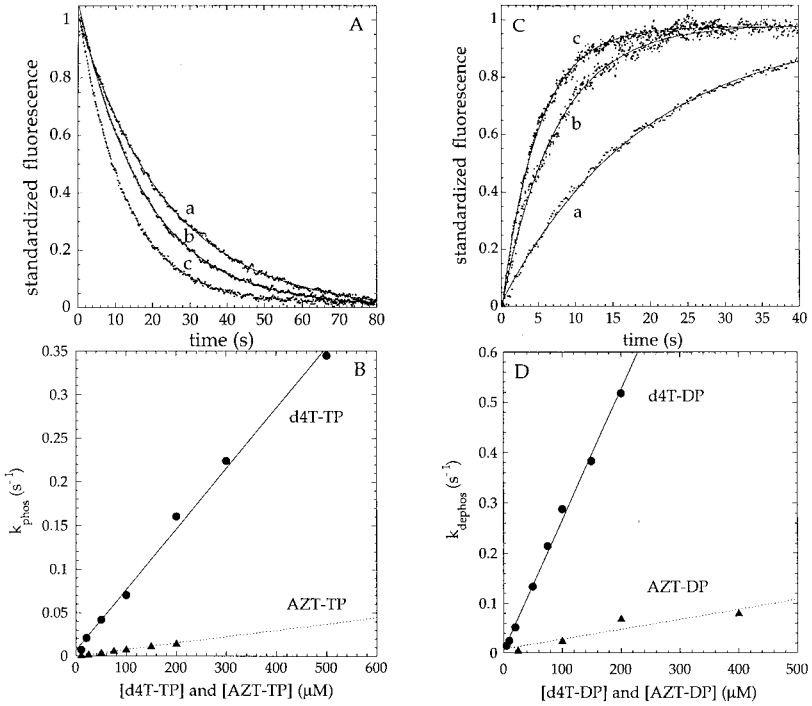

Fig. 3. Presteady state kinetics of phosphoryl transfer. (A) Kinetics of phosphorylation of NDP kinase A by d4T-TP. The enzyme (1 μM, final concentration) in T_2 buffer (Tris-HCl 50 mM, pH 7.5, $MgCl_2$ 5 mM, KCl 75 mM, DTE 1 mM, and glycerol 5%) was rapidly mixed at 20°C with d4T-TP (50, 100, and 200 μM for curves a, b and c, respectively). The decrease in fluorescence was monitored during 200 s on a stopped-flow apparatus (λ_{exc} = 304 nm, emission filter 320 nm cutoff). For clarity, the data were plotted on a 0–80 s scale (B) Concentration dependence of the phosphorylation rate constant with d4T-TP or AZT-TP concentration. The pseudo first-order rate constant for the reaction (k_{phos}) was plotted against the analog triphosphate concentration [d4T-TP (●) and AZT-TP (▲)]. The apparent second-order rate constant is 700 $M^{-1}s^{-1}$ for d4T-TP and 75 $M^{-1}s^{-1}$ for AZT-TP. (C) Kinetics of dephosphorylation or phosphorylated NDP kinase A by d4T-DP. The phosphorylated enzyme (1 μM, final concentration; Deville-Bonne *et al.*, 1996) in T_2 buffer at 20°C was rapidly mixed with d4T-DP (40, 100, and 150 μM, respectively, for curves c, b, and a). (D) Concentration dependence of the phosphotransfer rate constant with d4T-DP or AZT-DP concentration. The pseudo first-order rate constant for the reaction (k_{dephos}) was plotted against analog diphosphate concentration. d4T-DP (●) and AZT-DP (▲). Apparent rate constants are 2600 $M^{-1}s^{-1}$ for d4T-DP and 200 $M^{-1}s^{-1}$ for AZT-DP. (A) and (C), The solid lines represent the best fit of each curve to a monoexponential. (B) and (D), The linear fit indicates that data can be analyzed as a second-order reaction.

constant (k_{obs}). The symmetrical experiment with phosphorylated enzyme and d4T-DP leads to a similar pattern (Fig. 3C). k_{obs} increases with the nucleotide concentration, and varies linearly with the analog concentrations (Fig. 3B and D). In the case of dideoxynucleotides, k_{obs} reaches a plateau at high analog concentrations (Fig. 4). These data are compatible with a two-step mechanism with a fast binding step, followed by a slow phosphoryl transfer, according to one of the reaction schemes:

$$E + NTP \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrows}} E \cdot NTP \underset{k_{-2}}{\overset{k_{+2}}{\leftrightarrows}} E \sim P \cdot NDP$$

(forward reaction)

$$E \sim P + NDP \underset{k'_{+1}}{\overset{k'_{-1}}{\leftrightarrows}} E \sim P \cdot NDP \underset{k_{+2}}{\overset{k_{-2}}{\leftrightarrows}} E \cdot NTP$$

(backward reaction)

The pseudo first-order rate constant (k_{obs}) obeys one of the following relationships:

$$k_{obs} = k_{+2} \cdot [NTP] / [\ (k_{-1}/k_{+1}) + [NTP]\ ]$$

(forward reaction)

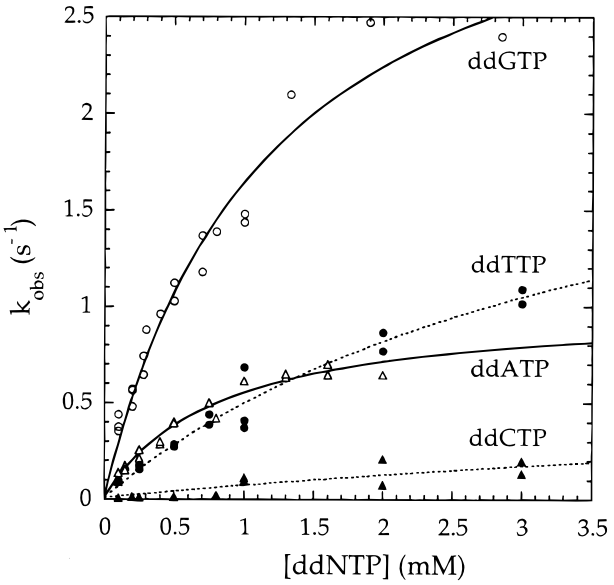

Fig. 4. Presteady-state kinetics of NDP kinase phosphorylation with ddNTP. Stopped-flow experiments were performed with ddGTP (○), ddTTP (●), ddATP (Δ), and ddCTP (▲) using *Dictyostelium* NDP kinase (1 μM) in T buffer (Tris-HCl 50 mM, pH 7.5, $MgCl_2$ 5 mM, KCl 75 mM). The pseudo first-order rate constant for the reaction is hyperbolically dependent on the ddNTP concentration.

$$k_{obs} = k_{-2} \cdot [NDP] / [\ (k'_{+1}/k'_{-1}) + [NDP]\ ]$$

(backward reaction)

For each reaction, k_{obs} increases with nucleotide concentration and reaches a plateau corresponding to the rate constant of the phosphotransfer (k_{+2} or k_{-2}). The equilibrium dissociation constant of the nucleotide is calculated at half saturation, for [NTP] = k_{-1}/k_{+1} = K_S or [NDP] = $k'_{+1}/k'_{-1} = K'_S$. For low concentrations of nucleotide, k_{obs} is expected to increase linearly with the nucleotide concentration. The slope represents the apparent bimolecular rate constant: respectively, k_{+2}/K_S and k_{-2}/K'_S in the forward and in the backward reaction. If the phosphoryl transfer reaction is the limiting step of the two-step mechanism, this bimolecular rate k_{+2}/K_S should be a measure of the catalytic efficacy k_{cat}/K_m of the enzyme for the considered analog.

When dideoxynucleosides triphosphates (ddNTP) were used as donor, the saturation was reached with millimolar concentration of nucleotides (Fig. 4), in agreement with the model. ddNTP phosphorylate 10,000 times slower the NDP kinase than their natural counterparts, with k_{+2} in the 1 to 3 s^{-1} range and K_S in the millimolar range. Interestingly, while NDP kinase is mostly nonspecific toward natural nucleotides, the enzyme discriminates between ddNTP with ddGTP > ddATP > ddTTP > ddCTP in catalytic efficiencies. AZT-DP exhibits the same behavior as ddADP. In contrast, d4T-DP has a catalytic efficiency of phosphorylation by NDP kinase A 1000 times less than dTDP (2.6×10^3 $M^{-1}s^{-1}$ compared to 2×10^6 $M^{-1}s^{-1}$, respectively). It should be noted that the catalytic efficiency k_{cat}/K_m found in steady state experiments is in excellent agreement with stopped-flow results k_{+2}/K_S (Fig. 4), strengthening the validity of the kinetic model proposed above (Schneider *et al.*, 2000, 1998).

Phosphorylation of AZT-MP was proposed to be a major bottleneck in the cellular phosphorylation pathway of AZT (Lavie *et al.*, 1997a), since the catalytic rate constant of TMP kinase for dTMP (35 s^{-1}) is strongly reduced with AZT-MP (0.175 s^{-1}), with no change in nucleotide K_m. This has been proposed to be due to a shift of the conserved P-loop by 0.5 Å (Lavie *et al.*, 1997b). The catalytic efficiency of TMP kinase for AZT-MP is 3×10^4 $M^{-1}s^{-1}$ (100 times lower than with dTMP). Our results indicate that the catalytic efficiency of human NDP kinase A with AZT-DP is even more strongly reduced (15,000 times lower than with dTDP) (Schneider *et al.*, 2000). Although it

is difficult to compare catalytic efficiencies of different enzymes purified from different species, these results clearly show that TMP kinase is one of the two bottlenecks in AZT activation.

In conclusion, our kinetic experiments show that the absence of the 3′-OH on the ribose moiety or its substitution have no effect on the equilibrium constant of the NDP kinase reaction, but that the rate of phosphorylation is impaired by a factor of 10^3 to 10^4, depending on the analog.

AFFINITY OF ANTI-HIV DRUGS FOR NDP KINASE

In case of AZT-DP and -TP or d4T-DP and -TP, the saturation could not be reached in presteady state experiments (Fig. 3) and thus the affinity of the nucleotide for the enzyme could not be estimated by stopped-flow experiments. In order to directly measure the binding constant of the nucleotide analogs, we engineered a mutant of *Dictyostelium* NDP kinase in which the Phe located near the surface of the protein and belonging to the active site (Phe60 in human NDP kinase numbering) was substituted by a Trp. The catalytic parameters of this F → W point mutant were similar to those of the wild-type enzyme, indicating that the presence of the additional Trp does not affect nucleotide binding and catalysis. A 10% decrease in intrinsic fluorescence of the mutant protein at 340 nm is observed upon NDP binding at the active site, due to the quenching of the fluorescence of the newly introduced Trp upon stacking with the base. We have used this signal to measure the substrate affinity for the protein. The affinity for ddNDP is reduced about tenfold as compared with the corresponding (d)NDP. In contrast, in case of AZT-DP, the affinity was similar to dTDP (Schneider *et al.*, 1998). It should be noted, however, that these K_d values do not correspond to the affinity of the substrate, but represent the binding affinities of enzyme inhibitors as dead-end complexes. Indeed, it is impossible to measure the affinity of a NTP substrate because of the catalytic phosphotransfer activity. To overcome this difficulty, a double mutant was designed in which the catalytic histidine was substituted by a glycine residue in the single mutant F → W described above. This H → G/F → W double mutant carries a very small NTPase activity, but has no phosphotransferase activity (Schneider *et al.*, 2000). Upon excitation at 310 nm the intrinsic fluorescence at 330 nm of the H → G/F → W mutant is enhanced by 50% (Fig. 5, inset) in presence of saturating concentration of d4T-TP. The dissociation constant at equilibrium (K_D) was calculated from the binding curve obtained by titrating the change in fluorescence at increasing concentrations of nucleoside analog (Fig. 5). Most nucleoside triphosphate analogs bind with a lower affinity compared to their natural counterparts. For example, the K_D for AZT-TP is 30 μM compared to 1 μM for dTTP. Similarly, ddNTP bind with an affinity 5 to 10 times weaker than the corresponding NTP. However, an important exception is d4T-TP, a dTTP analog that binds with a K_D similar to dTTP (Schneider *et al.*, 2000). It seems that the active site accommodates the planar configuration of the d4T ribose moiety as well as natural nucleotides. In this respect, it is particularly interesting to note that among the antiviral nucleoside analogs that we have tested, d4T-TP binds to the NDP kinase active site with the best affinity and is phosphorylated by the enzyme at the highest rate. Preliminary structural results with this analog (Meyer *et al.*, 2000, personal communication) indicate that the planar conformation of the ribose moiety in d4T could

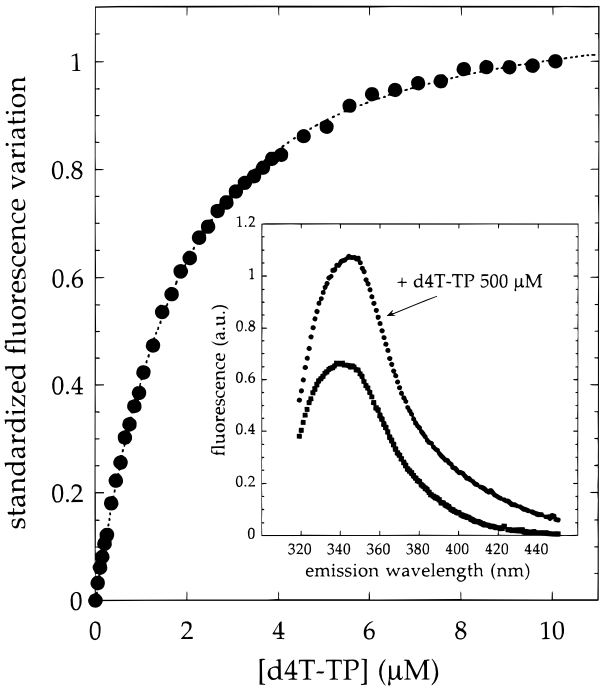

Fig. 5. Binding of d4T-TP to F → W/H → G NDP kinase. Fluorescence change of F→W/H→G NDP kinase (1 μM) upon binding of d4T-TP in buffer T (Tris–HCl 50 mM, pH 7.5, $MgCl_2$ 5 mM, KCl 75 mM), λ_{exc} = 310 nm and λ_{em} = 330 nm. The K_d value for d4T-TP is 1.2 +/− 0.1 μM. Inset: Fluorescence emission spectra of F→ W/H→G NDP kinase (1 μM) in the absence and presence (dotted line) of 0.5 mM d4T-TP.

authorize a unique nucleotide conformation stabilized by an unusual internal H-bond between the $C_3'H \cdots O_7$ instead of OH ⋯ H with the natural compound.

WHY ARE NRTI POOR SUBSTRATES FOR NDP KINASE?

We have shown that the absence or the substitution of the 3′-OH in a nucleoside impairs both binding of the nucleotide (2–30 fold higher dissociation constant) and catalysis (1000–50,000-fold decrease in catalytic efficiency). The crystal structure of NDP kinase complexed with several natural substrates provides an explanation for most of the properties described above. Indeed, the 3′-OH of the nucleotide sugar is involved in a network of hydrogen bonds with residues Asn115 and Lys12 of the active site, as well as with the oxygen bridging the β- and γ-phosphates of the nucleotide (Cherfils *et al.*, 1994; Morera *et al.*, 1994, 1995b; Xu *et al.*, 1997a; Janin *et al.*, 2000). Point mutations of Asn115 have little effect on the catalytic efficiency with natural nucleotides (Xu *et al.*, 1997b) and the change of Lys12 to Ala results in a residual activity of 1% (Schneider and Deville-Bonne, unpublished results, 2000). Therefore, the loss of the internal hydrogen bond between the 3′-OH and the bridging phosphate oxygen in ddNTP and AZT-TP appears to be the major reason for the low activity of the enzyme with these analogs (Schneider *et al.*, 1998). The structure of the complex of a point mutant Asn115 → Ala of *Dictyostelium* NDP kinase with AZT-DP provides additional evidence supporting this hypothesis (Xu *et al.*, 1997b). In this complex AZT-DP binds at the same site and in the same orientation as dTDP the natural substrate, with no change in the conformation of the phosphate chain. However, the sugar conformation is different since deoxyribose is C3′-*endo* in dTDP and C2′-*endo* in AZT-DP. The 3′ azido group moves away from the site normally occupied by the hydroxyl where it would collide with protein chain atoms. The mobile ε-amino group of Lys12 is strongly displaced by 2.6 Å, whereas the other active site residues are essentially undisturbed. The movement of Lys12 has an important functional consequence, for the ε-amino group can no longer interact with the γ-phosphate of the donor substrate during the transfer to the active site histidine (Xu *et al.*, 1997a). In case of d4T on the contrary, there is no steric hindrance at the 3′ position (Fig. 1) and the lysine likely stays in place, resulting in a better phosphorylation efficiency. It is possible that the conformation of d4T might play an important role in the formation of the transition state during catalysis, as proposed for DNA polymerases (Krayevsky and Watanabe, 1998).

CONCLUSION

A better understanding of the mechanism of nucleoside analogs phosphorylation by NDP kinase may be of particular interest for the design of improved nucleoside analogs that could be more efficient in anti-HIV therapies. Indeed, one of the main difficulties of the NRTI approach is that the requirement for a substitution of the 3′-OH position of the sugar leads to a paradox. On one hand, a NRTI must lack the 3′-OH group of the ribose moiety to be a chain terminator and block DNA elongation by reverse transcriptase (Huang *et al.*, 1998). On the other hand, we have shown that this same modification considerably decreases its ability to be phosphorylated as a triphosphate derivative by NDP kinase because of the absence of a crucial intramolecular H-bond between the 3′ position of the sugar and the oxygen bridging the β- and γ-phosphates. A compound that would conciliate these two requirements should be an excellent candidate for therapeutic use. To some extent, this is already the case of d4T: although it is missing a 3′-OH, the double bond on the sugar moiety of d4T-DP could orient the 3′ hydrogen in a proper direction toward the O_7. In the case of ddNDP, the low reactivity of these compounds may be related to the tetrahedral conformation of C3′ preventing the formation of this H-bond (Schneider *et al.*, 2000). Testing this hypothesis may await structural data. For unknown reasons, attempts to obtain cocrystals of NDP kinase with several ddNDP have, thus far, remained unsuccessful (Janin, personal communication, 2000).

Another important element to consider in using a nucleoside analog as a therapeutic agent is its propension to elicit resistances in the target reverse transcriptase (RT). Indeed, the accumulation of mutations in RT, leading to a decreased sensitivity to NRTI, is a major drawback in their use in therapies. The resistance of HIV-RT to several anti-HIV drugs is mainly due to the unstability of the incorporated analog. The anti-HIV analog monophosphate could be removed from DNA, allowing further viral DNA elongation: it was proposed recently that the cleavage of the phosphodiester bond between the 5′-phosphoryl of the incorporated analog-MP and the adjacent 3′-OH could

be due to a nucleophilic attack by pyrophosphate or ATP (Arion *et al.*, 1998; Meyer *et al.*, 1999). It is tempting to propose that a modification of the phosphate involved in the phosphodiester bond, for example, by substituting one oxygen with a borano (BH_3^-) or a thio group (Eckstein, 1985; He *et al.*, 1998), could decrease the sensitivity to pyrophorolysis and result in a better inhibition of RT. Indeed, oligonucleotides with a boranophosphate internucleotide linkage have been shown to be quite stable toward cleavage by exonucleases (Li *et al.*, 1995; Porter *et al.*, 1997). A α-borano substituted triphosphate nucleoside analog could, therefore, increase its stability toward excision mechanisms. Preliminary results indicate that such a modification increases the analog phosphorylation rate by the NDP kinase since the proSp oxygen of the α-phosphate does not interact either with any protein residue or with Mg^{2+} (Meyer *et al.*, 2000).

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