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NDP kinase reactivity towards 3TC nucleotides

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Abstract

Nucleoside diphosphate (NDP) kinase is usually considered as the enzyme responsible for the last step of the cellular phosphorylation pathway leading to the synthesis of biologically active triphospho-derivatives of nucleoside analogs used in antiviral therapies and in particular in the treatment of AIDS. NDP kinase lacks specificity for the nucleobase and can use as substrate both ribo- or 2′-deoxyribonucleotides. However, only nucleoside analogs with a sugar moiety in the D-configuration (e.g. 3′-deoxy-3′-azidothymidine (AZT), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T)) have so far been analyzed as substrates of NDP kinase. In contrast, β-L-2′,3′-dideoxy-3′-thiacytidine (3TC), also called lamivudine, is a nucleoside analog that is now widely used in AIDS therapy and has a sugar moiety in the L-configuration. Using protein fluorescence to monitor the phosphotransfer between the enzyme and the nucleotide derivative at the presteady state, we have studied the reactivity of 3TC triphosphate and of other L-dideoxynucleotides with NDP kinase. We found that L-dideoxynucleoside triphosphates have a poor affinity for NDP kinase and that the catalytic efficiency of the phosphorylation of L-dideoxyderivatives is very low as compared with their D-enantiomers. We discuss these results using a computer model of 3TC diphosphate bound to the NDP kinase active site. NDP kinase may not seem to be the major enzyme phosphorylating 3TC-DP, in contrast to current opinion.

Keywords: Antiviral analog; 3TC, lamivudine; NDP kinase, *nm*-23

Abbreviations: AZT, 3′-deoxy-3′-azidothymidine; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; 3TC, β-L-2′,3′-dideoxy-3′-thiacytidine; dCK, deoxycytidine kinase; dCMPK, deoxycytidylate kinase; ddCTP, 2′,3′-dideoxycytidine triphosphate; ddN, 2′,3′-dideoxynucleoside; Dd-NDPK, *Dictyostelium discoideum* nucleoside diphosphate kinase; NDP, nucleoside diphosphate; NDPK-A, human nucleoside diphosphate kinase type A; NTP, nucleoside triphosphate; HIV, human immunodeficiency virus.

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1. Introduction

2′,3′-dideoxynucleoside (ddN) analogs are extensively used as antiviral drugs, especially in anti-AIDS therapies. ddNs require phosphorylation by intracellular enzymes into their corresponding triphosphates to become active and reach their target, the viral reverse transcriptase. All the nucleoside analogs used in antiviral treatments have the natural β-D-configuration with the exception of β-L-2′,3′-dideoxy-3′-thiacytidine (3TC, lamivudine) which is licensed for use in human immunodeficiency virus (HIV) and hepatitis B virus (HBV) infections. The pharmacokinetics of 3TC phosphorylation in peripheral blood mononuclear cells from patients infected with HIV-1 have been thoroughly studied (Cammack et al., 1992; Kewn et al., 1997; Robbins et al., 1998; Solas et al., 1998; Moore et al., 1999). 3TC is well anabolized to 3TC-TP (several pmol per 10^6 cells) with an intracellular half-life of about 10–15 h. In mammalian cells, the activation pathway for 3TC presumably involves the salvage pathway enzymes for deoxycytidine, i.e. deoxycytidine kinase (dCK), UMP-CMP kinase and nucleoside diphosphate (NDP) kinase. One of the cellular kinases, the cytosolic dCK has been shown to phosphorylate several L-nucleosides that are efficient antiviral agents, including 3TC (Shewach et al., 1993; Van Draanen et al., 1992). The enantioselectivity of UMP-CMP kinase has been extensively studied and found to be low with all substrates (Wang et al., 1999; Maury, 2000). Using FTC-MP, the 5-fluoro analog of 3TC-MP, human UMP-CMP kinase has been proposed to be the enzyme responsible for the phosphorylation of 3TC-MP in cells (Furman et al., 1995). The human UMP-CMP kinase has been recently cloned, but its enzymatic parameters with 3TC-MP have not yet been measured (Van Rompay et al., 1999). The enzyme responsible for the phosphorylation of 3TC-DP into 3TC-TP has not been identified, but NDP kinase is usually proposed as the most likely candidate.

NDP kinase catalyzes the γ-phosphotransfer between nucleosides di- and triphosphates (Parks and Agarwal, 1973). NDP kinase, which is expressed in relatively high amounts in cells, presents a broad specificity for nucleotides and a high turnover number for natural nucleotides ($k_{cat} = 1000\ s^{-1}$). Therefore, NDP kinase is often considered as a non rate-limiting step in the phosphorylation of antiviral agents. However, we showed that AZT-DP, d4T-DP and dideoxynucleosides-DPs are poor substrates of NDP kinase, due to the absence of the 3′OH of the sugar (Bourdais et al., 1996; Schneider et al., 1998, 2000). Concerning 3TC phosphorylation, 3TC-DP was found to be the predominant anabolite in blood cells from patients infected with HIV-1 and treated with 3TC, indicating that the conversion of 3TC-DP into 3TC-TP can be regarded as the rate-limiting step (Moore et al., 1999).

The reaction catalyzed by NDP kinase involves the formation of a phosphohistidine intermediate (Parks and Agarwal, 1973) according to:

$$E + NTP \rightleftarrows E - NTP \rightleftarrows E \sim P - NDP \rightleftarrows E \sim P + NDP \quad \text{(Scheme 1)}$$

The crystal structures of NDP kinases from *Dictyostelium*, *Drosophila* and human, either free or complexed with a NDP demonstrate that eukaryotic enzymes are homohexamers made of trimers stacked in a staggered arrangement (Dumas et al., 1992; Chiadmi et al., 1993; Cherfils et al., 1994; Morera et al., 1995; Webb et al., 1995). The overall fold of the 17 kDa subunit and in particular the binding site for the nucleotide are highly conserved. Up to five isoforms of the human NDP kinase were found, with human nucleoside diphosphate kinase type A (NDPK-A) and human nucleoside diphosphate kinase type B (NDPK-B) being the major cytosolic species (88% identity). NDP kinases from the lower eukaryote *Dictyostelium discoideum* (Dd-NDPK) and from human origin (human NDP kinase type A) share 60% sequence identity (58% in the case of human NDP kinase type B) and present remarkably similar kinetic parameters (Gonin et al., 1999). Dd-NDPK is, therefore, a reliable model for eukaryotic NDP kinase. Here, we examined the phosphorylation of 3TC-DP by NDP kinase and compared the reactivity of the enzyme towards several D- and L-ddNTP analogs.

2. Materials and methods

2.1. Synthesis of nucleotides and purification of recombinant NDP kinase

β-L-3TC was extracted from Epivir® tablets (GlaxoWellcome) with methanol. After purification by silica gel column chromatography (dichloromethane/methanol 95/5), the nucleoside was phosphorylated into the 5′-monophosphate by overnight incubation at 20°C with GTP in the presence of dCK from *Bacillus subtilis* (a kind gift of Dr A.M. Gilles and Dr O. Barzu). 3TC-MP was then phosphorylated to the 5′-triphosphate according to the procedure used by Bourdais for AZT (Bourdais et al., 1996). The L-derivatives of d4T-TP, AZT-TP, ddATP and 2′,3′-dideoxycytidine triphosphate (ddCTP) were synthesized as described in (Faraj et al., 1994). All compounds were characterized by nuclear magnetic resonance (^{1}H, ^{31}P) spectroscopy and mass spectroscopy.

The NDPK-A, the wild type NDP kinase from *Dictyostelium discoideum* and its double mutant F64W-H122G were overexpressed in *E.coli* and purified as earlier described (Schneider et al., 1998, 2000).

2.2. Kinetic measurements of NDP kinase activity

The phosphorylation of 3TC-DP catalyzed by NDP kinase under steady state conditions was followed by measuring the formation of [γ-^{32}P]-3TC-TP in the presence of 1 mM [γ-^{32}P]-GTP (100 Ci per mmol) and various amounts of 3TC-DP (0.1–0.5 mM). The protocol was similar to that described in (Bourdais et al., 1996) except for the stop solution which was a mix of 20 mM GTP and 20 mM EDTA. The products of the reaction were separated on TLC plates and counted in a PhosphorImager. As the reaction was monitored during several hours in the presence of a high concentration of enzyme, a control was made with the enzyme and [γ-^{32}P]-GTP in the absence of 3TC-DP. The light signal (less than 1%) seen at the level of the 3TC-TP spot was used as background for each incubation time.

2.3. Binding studies

The affinity of nucleoside triphosphate (NTP) derivatives for NDP kinase was determined by following the variation of the intrinsic fluorescence of the NDP kinase double mutant F64W-H122G upon nucleotide binding as described (Schneider et al., 1998). The fluorescence was measured at 330 nm with excitation at 310 nm (2 nm excitation slit and 4 nm emission slit). Successive aliquots of the nucleotide were added to a 1 μM enzyme solution (in subunits) in buffer T1 (50 mM Tris–HCl, pH 7.5, 75 mM KCl and 5 mM $MgCl_2$) at 20°C. The inner filter effect was negligible. Experimental binding curves were fitted to quadratic equation for ligand–protein curve after correction for dilution. They allowed the determination of the dissociation constant K_D. When NTPs were used, the true NTP concentration was measured in order to take into account the small residual ATPase activity. The correction was less than 2%.

2.4. Presteady state kinetic experiments

Stopped-flow kinetic experiments were performed with an Hi-Tech DX2 microvolume stopped-flow reaction analyzer equipped with a high intensity xenon lamp as described (Schneider et al., 1998). The excitation wavelength was 304 nm, with a 2 mm excitation slit and a 320 nm cutoff filter at the emission. Mixing was achieved in less than 2 ms. After mixing NDP kinase (1 μM) and NTP (10–500 μM) in buffer T1, the decrease of the intrinsic protein fluorescence was monitored for 10–1000 s. In each experiment 400 pairs of data were recorded, and the data from three to four identical experiments were averaged and fitted to a number of non-linear analytical equations using the software provided by Hi-Tech. All curves fitted single exponentials. In some time-course experiments a Photon Technology International (PTI) spectrofluorometer was used. The data were analyzed using the reaction scheme earlier described:

$$E + NTP \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} E \cdot NTP \underset{k_{-2}}{\overset{k_{+2}}{\leftrightarrow}} E \sim P \cdot NDP \underset{k_{-3}}{\overset{k_{+3}}{\leftrightarrow}} E \sim P + NDP \quad \text{(Reaction 1)}$$

where NTP and NDP referred to any natural nucleotides or antiviral tri- and di-phosphate derivatives, respectively. The observed single step could be attributed to the phosphotransfer between the nucleotide and the enzyme in both directions. In both the forward and the reverse reaction, the product concentration remained very low, and the contribution of the product binding could be neglected. The binding steps in both directions were fast and not detectable in the time range of the stopped-flow experiments. The observed rate of phosphorylation k_{obs} increased with [ddNTP] to reach a plateau value equal to k_{+2}, the true rate of histidine phosphorylation (Eq. (1)). The half-saturation occurred for [ddNTP] = $k_{-1}/k_{+1} = K_D$, the equilibrium constant of the nucleotide substrate evaluated by fast kinetic experiments. At low nucleotide concentration, k_{obs} increased linearly with [ddNTP] with an apparent bimolecular rate constant k_{+2}/K_D.

$$k_{obs} = \frac{k_{+2} \cdot [\text{ddNTP}]}{K_D + [\text{ddNTP}]} \quad (1)$$

Saturation could not be obtained with the concentrations of analogs used here. Therefore, we measured the catalytic efficiency of the enzyme phosphorylation (CE_{phos}), equivalent to a second order constant, allowing a reliable comparison of a variety of NDP kinase substrates.

2.5. Computer modeling

Molecular modeling of the enzyme complex was carried out on a Silicon Graphics O2 workstation using INSIGHT II 98 software (Biosym/MSI). In our model, the analog β-L-3TC-DP was built using the Biopolymer program. We used 1NDC (TDP bound to Dd-NDP kinase) (Cherfils et al., 1994) rather than 1NUE (GDP bound to human NDP kinase) (Morera et al., 1994) as a starting structure, as TDP has a pyrimidine base as 3TC and no structural data were available for a NDP kinase complexed with a cytosine derivative. Note that the active site residues in all known NDP kinase structures are totally conserved. The refinement procedure was simple energy minimization using the Discover 3 program.

3. Results and discussion

3.1. Phosphorylation of 3TC-DP by NDP kinase at the steady state

The rate of phosphoryl transfer catalyzed by human NDPK-A on β-L-3TC-DP was measured using the classical enzymatic assay as described in the Section 2. A high concentration of enzyme (18 μM subunits) and long incubation times (several hours) were required to detect the appearance of [γ-^{32}P]-3TC-TP from 3TC-DP and [γ-^{32}P]-GTP (Fig. 1). As shown in Fig. 1, inset, the reaction was measured under initial rate conditions. The

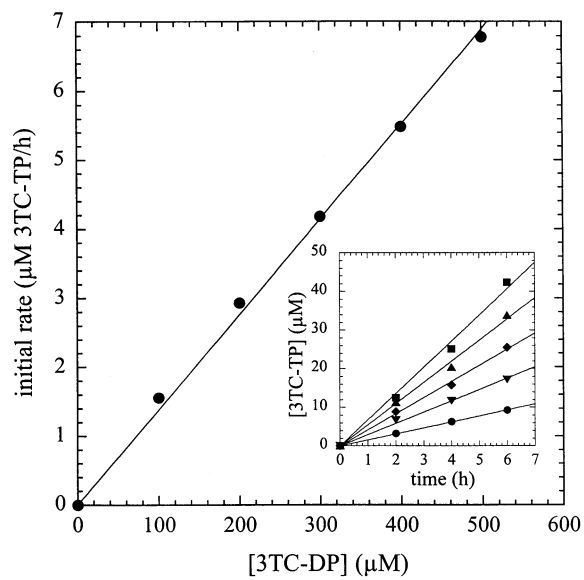

Fig. 1. Steady state kinetic measurements of NDP kinase activity with β-L-3TC-DP. The initial rate of 3TC-TP formation was determined in the presence of 1 mM [γ-^{32}P]-GTP from the slope of the curves shown in inset. The specificity constant (k_{cat}/K_M) was calculated from this plot with a high enzyme concentration of 18 μM. Inset, time course of [γ-^{32}P]-3TC-TP formation from [γ-^{32}P]-GTP in the presence of 0.1 mM (●), 0.2 mM (▼), 0.3 mM (◆), 0.4 mM (▲), or 0.5 mM (■) 3TC-DP.

reaction velocity was found to vary linearly with the concentration of the substrate 3TC-DP, with no evidence for saturation below 0.5 mM 3TC-DP, and was found to be very low. The rate of spontaneous GTP hydrolysis was found in a similar range (not shown). NDP kinase presents a weak specificity constant for 3TC-DP, $k_{cat}/K_M = 0.22\ M^{-1}\ s^{-1}$. Indeed, NDP kinase presents a high catalytic efficiency for CDP $k_{cat}/K_M = 5 \times 10^5\ M^{-1}\ s^{-1}$. The ability of 3TC-DP to be phosphorylated by NDPK-A is at least one million times lower than that of the natural nucleotide. Likewise, ddCTP is also known to be a weak substrate for NDP kinase, the weakest among the dideoxynucleotides (Schneider et al., 1998). The other major human isoform of NDP kinase, NDPK-B, and the mitochondrial form H4 (Milon et al., 2000) also had a very low activity with 3TC-DP as a substrate (not shown).

3.2. Phosphoryl transfer between antiviral analogs and NDP kinase at the presteady state

The rate of phosphotransfer between the enzyme and a nucleotide analog was also analyzed by stopped flow experiments. As the intrinsic enzyme fluorescence was found sensitive to the phosphorylation state of the catalytic histidine, the fluorescence signal is related to the phosphoryl transfer either from the phosphoenzyme to a NDP acceptor, or from a NTP donor to the enzyme, i.e. the two half reactions of Scheme 1 (Deville-Bonne et al., 1996). The rate of the phosphoryl transfer from the enzyme to the NDP is always five times faster than the rate of formation of the phosphointermediate, indicating that NDP kinase is working close to the thermodynamic equilibrium (Garces and Cleland, 1969; Parks and Agarwal, 1973). The equilibrium constant of the enzyme with antiviral dideoxyderivatives was earlier reported to be the same as with natural nucleotides (Schneider et al., 1998). Therefore, the reaction can be monitored in either direction as both rates (forward and reverse) are affected similarly. When a fast mixing experiment was performed with NDPK-A (1 μM in subunits, final concentration) and β-D-ddCTP or β-L-3TC-TP (300 μM, final concentration), the phosphotransfer rate was very low and the catalytic efficiency of phosphotransfer could be estimated to be less than $10\ M^{-1}\ s^{-1}$ (Table 1).

Table 1
Pre-steady state reaction of NDPK-A with D- and L-nucleotides[a]

Phosphotransfer from D- or L-XTP to NDPK A k_{+2}/K_S ($M^{-1}\ s^{-1}$)		
	D-XTP	L-XTP
DTTP	10^{6}[b]	n.d.
d4T-TP	750 ± 100[b]	<10
AZT-TP	75 ± 5[b]	<10
DdATP	65 ± 5	<10
DdCTP	<10	<10
3TC-TP	–	<10

[a] All experiments were performed at 21°C using a stopped flow as described in Fig. 2. n.d., not determined. Values <10 $M^{-1}\ s^{-1}$ are not significant.

[b] The activity of the enzyme with the D-isomers of these analogs has been determined earlier (Schneider et al., 1998, 2000).

Given this very low activity of NDPK-A with 3TC-DP, it was interesting to compare the activity of NDP kinase for the D- and the L-isomers of a nucleotide analog. Since, unfortunately the D-isomer of 3TC was not available, we analyzed the time-course of the reaction of NDPK-A with the L-enantiomers of several other antiviral analogs, i.e. AZT-TP, ddATP, ddCTP and d4T-TP (Fig. 2). As shown in Table 1, d4T-TP was found the best phosphodonor, AZT-TP and ddATP had a similar intermediate catalytic efficiency while ddCTP was a very poor substrate. For natural nucleotides, CTP was also less reactive than GTP by a factor of ten (Schaertl et al., 1998), no structural explanation was available in absence of structural data for complexes of the enzyme with C derivatives. All L-enantiomers of the dideoxynucleotides tested were very poor substrates of NDPK-A, with a rate constant, k_{obs}, below the limit of detection of the assay ($k_2/K_D < 10\ M^{-1}\ s^{-1}$) (Table 1).

The specific activity of NDP kinase from *Dictyostelium* has been reported to be about ten times higher than that of human NDPK-A (Gonin et al., 1999). When the rate of phosphotransfer of

3TC-TP was measured using Dd-NDPK, it was detectable (Fig. 3a). For all concentrations of 3TC-TP, a single exponential decay was observed, characterized by the first order rate constant k_{obs}. This constant k_{obs} is expected to vary according to the amount of enzyme–substrate complex and to reach a maximum value in the presence of excess substrate (Eq. (1)). For 3TC-TP, saturation was not reached but k_{obs} could be extrapolated to a maximum rate of phosphotransfer $k_{cat} = k_{+2} = 0.014\ s^{-1}$. This value is extremely low, when compared with k_{cat}^{ATP} (1000 s^{-1}) and even to $k_{cat}^{AZT\text{-}TP}$ (0.7 s^{-1}) (Bourdais et al., 1996). Similar monoexponential decays were found for the natural D-isomers of ddCTP. The catalytic efficiency of Dd-NDPK for D-ddCTP and L-3TC-TP was 35 and 10 $M^{-1}\ s^{-1}$, respectively (Fig. 3b). We conclude that the catalytic activity of NDP kinase (human or Dd) is strongly dependent on the configuration of the sugar moiety of the dideoxynucleotide with a high preference for D-enantiomers.

3.3. Affinity of 3TC-TP for NDP kinase

The binding affinity of nucleosides triphosphates to NDP kinase, in the absence of catalysis, was investigated using the fluorescence titration of the double mutant (F64W-H122G in Dd-NDPK) lacking the catalytic histidine and in which a tryptophan replaces the phenylalanine stacking to the nucleobase (Schneider et al., 2000). It can be noted that D-ddCTP presented a weak affinity ($K_D = 150\ \mu M$) while D-CTP bound to NDP kinase with $K_D = 7\ \mu M$. The affinity for L-analogs (3TC-TP and d4T-TP) was measured by a competition assay, the binding of CTP to the mutant enzyme was followed in the presence of various β-L-3TC-TP concentrations, as 3TC-TP alone induced no signal (Fig. 4). The plot of the $^{app}K_D$ for CTP against 3TC-TP concentration indicated a K_D value for β-L-3TC-TP of 1.5 mM (Fig. 4, inset). The competition assay gave a K_D for L-β-d4T-TP of 220 μM, i.e. more than 200 fold higher than the value earlier found for β-D-d4T-TP and for D-dTTP (Schneider et al., 2000). These results

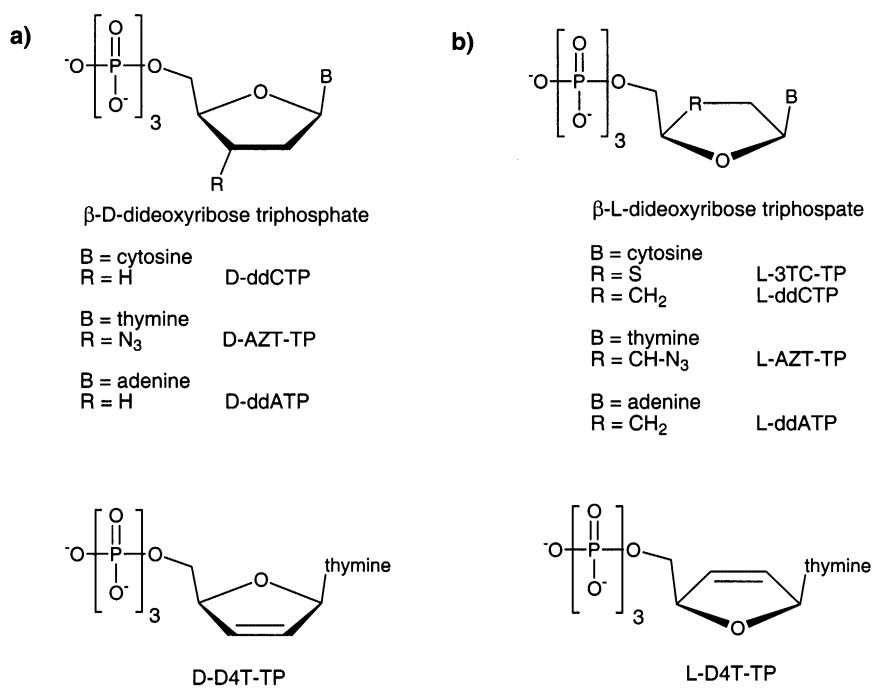

Fig. 2. Structure of (a) β-D- and (b) β-L-dideoxynucleotide analogs.

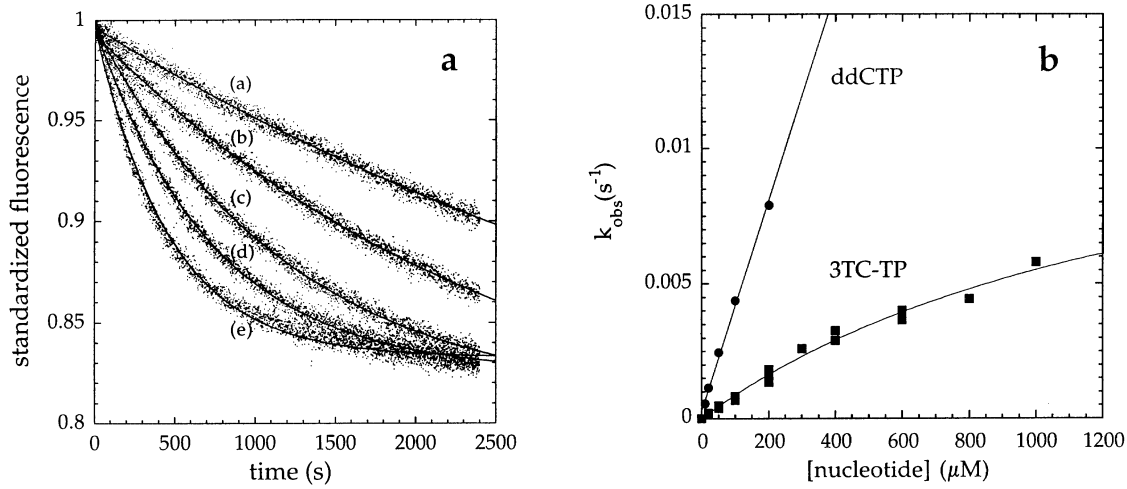

Fig. 3. Presteady state kinetics of NDP kinase phosphorylation by β-L-3TC-TP. (A) 1 μM of Dd-NDPK was rapidly mixed with 20 – 400 μM L-β-3TC-TP and the decrease of fluorescence was monitored in a time scale up to 40 min. Representative traces are shown for 20 (a), 50 (b), 100 (c), 200 (d) and 400 (e) μM β-L-3TC-TP. The solid line represents the best fit of each curve to a monoexponential. (B) Concentration dependence of the pseudo-first order rate constant (k_{obs}) for phosphorylation versus [β-L-3TC-TP] (■) and [β-D-ddCTP] (●). The linear fit indicates a second order reaction, the slope represents the catalytic efficiency.

support the conclusion that the configuration of the sugar moiety is critical for the correct binding of the nucleotide in the active site of the enzyme.

To understand the effect of the sugar configuration on catalysis, 3TC-DP was built with the phosphate chain having the same conformation as dTDP in the structure of its complex with Dd-NDPK (PDB code1NDC) and docked into the NDP kinase active site. We assumed that, as for natural nucleotides, the phosphate chain was oriented towards the catalytic His and the nucleobase positioned at the surface of the active site. After minimization of the complex between Dd-NDPK and β-L-3TC-DP, we observed that the position of the cytidine was significantly displaced preventing a proper stacking with Phe 64 (Fig. 5). This could explain why no fluorescence modification was observed in binding experiments performed with H122G-F64W NDP kinase. The α- and β-phosphates did not fit closely in the active site and adopted a different conformation from that of a natural nucleotide. This resulted in a predicted movement of Arg 92 side chain, while other surrounding amino acids were only slightly displaced. Arg 92 is known to stabilize the phosphate during the transition state of the reaction (Xu et al., 1997). Moreover, in NDP kinase bearing the mutant R92A, only 1% of activity was found showing that Arg 92 is indeed important for catalysis (Tepper et al., 1994). The NDP kinase active site has been compared with a preformed rigid template for the D-nucleotides (Cherfils et al., 1994). L-3TC-DP can only be accomodated upon a shift of Arg-92, explaining the weak binding and the low reactivity of the L-derivatives with NDP kinase.

This study showed that NDP kinase presents a strong preference for D-antiviral nucleotides. The enzyme bound L-enantiomers of 3TC-TP and d4T-TP very weakly and phosphoryl transfer was extremely slow. There was a factor of 200 between the binding constants of D-d4T-TP and the corresponding L compound. NDP kinase from beef liver was earlier reported to phosphorylate D-carbovir diphosphate (a guanosine analog) 200 times faster than the L-derivative (Miller et al., 1992). The stereospecificity of NDP kinase is in contrast with that of dCK, the first enzyme of the pathway. dCK, like other deoxynucleoside kinases, TK2 and dGK, were shown to present a relaxed enantioselectivity and to phosphorylate D and L derivatives at similar velocities (Maury, 2000).

The herpes simplex virus type 1 thymidine kinase and several other viral enzymes (e.g. HIV-1 reverse transcriptase, hepatitis B virus DNA polymerase) even recognize the β-L enantiomers better than the D-enantiomers of analogs (Faraj et al., 1994; Wang et al., 1998; Spadari et al., 1998).

The present study quantified the catalytic efficiency of NDPK-A ($k_{cat}/K_M = 0.22\ M^{-1}\ s^{-1}$) and the dissociation constant ($K_D = 1.5$ mM) for 3TC-DP. As K_D can be approximated to an estimation of the K_M value for 3TC-DP, we calculate that the maximum rate of phosphorylation of 3TC-DP by NDPK-A proceeds with a k_{cat} of 1.2 h^{-1}. The analysis of the content in 3TC derivatives in blood mononuclear cell extracts showed an intracellular 3TC-DP concentration less than 10 μM (10 pmol per 10^6 cells) (Cammack et al., 1992; Robbins et al., 1998; Solas et al., 1998; Moore et al., 1999). The amount of 3TC-TP formed was estimated at a mean value of 2 pmoles per 10^6 cells per h, which, given a cell volume of one picoliter, should correspond to a rate of 2 μM h^{-1}. The intracellular production of 3TC-TP at such a rate (2 μM h^{-1}) by NDP kinase with a catalytic efficiency of 0.22 $M^{-1}\ s^{-1}$ could be achieved if the kinase amounts to 25 μM i.e. 0.4 mg ml^{-1}, which appeared highly improbable. It is unlikely that NDP kinase (A, B or H4) would be the enzyme catalyzing the formation of 3TC-TP in cells. This raises the likelihood that other phosphoryl transferases should be responsible for the phosphorylation in vivo of β-L-3TC. Newly discovered isoforms like NDP kinase H5 which has no enzymatic activity with D-nucleotides (Munier et al., 1998) could be active with L-derivatives, although this seems improbable as similar kinetic parameters were observed for several human NDP kinases with thymidine derivatives (Gonin et al., 1999). It has been shown that L-carbovir is phosphorylated in vitro at a low rate to the triphosphate derivative by several enzymes able to react with GTP, including pyruvate kinase, phosphoglycerate kinase, phospho-*enol*pyruvate carboxykinase and creatine kinase (Miller et al., 1992). Experiments are currently being designed in the laboratory to identify human enzymes in-

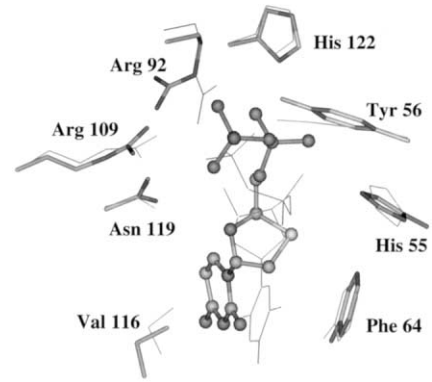

Fig. 5. Superposition of 3TC-DP and dTDP into NDP kinase active site. The computer modeling is performed on a Silicon Graphics workstation using the Insight II 98 software. The X-ray crystal structure of Dd-NDPK complexed with dTDP (pdb 1NDC, solid thin lines) is used. β-L-3TC-DP is presented in ball and sticks, and the amino acid residues interacting with the nucleotide are in thick lines. Note the shift by 1.8 Å of the guanidinium group of Arg 92.

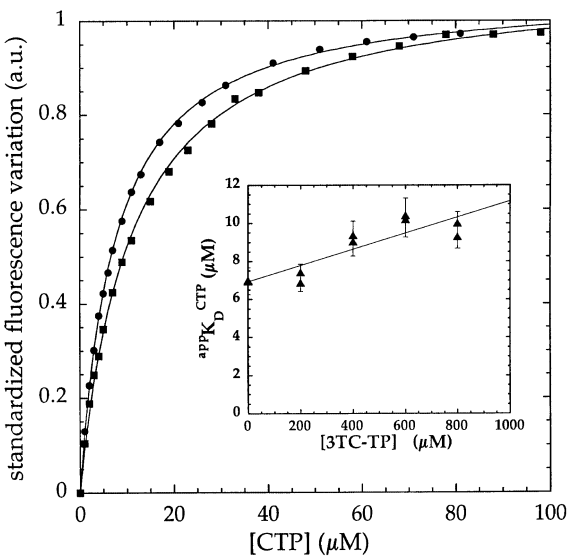

Fig. 4. Binding affinity of β-L-3TC-TP for NDP kinase. Fluorescence titration curve of F64W-H122G mutant NDP kinase (1 μM) by CTP in buffer T1 at 20°C in the absence (●) or in the presence of 800 μM 3TC-TP (■). Solid lines represent the best fit of the data to a quadratic saturation curve. They give the values of the apparent K_D of CTP in the presence of various concentrations of 3TC-TP. Inset: the apparent K_D for CTP are plotted against [β-L-3TC-TP]. The true K_D for 3TC-TP is determined from the slope ($K_D^{CTP}/K_D^{3TC\text{-}TP}$) and the intercept with the ordinate axis (K_D^{CTP}). The found value is $K_D^{3TC\text{-}TP} = 1.5 \pm 0.2$ mM.

volved in the activation of antiviral L-nucleoside analogs into their triphosphate derivatives.

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