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Broad specificity of human phosphoglycerate kinase for antiviral nucleoside analogs

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Abstract

Nucleoside analogs used in antiviral therapies need to be phosphorylated to their tri-phospho counterparts in order to be active on their cellular target. Human phosphoglycerate kinase (hPGK) was recently reported to participate in the last step of phosphorylation of cytidine L-nucleotide derivatives [Krishnan PGE, Lam W, Dutschman GE, Grill SP, Cheng YC. Novel role of 3-phosphoglycerate kinase, a glycolytic enzyme, in the activation of L-nucleoside analogs, a new class of anticancer and antiviral agents. J Biol Chem 2003;278:36726–32]. In the present work, we extended the enzymatic study of human PGK specificity to purine and pyrimidine nucleotide derivatives in both D- and L-configuration. Human PGK demonstrated catalytic efficiencies in the 10^4–10^5 M^{-1} s^{-1} range for purine ribo-, deoxyribo- and dideoxyribonucleotide derivatives, either in D- or L-configuration. In contrast, it was poorly active with natural pyrimidine D-nucleotides (less than 10^3 M^{-1} s^{-1}). Pyrimidine L-enantiomers, which are promising therapeutic analogs against B hepatitis, were 2–25 times better substrates than their D-counterparts. The broad specificity of substrate of human PGK suggests that this enzyme may be involved in the cellular activation of several antiviral nucleoside analogs including dideoxyinosine, acyclovir, L-2′-deoxycytosine and L-2′-deoxythymidine.

Keywords: Phosphoglycerate kinase; NDP kinase; Antiviral analog; Acyclovir; L-dC; L-dT

1. Introduction

Antiviral and anticancer nucleoside analogs require activation to triphosphate derivatives within cells to inhibit DNA elongation and to exert their biological effects. In mammalian cells, four nucleoside kinases and four nucleoside monophosphate (NMP) kinases from the salvage pathway with partially overlapping specificities for the nucleobase, catalyse the addition of α- and β-phosphates to nucleosides and nucleoside monophosphates, respectively.

Nucleoside diphosphate (NDP) kinase catalyses the reversible transfer of phosphate between natural nucleoside di- and triphosphates, whether ribo- or deoxyribo-, with little base specificity [2]. While extremely high with natural nucleotides, the catalytic efficiency of NDP kinase decreases by a factor of 10^4 with AZT diphosphate and ddNDPs, as compared to dTDP or other NDPs [3,4]. This is due to the absence of the 3′hydroxyl group in antiviral chain terminating analogs which is crucial for NDP kinase activity. Indeed, kinetic and structural studies showed that this 3′-hydroxyl is involved in a key hydrogen bonding network with two residues of the active site, Lys 12 and Asn 115 in the human enzyme. In addition, it participates in an intra-nucleotide bond with the oxygen bridging the

Abbreviations: AZT, 3′-deoxy-3′-azidothymidine; d4T, 2′,3′-didehydro-2′,3′-dideoxythymidine; 3TC, β-L-2′,3′-dideoxy-3′-thiacytidine; L-FMAU, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; dNDP, 2′-deoxynucleoside diphosphate; dNTP, 2′-deoxynucleoside triphosphate; ddNDP, 2′,3′-dideoxynucleoside diphosphate; ddNTP, 2′,3′-dideoxynucleoside triphosphate; DTT, dithiothreitol; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NDPK-A, human nucleoside diphosphate kinase type A; hPGK, human phosphoglycerate kinase; HIV, human immunodeficiency virus; HBV, hepatitis B virus.

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β- and γ-phosphates which is essential for the catalytic efficiency of the enzyme[4–7].

β-L-nucleoside derivatives belong to a new generation of analogs with high antiviral efficiency and little cytotoxicity. In addition to β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) currently used in AIDS and B hepatitis therapies, a number of β-L-nucleoside analogs display anti-HIV and anti-HBV activities, including L-ddC, L-FddC [8,9], and 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU) [10]. It was recently shown that β-L-2′-deoxythymidine (L-dT, Telbivudine) and β-L-2′-deoxycytidine (L-dC) provided as the prodrug Val-LdC (Valtorcitabine), specifically inhibit HBV replication. These compounds are currently being tested clinically [11]. L-deoxynucleosides, as well as 3TC, are efficiently phosphorylated to triphosphate derivatives within the cell [12,13]. In addition, human degradation enzymes like cytidine deaminase and phosphatases are strictly specific for D-nucleotides and this probably contributes to the high cellular level of L-dCTP, L-dTTP and 3TCTP [11–14].

In this context, the study of the enantioselectivity of the enzymes involved in the metabolism of nucleoside analogs is of interest. The enzymes catalyzing the first step of phosphorylation of L-nucleoside analogs including human deoxycytidine kinase, thymidine kinase 2 and deoxyguanosine kinase show a relaxed enantioselectivity (reviewed in [14]). For the second step, UMP–CMP kinase is active with both D- and L-conformers and in particular with 3TC monophosphate [15–17]. In contrast, NDP kinase activity is low with L-carbovir-diphosphate [18] and 3TC-diphosphate [19], indicating that it may not account for the phosphorylation of L-nucleoside diphosphates within the cell [20]. This hypothesis has gained support by the recent report that human phosphoglycerate kinase (hPGK), a key enzyme in glycolysis, is able to phosphorylate L-FMAU diphosphate as well as L-CDP, L-dCDP, L-ddCDP and 3TCDP [20,21,1]. PGK is a typical two-domain kinase, catalysing the transfer of one phosphate from 1,3-biphosphoglycerate to ADP to produce ATP in the presence of magnesium ions. Each domain binds one of the two substrates and undergoes a conformational change, the direct phosphotranfer proceeding in the ternary complex only [22].

In this work, we extended the study of human PGK specificity to all natural, ribo- and deoxyribonucleotides in D-configuration, as well as for their L-enantiomers. The catalytic efficiency of hPGK for the D-dideoxynucleotides and for the antiviral analogs, AZT, d4T and acyclovir (Fig. 1), was also measured.

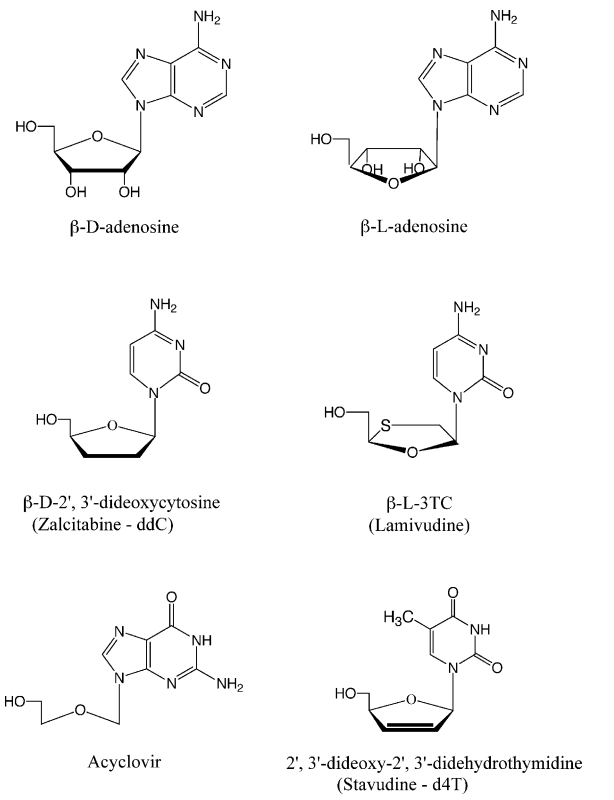

Fig. 1. Structure of β-D- and β-L-ribonucleosides and nucleoside analogs.

2. Material and methods

2.1. Materials

β-L-nucleoside 5′-triphosphate derivatives were prepared from their unprotected β-L-nucleoside parent as previously described [23]. Acyclovir triphosphate was synthesized as described [23]. All compounds were characterized by HPLC, nuclear magnetic resonance (^{1}H, ^{31}P), spectroscopy and mass spectroscopy. The purity of all nucleotides was >98%. Natural nucleotides were obtained from Sigma–Aldrich. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and ddNTP were purchased from Roche. 3TC-di- and triphosphate were synthesized by Biolog Life Science from 3TC extracted from Epivir tablets (Glaxo Smith Kline).

2.2. Cloning, expression and purification of human His-tagged phosphoglycerate kinase

The 1251 bp fragment corresponding to the gene coding for the human PGK was amplified by PCR [24] with Human Liver Marathon-Ready cDNA bank (Clontech) using two synthetic oligonucleotides: 5′-GGAATTC**CATATG**TCGCTTTCTAACAAGCTGACG-3′ and 5′-CGC**GGATCC**TTAAATATTGCTGAGAGCATCCACC-3′. During amplification, *Nde*I and *Bam*HI restriction sites (in bold letters in the oligonucleotide sequences) were created at both ends of the amplified fragment. After digestion by *Nde*I and *Bam*HI, the amplified gene was inserted into the pET28a plasmid (Novagen) digested with the same enzymes. The DNA insert was sequenced [25] to verify the absence of any mutational event in the course of amplification.

BL21(DE3)/pDIA17 *E. coli* cells transformed with the *pgk*-pET28a plasmid were grown in 2YT medium [24] containing 30 μg/mL chloramphenicol and 70 μg/mL kanamycin until E^{600} = 1. After induction with 1 mM isopropyl thio-β-D-galactoside and growth for 3 h at 30 °C, cells were harvested, resuspended in 40 mL lysis buffer made of sodium phosphate 50 mM pH 8, containing 300 mM NaCl, 10 mM imidazole, protease inhibitors (Complete EDTA-free, Roche) and 1 mM DTT, and kept at −80 °C. Cells were broken twice in a French Press at 100 MPa and centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was added onto a 20 mL nickel–nitrilotriacetic acid column (Quiagen) preequilibrated with lysis buffer. The column was washed with lysis buffer (100 mL). The protein was then eluted by a linear imidazole gradient (10–250 mM, pH 8). Fractions containing the enzymatic activity were pooled and dialysed against *Tris*-HCl 50 mM pH 7.5, containing 20 mM NaCl, 1 mM DTT and 50% glycerol and then kept at −20 °C. The protein was >99% pure as judged by 15% SDS–PAGE (Biorad). Protein concentration was determined using an extinction coefficient of ΔE^{280}= 0.633 for a 1 mg/mL solution. The homogeneity of the enzyme preparation, in *Tris*-HCl 50 mM pH 7.5, containing 20 mM NaCl, was measured by dynamic light scattering experiments using a Dynapro-800 instruments (Proteinsolutions). The enzyme was stable for several months in 50% glycerol at −20 °C.

2.3. Assay of phosphoglycerate kinase

$$1,3-\text{diphosphoglycerate} + \text{ADP} \overset{\text{forward}}{\underset{\text{reverse}}{\Leftrightarrow}} \text{ATP} + 3-\text{phosphoglycerate}$$

The reaction catalysed by phosphoglycerate kinase (above) was followed in a coupled spectrophotometric assay using G3PDH as previously described [26,27]. In the forward reaction, the assay contained 50 mM *Tris*-HCl, pH 7.5, 50 mM K_2HPO_4, 5 mM $MgCl_2$, 5 mM NH_4Cl, 5mM NAD^+, 5 mM DTT, 80 μg/mL G3PDH, 10 mM D,L-glyceraldehyde-3-phosphate and NDP-Mg^{2+} at the desired concentration. The glyceraldehyde-3-phosphate was prepared from D,L-glyceraldehyde-3-phosphate-diethylacetal (monobarium salt) (Sigma–Aldrich) according to the manufacturer instructions. The reaction buffer was preincubated 12 min at 25 °C, allowing formation of 1,3-bisphosphoglycerate. The reaction was started with addition of hPGK at the desired concentration (50 ng–120 μg).

In the reverse reaction, the assay contained 50 mM *Tris*-HCl, pH 7.5, 50 mM Na_2SO_4, 1 mM $MgCl_2$, 0.2 mM NADH, 5 mM 3-phosphoglycerate, 1 mM DTT, 150 μg/mL G3PDH and NTP-Mg^{2+} at the desired concentration. G3PDH was previously dialysed overnight with three buffer changes against 50 mM *Tris*-HCl pH 7.5, containing 1 mM DTT. The concentration of G3PDH was measured spectrophotometrically using E^{280} = 1.27 for a 1 mg/mL solution. The reaction was started at 25 °C in the spectrophotometer cell (Uvikon 932) by addition of PGK (20 ng–320 μg) diluted in *Tris*-HCl pH 7.5, containing 1 mM DTT. Alternatively the enzyme-coupled assay was performed in 96-wells microplates in 250 μL (final volume) with four times less enzyme. The reaction was monitored during 20–40 min with a microplate reader (BIO-TEK Elx808). The reaction rates were calculated using the KC4 software (BIO–TEK). In all assays, the reactions were measured under steady state conditions (rates in the 0.01–0.1 ΔA min^{-1} range). Standardized rates were expressed in μmol of product per minute per μmol of PGK. Data were fitted to a hyperbolic equation using Kaleidagraph sofware to determine the kinetic parameters, the turnover number k_{cat}, and the Michaelis constant K_M. When saturation was not attained, the catalytic efficiency k_{cat}/K_M was measured from the initial slope. In all other cases, the ratio k_{cat}/K_M deduced from the fit of the saturation curve was in agreement with the catalytic efficiency measured from the initial slope. All of the values shown in Tables 1–4 are the means and standard deviations from at least three independent experiments.

2.4. Purification of recombinant human NDP kinase A and enzymatic activity measurement

Nucleoside diphosphate kinase type A from Human (NDPK-A) was overexpressed in *E. coli* and purified as previously described [6]. Enzymatic activity was measured according to the assay described in [3]. The formation of [^{14}C]-ATP was measured under initial rate conditions in a final volume of 3 μL during 3–10 min in the presence of 0.2 mM [^{14}C]-ADP (NEC) (2 GBq/mmol, 54 mCi/mmol) and various amounts of D- or L-dNTP and NTP (1–3 mM). The amount of enzyme was usually 0.5 nM and 10 μM for D- and L-nucleoside triphosphates, respectively. The products of the reaction were separated on TLC plates (Macherey-Nagel) and counted in a PhosphorImager (Amersham Bioscience). Alternatively, the reactivity of NDP kinase with nucleoside triphosphates was monitored by rapid mixing in a stopped-flow fluorometer (Hi Tech) as described [4].

Table 1
Reaction of hPGK with D- and L-purine triphosphate derivatives

Catalytic efficiency k_{cat}/K_M (M^{-1} s^{-1})		
Substrate	D-Enantiomer	L-Enantiomer
ATP	1.7 (±1.5) × 10^6	2.0 (±0.5) × 10^5
2′dATP	3.0 (±0.5) × 10^4	2.6 (±0.4) × 10^4
2′3′ddATP	2.3 (±0.3) × 10^4	–
GTP	6.0 (±1.0) × 10^5	4.5 (±0.5) × 10^5
2′dGTP	1.3 (±0.3) × 10^5	1.2 (±0.5) × 10^5
2′3′ddGTP	7.0 (±0.7) × 10^4	–
AcyclovirTP	2.5 (±0.3) × 10^4	–

Catalytic efficiencies were measured using a spectrophotometric assay under initial rate conditions and calculated from the slope of the plot at low substrate concentration. Value are means of three to five independant measurements.

Table 2
Reaction of hPGK with D- and L-pyrimidine triphosphate derivatives

Catalytic efficiency k_{cat}/K_M (M^{-1} s^{-1})		
Substrate	D-Enantiomer	L-Enantiomer
UTP	2.0 (±0.3) × 10^3	4.5 (±0.5) × 10^3
2′dTTP	110 (±10)	500 (±100)
2′3′ddTTP	50 (±5)	–
AZTTP	15 (±3)	–
d4TTP	15 (±3)	–
CTP	150 (±30)	5200 (±1000)
2′dCTP	<5	1200 (±200)
2′3′ddCTP	<5	–
3TCTP	–	600 (±100)

Catalytic efficiencies were measured under conditions described in Table 1

Table 4
Reaction of hNDPK with D- and L-triphosphate derivatives

Catalytic efficiency k_{cat}/K_M ($M^{-1}s^{-1}$)		
Substrate	D-Enantiomer	L-Enantiomer
ATP	1.7 (±0.2) × 10^6	<10[a]
2′dATP	1.4 (±0.2) × 10^6	32 (±2)[a]
GTP	8.0 (±0.5) × 10^6	16 (±1)[a]
2′dGTP	4.0 (±0.5) × 10^6	40 (±2)[a]
AcyclovirTP	30 (±5) [31]	–
UTP	–	1[a]
2′dTTP	1.2 (±0.2) × 10^6	1.7[a]
AZTTP	75 (±5) [6]	<10 [19]
d4TTP	750 (±100) [6]	<10 [19]
CTP	4.0 (±0.3) × 10^5	1.2[a]
2′dCTP	4.0 (±0.2) × 10^4	1[a]
3TCTP	–	0.2 [19]

[a] Catalytic efficiencies were measured with the radioactive assay as described in Section 2.

Table 3
Kinetic parameters of hPGK with D- and L-NTP derivatives calculated from saturation curves

Substrate	D-Enantiomer			L-Enantiomer		
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
ATP	500 ± 100	0.5 (± 0.1)	1.0 (± 0.2) × 10^6	100 ± 10	0.3 (± 0.1)	3.3 (± 1.2) × 10^5
GTP	420 ± 100	0.5 (± 0.1)	0.8 (± 0.2) × 10^6	120 ± 10	0.15 (± 0.05)	8 (± 4) × 10^5
2′dGTP	–	–	–	110 ± 20	1.0 ± 0.3	1.1 (± 0.7) × 10^5
CTP	–	–	–	10 ± 3	1.5 ± 0.5	6600 ± 3000
2′dCTP	–	–	–	4 ± 1	2.5 ± 0.5	1600 ± 600

3. Results and discussion

3.1. Enzymatic reaction of purified recombinant human PGK with natural substrates

Highly purified recombinant human PGK, expressed as a His-tag fusion protein, was analysed by dynamic light scattering. The dynamic light scattering assessment of free PGK (5 mg/mL) showed a multimodal size distribution, indicating that the enzyme non-specifically aggregates under these conditions. A similar aggregation of PGK from *Trypanosoma brucei* PGK has been reported [28]. In contrast, in the presence of ADP (1 mM) and 3-phosphoglycerate (1 mM), a hydrodynamic radius of 2.7 nm was observed, corresponding to the monomeric state of the protein (data not shown).

The activity of hPGK with nucleoside analogs was characterized in the reverse direction in most of the experiments reported below because 5′-triphosphate nucleoside derivatives are more easily available than their diphosphates. In addition, assaying the enzyme in this direction avoids using 1,3-bisphosphoglycerate, a very labile substrate of the forward reaction. We verified for substrates that could be obtained as di-phospho derivatives, that the relative catalytic efficiencies were similar when the reaction was measured in both directions in the presence of the other substrate in saturating amounts (Fig. 4). Under these conditions, the catalytic parameters of hPGK for its natural substrates are K_M^{ADP} = 0.4 (±0.1) mM and k_{cat} = 1300 (±200) s^{-1}, and K_M^{ATP} = 0.5 (±0.1) mM and k_{cat} = 500 (±100) s^{-1}. The reaction with GTP showed similar kinetic parameters. These kinetic parameters are in agreement with those previously reported for yeast [29] and rabbit muscle PGK [26].

3.2. Activity of hPGK towards purine nucleoside triphosphate analogs

Fig. 2A–B and Tables 1 and 3 show the activity of hPGK with triphosphate derivatives of purine D-nucleosides, including 2′-deoxy-ATP (dATP), 2′-deoxy-GTP (dGTP), 2′,3′-dideoxy-ATP (ddATP), 2′,3′-dideoxy-GTP (ddGTP) and acyclovir triphosphate. The deoxynucleotides were efficient substrates of hPGK, with catalytic efficiencies (k_{cat}/K_M) decreased only by a factor of 6 for dGTP (Fig. 2A) and 20 for dATP (Fig. 2B) as compared to GTP and ATP, respectively. Both D-ddATP and D-ddGTP were almost as efficient as the 2′-deoxynucleotides, suggesting a weak influence of the sugar 3′-hydroxyl of the nucleotide substrate on enzyme activity (Table 1). This was also the case for acyclovir, a derivative of guanosine lacking a sugar moiety (Fig. 1).

Next, we analysed the activity of hPGK with purine L-nucleotides. β-L-ATP and β-D-ATP are enantiomers resulting from the inversion of configuration of asymmetric carbons of the ribose (Fig. 1). Fig. 2 C–D show the activity

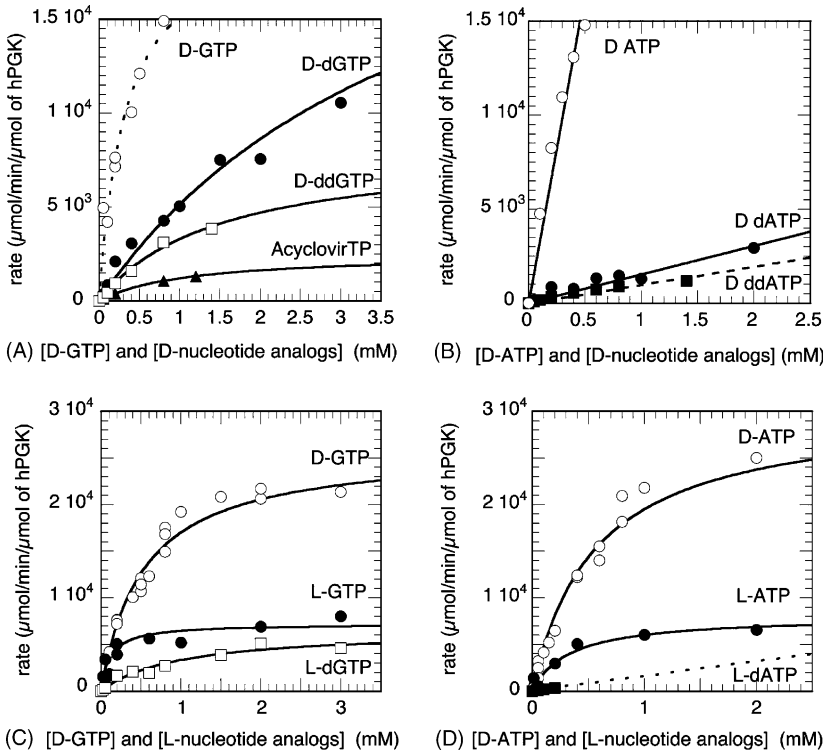

Fig. 2. Reaction of human phosphoglycerate kinase with purine NTPs and their analogs The initial rate of dephosphorylation of NTP was determined in the presence of excess phosphoglycerate and plotted as a function of NTP concentration. K_M and k_{cat} were obtained from the fit of the data to Michaelis curves (shown on Table 3). When saturation could not be attained, the specificity constant (k_{cat}/K_M) was calculated from the slope of the plot at low substrate concentration (Table 1). (A):(○) D-GTP, (●)D-dGTP, (□) D-ddGTP, (▲) AcyclovirTP; (B): (○) D-ATP, (●) D-dATP, (■) D-ddATP; (C): (○) D-GTP, (●) L-GTP, (□) L-dGTP; (D): (○) D-ATP, (●) L-ATP, (■) L-dATP.

of hPGK with L-ribo and L-2′deoxyribo-nucleoside triphosphates (L-NTP and L-dNTP). L-GTP and L-ATP were very efficient substrates (Tables 1 and 3). The catalytic parameters were only slightly affected by the ribose configuration, with K_M values for L-nucleotides around 0.2 mM (0.4 mM for their D-counterparts). The V_{max} is decreased only 4-fold. This high activity of PGK towards L-nucleotides is conserved with deoxy-derivatives since catalytic efficiencies of L-dATP and L-dGTP were decreased by 4-fold only, as compared to L-ribonucleotides. These activities are significantly higher than previously reported for triphospho-derivatives of purine nucleotides in [21]. Indeed, the authors reported an activity with ATP 4,000 fold lower than values obtained with yeast and rabbit muscle PGK [26] and 2,000-fold lower than reported here with hPGK. Several reasons may explain this discrepancy, the most credible being the difference in the enzymatic assays. Our data were acquired in initial rate conditions in the presence of a coupled system that avoids the accumulation of ADP. Cheng's laboratory data were measured in similar conditions but in the absence of the auxiliary enzymes [21]. The ADP produced in the reaction is likely to accumulate and strongly inhibit the enzyme, leading to an underestimate reaction rate (Corinne Lionne, personal communication). The assay used here is more confident for measuring the initial rates.

3.3. Activity of hPGK towards pyrimidine nucleotide analogs

The activity of hPGK with pyrimidine nucleotides was strikingly different than with the purine nucleotides analysed above. The catalytic efficiency of the enzyme with D-UTP and D-CTP was respectively 300 and 3,500-fold decreased compared to D-ATP (Tables 2 and 3). In addition, and contrary to purine nucleotides, presence of the hydroxyl groups on the ribose moiety is important since both 2′ deoxy and 2′,3′ dideoxy derivatives showed very low activity if any. Consistent with the importance of the OH groups on the ribose moiety, hPGK also displayed very low activity with AZTTP and d4TTP with k_{cat}/K_M values of 15 M^{-1} s^{-1} (Table 2).

When we examined the activity of hPGK with L-derivatives of pyrimidine nucleotide analogs (Fig. 3, Tables 2 and 3), the L-enantiomers were better substrates than the corresponding D-enantiomers. Indeed, the efficiency of phosphotransfer increased 26-fold from D-CTP to L-CTP and 2.2-fold from D-UTP to L-UTP. As for L-nucleotides in the purine series, the absence of the 2′-OH in L-2′-dCTP and L-2′-dUTP resulted in a decrease of activity by 4–10 fold compared to L-CTP and L-UTP. 3TCTP is missing both 2′-OH and 3′-OH groups and also has an inversion of the sugar configuration. It was the least efficient substrate among L-cytidine derivatives.

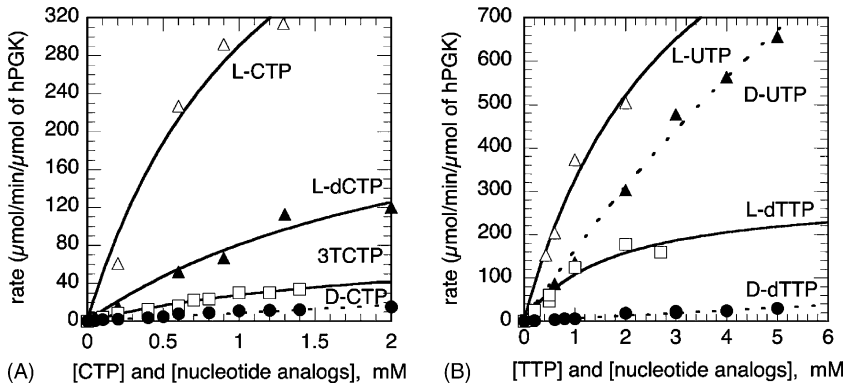

Fig. 3. Reaction of human phosphoglycerate kinase with pyrimidine NTPs and their analogs The initial rate of dephosphorylation of pyrimidine triphosphate was determined as in Fig. 2. (A): (Δ) L-CTP, (▲) L-dCTP, (□) 3TCTP, (●) D-CTP; (B): (Δ) L-UTP, (▲) D-UTP, (□) L-dTTP, (●), D-dTTP.

The activity of hPGK was also tested with ADP and some cytidine diphosphate derivatives (Fig. 4). Values calculated for the catalytic efficiency was $3.0 \times 10^6\ M^{-1}\ s^{-1}$ for D-ADP (compared to $1.7 \times 10^6\ M^{-1}\ s^{-1}$ for D-ATP). In the cytidine series, catalytic efficiencies were found lower: $800\ M^{-1}\ s^{-1}$ (600), $250\ M^{-1}\ s^{-1}$ (150) and $< 10\ M^{-1}\ s^{-1}$ (<5) for 3TCDP, D-CDP and D-dCDP respectively (number in brackets, from Table 2, are the values for the corresponding triphosphates). We assumed that the reaction rates of hPGK measured for other substrates, either purine or pyrimidine derivatives, are similar in the forward and in the reverse assay.

3.4. Reaction of L-nucleoside analogs with human nucleoside diphosphate kinase

The activities of human NDP kinase with L-ribo- and L-deoxyribonucleoside triphosphates are shown in Table 4. While the efficiencies of phosphotransfer with D-NTP and D-dNTP were high (in the $10^7\ M^{-1}\ s^{-1}$ and $10^6\ M^{-1}\ s^{-1}$ range respectively), NDP kinase was almost inactive with L-NTP and L-dNTP. Although L-2′-deoxyderivatives were metabolized 5-times more efficiently than L-ribonucleotides, in particular in the purine series, their catalytic efficiencies remained less than $40\ M^{-1}\ s^{-1}$. This confirms that, even in the presence of a 3′-hydroxyl group on the ribose, the stereospecificity of NDP kinase is restricted to nucleosides in the D-configuration. The lack of reactivity of NDP kinase with L-(d)NTP demonstrates that the L-nucleotide preparations are indeed chemically devoid of D-contaminants. In the case of the presence of D-NTP traces, the activity measured with NDP kinase would be higher due to the high k_{cat} of this enzyme.

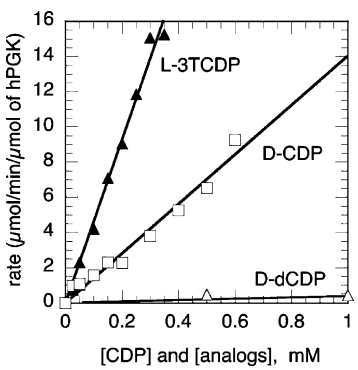

Fig. 4. Reaction of human phosphoglycerate kinase with D-CDP, D-dCDP and 3TCDP The initial rate of phosphorylation of NDP was measured in presence of excess of DL glyceraldehyde-3-phosphate and plotted as a function of NDP. Data were fitted according to a linear curve and the specificity constant (k_{cat}/K_M) was calculated from the slope of the plot.(▲) L-3TCDP, (□) D-CDP, (Δ) D-Dcdp.

3.5. Binding of nucleotide to hPGK

Fig. 5A shows a schematic representation of D-ADP-Mg^{2+} bound in the active site of human PGK. The phosphate chain interacts with several active site residues along with a magnesium ion that is essential for catalysis. The adenine base only shows hydrophobic interactions with the

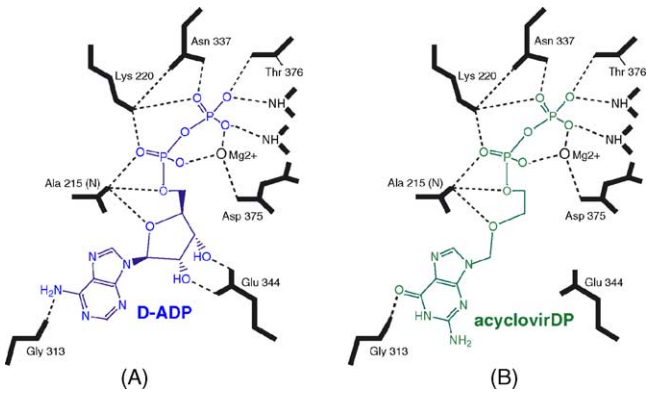

Fig. 5. A scheme for binding of D-ADP and acyclovirDP in the nucleotide binding site of human PGK. (A) The binding of D-ADP in the active site of hPGK. D-ADP was drawn bound in the active site of PGK on the basis of the X-ray structure of Mg^{2+}-ADP complexed to pig muscle PGK [28]. Residue numbering corresponds to the human enzyme. The picture was drawn using ChemDraw software. (B) The binding of acyclovir diphosphate in the active site of hPGK. The model was obtained by replacing the adenine base by a guanine and by modifying the ribose moiety of ADP in the model shown in (A).

protein. The only moderate decrease observed in hPGK activity with deoxy- and dideoxypurines as well as with acyclovir, as compared to D-GTP and D-ATP (Tables 1 and 3), suggests that the hydrogen bonds between Glu 344 and the 2′-OH and 3′-OH of the sugar are not essential for catalysis. The proposed configuration of acyclovir diphosphate in the nucleotide binding site (Fig. 5B) provides an explanation for the fact that, in spite of a major destructuration of the ribose moiety, only a small decrease (24-fold) was observed in acyclovir diphosphate phosphorylating efficiency as compared to GTP. The inversion of the analog configuration is likely to disrupt the interactions between the ribose and Ala 215 and Glu 344 (Fig. 5A). However, the kinetic parameters for L- and D-purine nucleoside diphosphate analogs are not significantly different although L-pyrimidine nucleotides diphosphate are better substrates as compared to their D enantiomers.

In conclusion, we have shown that human PGK is not specific for the ribose configuration and for the presence of an hydroxyl group at the 2′ or 3′ position of the sugar. In contrast, it shows specificity for purine base. A role for hPGK in the cell phosphorylation of 3TC has been reported [1]. Our data suggest that hPGK may be involved in the cellular activation of several other clinically relevant compounds. This includes diphosphate derivatives of D-dideoxyinosine, active against AIDS, acyclovir, used against Herpes virus and LdT and LdC, active against B hepatitis. This wide specificity for purine substrates points to the possible involvement of hPGK in the phosphorylation of ganciclovir or abacavir, as well as of several anticancer drugs [30]. In the context of suicide enzyme therapy, improvement of herpes thymidine kinase towards acyclovir and ganciclovir is currently under investigation in several laboratories. The high activity of hPGK with purine nucleoside diphosphate analogs suggests that its co-expression with herpes thymidine kinase could result in a better activation of acyclovir and ganciclovir in target cells.

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