Phosphorylation of Pyrimidine L-Deoxynucleoside Analog Diphosphates

KINETICS OF PHOSPHORYLATION AND DEPHOSPHORYLATION OF NUCLEOSIDE ANALOG DIPHOSPHATES AND TRIPHOSPHATES BY 3-PHOSPHOGLYCERATE KINASE*

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Anticancer and antiviral D- and L-nucleoside analogs are phosphorylated stepwise in the cells to the pharmacologically active triphosphate metabolites. We recently reported that in the last step, L-deoxynucleoside analog diphosphates are phosphorylated by 3-phosphoglycerate kinase (PGK). To explain the preference of PGK for L- over D-deoxynucleoside analog diphosphates, the kinetics of their phosphorylation were compared with the dephosphorylation of the respective triphosphates using recombinant human PGK. The results attributed favorable phosphorylation of L-deoxynucleoside analog diphosphates by PGK to differences in $k_{cat}$, which were consequences of varied orientations of the sugar and diphosphates in the catalytic site of PGK. The amino acids involved in the catalytic reaction of PGK (including Glu344, Lys220, and Asn337) were therefore mutated. The impact of mutations on the phosphorylation of L- and D-deoxynucleoside analog diphosphates was different from those on dephosphorylation of the respective triphosphates. This suggested that the interactions of the nucleoside analogs with amino acids during the transition state were different in the phosphorylation and dephosphorylation reactions. Thus, reversible action of the enzyme may not involve the same configuration of the active site. Furthermore, the amino acid determinants of the action of PGK for L-deoxynucleotides were not the same as for the D-deoxynucleotides. This study also suggests the potential impact of nucleoside analog diphosphates and triphosphates on the multiple cellular functions of PGK, which may contribute to the action of the analogs.

Nucleoside analogs are an important class of anticancer and antiviral agents. Among these the L-deoxynucleoside analogs are emerging as a new class of compounds (1, 2). Some examples are L-SddC1 (lamivudine), which is in clinical use for the treatment of HIV and hepatitis B virus (3–5); L-FMAU (6) and 2',3'-dideoxy-2',3'-didehydro-β-D-5-fluorodeoxycytidine (7–9), which are in phase II clinical trials as anti-hepatitis B virus agents; and β-D-1(3)-dioxolane-2',3'-dideoxythymidine and ddC are some examples of anti-HIV drugs (13, 14), and L-deoxynucleoside analogs like β-D-arabinofuranosylcytosine and gemcitabine are antitumor drugs (15). The last step in the phosphorylation of L-deoxynucleoside analog diphosphates to the respective triphosphates, which are the pharmacologically active metabolites, remained largely unexplored. Through comparison of phosphorylation of several clinically relevant D-deoxynucleoside, D-dideoxynucleoside, and L-deoxynucleoside analog diphosphates by nucleoside-metabolizing enzymes purified from the human hepatic carcinoma cell line HepG2, we recently reported that L-deoxynucleoside analog diphosphates could be phosphorylated by 3-phosphoglycerate kinase (PGK); D-dideoxynucleoside analog diphosphates were likely to be phosphorylated by nucleoside diphosphate kinase; and L-dideoxynucleoside analog diphosphates were excellent substrates for creatine kinase (16). Moreover, L-deoxynucleoside analog diphosphates were better substrates for PGK than the corresponding D-deoxynucleoside and D-dideoxynucleoside analog diphosphates (at 200 μM). Other nucleoside-metabolizing enzymes have not exhibited a similar preference for L-deoxynucleoside analog diphosphates, and the property is unique to PGK.

Human PGK (~46 kDa) is a glycolytic enzyme that catalyzes the conversion of 1,3-biphosphoglycerate to 3-phosphoglycerate and during the process generates one molecule of ATP (17). The reaction is reversible. In addition to its participation in the glycolytic cycle, cytoplasmic PGK is known to stimulate viral mRNA synthesis (18). It is also expressed in the nuclei where it modulates DNA synthesis and repair (19–21). Since PGK in nuclei retains its ability to bind to its natural substrates, it has been proposed that its activity in nuclei may be regulated by the energy state of the cell (21). Extracellular PGK was recently shown to have a thiol-reductase activity (22). Reduction of plasmin by PGK results in proteolysis of plasmin to angiotatin fragments, which are inhibitors of angiogenesis (23–25). ATP and 3-phosphoglycerate could inhibit reduction of plasmin, presumably through inducing a conformational change that was not favorable to the reduction of plasmin (25). This suggested that the level of nucleoside diphosphates or triphosphates (natural or otherwise) might have a regulatory impact on the multiple cellular functions of PGK.

The sequences of mammalian PGKs are conserved over 96%, and there is also a high level of tertiary structure homology (26). The crystal structures of horse muscle PGK in...
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In this study the amino acids in the catalytic site of human PGK have been extrapolated from the model proposed by May et al. (29), which was based on the crystal structure of pig muscle PGK in ternary complex with ATP-PNP and 3-phosphoglycerate. The modified catalytic reaction is shown in Fig. 1.

PGK comprises of a single subunit with two domains; the N-terminal domain has a basic patch region (rich in arginines and histidines) for binding to 1,3-biphosphoglycerate (or 3-phosphoglycerate), and the C-terminal domain contains the nucleotide-binding site. The nucleobase binds to a hydrophobic groove on the surface of the C terminus of the enzyme. The oxygen on the α-phosphate interacts with Lys220, and the oxygen on the β-phosphate (or the bridge oxygen between β- and γ-phosphates) hydrogen bonds to Asn337. Asn337 also forms a hydrogen bond with the amino group of Lys220 and helps to stabilize its ion pair interaction with α-phosphate of the nucleotide (28–30). Fig. 1 depicts the catalytic reaction of PGK with ATP as the substrate and 3-phosphoglycerate as the phosphate acceptor. Enzyme-substrate(s) interactions induce a conformational change in PGK resulting in the reduction of distance between the N- and C-terminal domains for associative phosphate transfer. The γ-phosphate is then in the proximity of Arg339, and additional interaction of bridge oxygen between β- and γ-phosphate with Asn337 creates a positive charge on the phosphorus atom of γ-phosphate, facilitating nucleophilic attack by 3-phosphoglycerate. This disturbs the coordination of the metal ion and its linkage with α-, β-, and γ-phosphates and results in interaction of metal ion with Asp775 and α- and β-phosphates. This leads to the collapse of the transition state to form metal-ADP and 1,3-biphosphoglycerate complexes bound to PGK, which then reverses the conformational change and releases the products (27–29). As with any reversible enzyme it is generally assumed that the phosphorylation of ADP to ATP using 1,3-biphosphoglycerate as a phosphate donor would also go through a similar transitional state (ED* = EDP*).

To understand the preference of PGK for l-deoxynucleoside analog diphosphates over their d-deoxynucleoside analog counterparts, the kinetics of their phosphorylation were compared with the kinetics of dephosphorylation of the respective triphosphates using recombinant human PGK. The preference observed was further explained through kinetic analysis of phosphorylation and dephosphorylation of nucleoside analog diphosphates and triphosphates by single amino acid mutants of PGK. These included mutations of PGK at Glu344, Lys220, and Asn337, all of which are involved in hydrogen bonding with the sugar and phosphate side chain of nucleoside analog diphosphates or triphosphates during the catalytic reaction.

**EXPERIMENTAL PROCEDURES**

**Nucleoside Analog Diphosphates and Triphosphates—**Nucleoside analogs l-FMAUDP, d-FMAUDP, l-3′-deoxyadenosine diphosphate, l-3′-deoxyguanosine diphosphate, were synthesized according to the following procedure (which is a modification of the protocol published by Ruth and Cheng (31)). Nucleoside analogs were dissolved in trimethylphosphate (10 μl/mg of nucleoside) for 10 min at −10 °C. Phosphochoychoxylate (10 equivalents of trimethylphosphate) was added at the same temperature to generate nucleoside analog monophosphates. Ten parts of the mixtures of 2 mmol of phosphoric acid (in N,N-dimethylformamide) and 6 mmol of tributylamine were added to the solution containing nucleoside analog monophosphates. This resulted in the stepwise synthesis of nucleoside analog diphosphates and triphosphates. The reactions were stopped after 1 h by neutralization of the mixture with NaOH to obtain the diphosphates and triphosphates with high purity. The kcat values of nucleoside analog diphosphates are also converted to triphosphates. Nucleoside analog diphosphates and triphosphates were purified using DEAE Sephadex A-25 (Amersham Biosciences) and eluted with step gradients between 0 and 300 mM KCl. The purity of the respective nucleoside analog diphosphates and triphosphates was confirmed with a BioRad Protein Assay (Hercules, CA) before use. The nucleoside analog diphosphates and triphosphates included in this study were purchased from Amersham Biosciences.

Cloning PGK and Single Amino Acid Mutants of PGK—Total RNA was extracted from HepG2 cells (human hepatoma) using TRIzol and reverse transcribed using Superscript II R Nase H reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. An aliquot of cDNA was amplified using DNA polymerase and specific primers for human PGK, including 5′-GGA ATT CCA TAT GTC GCT TCT TAA CAA GAT GAC G, and 5′-CGC GGA TCC CTA ATT GAT GAG AGC ATC CAC. The PCR product was digested with Ndel and BamHI enzymes, ligated with Ndel-BamHI-digested pET28a bacterial expression vector (the sequence of PGK was confirmed by DNA sequencing), and transfected into Escherichia coli strain BL21 (DE3). The resulting PGK was an N-terminal histidine fusion protein, which was purified using nickel-nitrioltriacetic acid-agarose (Qiagen) column according to the manufacturer’s protocol. Single amino acid mutations of the wild-type PGK-pET28a plasmid were carried out using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The specific primers (and their complimentary oligomers) used were 40–50-mer, and the positions for the beginning and end of primers and the base pairs around the site of mutation (shown in bold type) are listed: (i) E344A, 5′-TGT GCC GGC TAA GCC-3′(ii) K220A, 5′-GCA GAC GCG ATC AGC-3′; (iii) K220R, 5′-GCA GAC GCG ATC AGC-3′; (iv) N337D, 5′-GCG TGG GAC GCT AGC-3′; (v) N337Q, 5′-CGT GGG TGG GAC GCT AGC-3′. The sequences were confirmed by DNA sequencing. The mutant enzymes were purified using the same method as for the wild-type enzyme.

**Phosphorylation and Dephosphorylation of Nucleoside Analog Phosphates—**Phosphorylation of nucleoside analog diphosphates by PGK and the mutant enzymes was evaluated in a buffer containing 50 mM Tris acetate (pH 7.5), 5 mM MgCl2, 1 mM NaF, 1 mM dithiothreitol, 10 mM sodium phosphate, 4 mM NAD+, and 4 mM 1,1,3-trichlorotrifluoroethane in a ratio of 45:55. The diphosphate of the respective nucleoside analogs was converted to triphosphate by the addition of 0.5 units/0.1 ml of glyceraldehyde-3-phosphate dehydrogenase (16). Dephosphorylation of nucleoside analog triphosphate by PGK or the mutant enzymes was evaluated in a buffer containing 50 mM Tris acetate (pH 7.5), 5 mM MgCl2, 1 mM NaF, 1 mM dithiothreitol, and 4 mM 3-phosphoglycerate. All of the samples were incubated at 37 °C. The reactions were stopped on ice, and the samples were deproteinized by trichloroacetic acid precipitation. The samples were then extracted in a mixture of trioctylamine and 1,1,3-trichlorotrifluoroethane in a ratio of 45:55. The diphosphate and triphosphate forms of nucleoside analogs were analyzed by HPLC as described above. The Km and kcat values were calculated from Lineweaver-Burk double reciprocal plots. All of the values shown in Tables I–VI are the means and standard deviations from at least three independent experiments.

**RESULTS**

**Phosphorylation of Nucleoside Analog Diphosphates by PGK—**The Km and kcat values of nucleoside analog diphosphates for recombinant human PGK are shown in Table I. This included diphosphates of l-deoxynucleoside analogs and d-ribonucleoside, d-deoxynucleoside, and d-ribofuranoside analogs. As can be seen, the results were not as major impact on binding; the Km values of all of the purine and pyrimidine analog diphosphates were within a 3-fold range. The kcat for purines, represented by ADP, which is the natural substrate of the enzyme, were higher than those of pyrimidines, as represented by TDP and CDP. In addition, within the same base, the kcat for d-ribonucleoside was greater than...
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The Km and kcat values of the nucleoside analog diphosphates for wild-type PGK were evaluated using 1,3-biphosphoglycerate as a phosphate donor.

|            | Km (µM) | kcat (s⁻¹) |
|------------|---------|------------|
| ADP        | 232.5 ± 28.7 | 600.0 ± 2073.7 |
| dADP       | 488.1 ± 12.5 | 845.4 ± 230.4 |
| TDP        | 160.5 ± 13.4 | 0.3 ± 0.003 |
| d-FMAUDP   | 341.0 ± 17.8 | 0.4 ± 0.007 |
| l-FMAUDP   | 666.5 ± 40.7 | 84.5 ± 3.8 |
| CDP        | 885.0 ± 11.0 | 0.9 ± 0.1 |
| ddCDP      | —        | —          |
| l-ddCDP    | 388.6 ± 13.3 | 0.5 ± 0.07 |
| l-SddCDP   | 325.1 ± 16.4 | 1.3 ± 0.07 |

* kcat lower than 0.04 min⁻¹, which is the limit for detection by HPLC.

The Km and kcat values for the diphosphates of nucleotides in transition state. Lys220 was replaced with alanine (to abolish hydrogen bonding) and with arginine (to evaluate the steric impact), and Asn337 was replaced with aspartate (to abolish hydrogen-bonding) and glutamine (to study the steric impact). The Km and kcat values are shown in Table IV, and the values in parentheses represent the changes with respect to the wild-type PGK. The rate of phosphorylation of diphosphates of cytidine analogs by both Lys220 and Asn337 mutants decreased to less than 0.04 min⁻¹, which is the limit for detection by HPLC. A similar decrease was observed with TDP and d-FMAUDP for all enzymes except K220R.

**Phosphorylation of l-Nucleoside Analog Diphosphates by PGK**—The effects of γ-phosphate of nucleoside analog triphosphates on Km and the rate of phosphorylation were evaluated, and the results are shown in Table II. The nucleobase had no significant impact on the Km value, however, the Km values for nucleoside analog triphosphates were 5–8-fold lower than those of the respective diphosphates. The kcat values for phosphorylation of d-ribonucleoside and l-deoxynucleoside analog triphosphates examined were 100–1000-fold lower than that for the phosphorylation of the respective diphosphates. The kcat values for all of the nucleoside analog triphosphates were, however, similar, indicating that the base configuration of sugar and the presence or absence of 2'- and 3'-hydroxyl groups had no impact on the rate of phosphate transfer. Interestingly, the kcat value for phosphorylation of dCTP and ddCTP was better than the kcat value for phosphorylation of the respective diphosphates. The ratios of the efficiency of phosphorylation of nucleoside analog triphosphates and the efficiency of phosphorylation of the respective diphosphates (derived from Table I) are also shown in Table II. Phosphorylation of the diphosphates of ribonucleosides and l-deoxynucleoside analogs were favored at least a 100-fold over the dephosphorylation of the respective triphosphates. This implied that phosphorylation of l-deoxynucleoside analog diphosphates would not be limited by dephosphorylation of the triphosphates generated during the reaction. Phosphorylation of d-FMAUDP and TDP were favored only by 2–10-fold over the dephosphorylation of the respective triphosphates. These results supported the observation that favorable phosphorylation of l-deoxynucleoside analog diphosphates as compared with the corresponding d-ribonucleoside analogs were due to differences in the kcat values.

**Phosphorylation of Nucleoside Analog Diphosphates by E344A**—The 2',3'-hydroxyl groups, which hydrogen bond to Glu344, had an impact on the phosphorylation of nucleoside analog diphosphates (Table I). To evaluate the role of Glu344 in the orientation of nucleoside analog diphosphates in the catalytic site, glutamic acid was replaced with alanine. The Km and kcat values for E344A are shown in Table III. The values in parentheses show the changes in Km and kcat in comparison with the wild-type PGK. As expected, the kcat value for d-ribonucleoside analogs ADP (over 10-fold) and CDP (at least 1000-fold) decreased significantly. The kcat value for the d-deoxynucleoside analogs TDP and d-FMAUDP increased slightly, similar to that of l-deoxynucleoside analog, l-SddCDP, whereas l-FMAUDP remained unaffected. There were no discernible patterns in the changes associated with the Km values. These results indicated that the hydrogen bonds between ribonucleoside diphosphates and the carboxyl group of Glu344 are important for the optimal orientation of the substrate in the catalytic site for efficient phosphate transfer. In the analogs lacking either or both 2'- and 3'-hydroxyl groups, substitution with alanine at position 344 had no significant effect on the rate of phosphorylation.

**Phosphorylation of Nucleoside Analog Diphosphates by K220A, K220R, N337D, and N337Q**—Lys220 and Asn337 stabilize the phosphate side chain by forming hydrogen bonds with oxygen on α-phosphate and β-phosphate, respectively. These amino acids are therefore directly involved in the interactions with diphosphates or triphosphates of nucleotides in transition state. Lys220 was replaced with alanine (to abolish hydrogen bonding) and with arginine (to evaluate the steric impact), and Asn337 was replaced with aspartate (to abolish hydrogen-bonding) and glutamine (to study the steric impact). The Km and kcat values are shown in Table IV, and the values in parentheses represent the changes with respect to the wild-type PGK. The rate of phosphorylation of diphosphates of cytidine analogs by both Lys220 and Asn337 mutants decreased to less than 0.04 min⁻¹, which is the limit for detection by HPLC. A similar decrease was observed with TDP and d-FMAUDP for all enzymes except K220R.

Mutation to K220A increased the Km of ADP over 5-fold; it, however, did not affect the Km of dADP and l-FMAUDP. The kcat value for all three analogs decreased significantly (ranging from 100- to 3000-fold). Replacement of Lys220 with arginine, which can form hydrogen bonds with oxygen on α-phosphate, resulted in a slight recovery of activity, without affecting the Km. The Km value of ADP for K220R also improved over 2-fold as compared with K220A. Replacement of Asn337 with aspartate resulted in a 3-fold decrease in Km of ADP and l-FMAUDP, whereas decreases in kcat ranged from 100- to 750-fold as compared with the wild-type enzyme. Substitution of Asn337 with glutamine resulted in a slight recovery of activity as compared with N337D. Since the rates of phosphorylation of most of the pyrimidine analogs could not be determined, changes in Km and kcat values observed with ADP, dADP, and l-FMAUDP could not be categorized. These results, however, indicate that hydrogen bonds stabilized by Lys220 and Asn337 are essential for the phosphorylation of d- and l-nucleoside analog diphosphates. Replacement of Lys220 and Asn337 with arginine and glutamine, respectively, decreased the kcat value significantly, indicating that these amino acids are not sterically accommodated in the catalytic cleft. Varied impacts of mutation on nucleoside analog diphosphates could be attributed to different conformations of the analogs in the catalytic site.

**Dephosphorylation of Nucleoside Analog Triphosphates by PGK**—The effects of α-phosphate of nucleoside analog triphosphates on Km and the rate of dephosphorylation were evaluated, and the results are shown in Table I. The nucleobase had no significant impact on the Km value, however, the Km values for nucleoside analog triphosphates were 5–8-fold lower than those of the respective diphosphates. The kcat values for dephosphorylation of d-ribonucleoside and l-deoxynucleoside analog triphosphates examined were 100–1000-fold lower than that for the phosphorylation of the respective diphosphates. The kcat values for all of the nucleoside analog triphosphates were, however, similar, indicating that the base configuration of sugar and the presence or absence of 2'- and 3'-hydroxyl groups had no impact on the rate of phosphate transfer. Interestingly, the kcat value for dephosphorylation of dCTP and ddCTP was better than the kcat value for phosphorylation of the respective diphosphates. The ratios of the efficiency of dephosphorylation of nucleoside analog triphosphates and the efficiency of phosphorylation of the respective diphosphates (derived from Table I) are also shown in Table II. Phosphorylation of the diphosphates of ribonucleosides and l-deoxynucleoside analogs were favored at least a 100-fold over the dephosphorylation of the respective triphosphates. This implied that phosphorylation of l-deoxynucleoside analog diphosphates would not be limited by dephosphorylation of the triphosphates generated during the reaction. Phosphorylation of d-FMAUDP and TDP were favored only by 2–10-fold over the dephosphorylation of the respective triphosphates. These results supported the observation that favorable phosphorylation of l-deoxynucleoside analog diphosphates as compared with the corresponding d-ribonucleoside analogs were due to differences in the kcat values.
Phosphorylation of L-Nucleoside Analog Diphosphates by PGK

The $K_m$ and $k_{cat}$ values of the nucleoside analog triphosphates for wild-type PGK were evaluated using 3-biphosphoglycerate as a phosphate acceptor.

Table II

| Triphosphate          | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) |
|-----------------------|------------|------------------------|
| ATP                   | 58.8 ± 11.5| 0.9 ± 0.13             |
| dATP                  | 73.6 ± 14.2| 1.8 ± 0.3              |
| TTP                   | 101.8 ± 18.9|1.0 ± 0.2              |
| d-FMAUTP              | 53.8 ± 9.6| 1.6 ± 0.3              |
| L-FMAUTP              | 81.5 ± 17.4|0.3 ± 0.03             |
| CTP                   | 86.7 ± 1.7 | 0.5 ± 0.09             |
| dCTP                  | 109.8 ± 3.9| 0.4 ± 0.04             |
| ddCTP                 | 692.8 ± 60.1|0.9 ± 0.04            |
| L-SddCTP              | 74.3 ± 5.9 | 0.3 ± 0.01             |

$\frac{K_m}{k_{cat}}$ values for forward reactions were lower than $0.04$ min$^{-1}$, which is the limit for detection by HPLC.

$*$Because $k_{cat}$ values for forward reactions were lower than $0.04$ min$^{-1}$, the efficiencies could not be compared.

Table III

Phosphorylation of nucleoside analog diphosphates by E344A

The $K_m$ and $k_{cat}$ values of the nucleoside analog diphosphates for E344A were evaluated using 1,3-biphosphoglycerate as a phosphate donor. The values in parentheses represent the $K_m$ or $k_{cat}$ values for the mutant enzyme divided by the values for wild-type PGK.

| Nucleoside Analog Diphosphate | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) |
|-------------------------------|------------|---------------------|
| ADP                           | 699.7 ± 13.9| 752.7 ± 161.3 (0.1)|
| dADP                          | 229.4 ± 16.8 (0.4)| 99.8 ± 7.7 (0.2) |
| TDP                           | 635.4 ± 24.4 (4)| 1.0 ± 0.2 (3)   |
| d-FMAUDP                      | 429.3 ± 64.3 (1.2)| 1.0 ± 0.07 (2.8)|
| L-FMAUDP                      | 1240.0 ± 90.0 (1.8)| 59.9 ± 7.7 (0.8)|
| CDP                           | 329.6 ± 86.6 (1)| 3.4 ± 0.6 (2.6) |
| L-SddCDP                      | 743.3 ± 59    | 0.3 ± 0.01         |

$^a$ $k_{cat}$ lower than 0.04 min$^{-1}$, which is the limit for detection by HPLC.

Phosphorylation of L-nucleoside analog diphosphates by recombinant human PGK showed that the nucleobase did not have a major impact on the $K_m$ of purine and pyrimidine analog diphosphates; however, it was apparent that the $k_{cat}$ value varied significantly (with purines faring better than the pyrimidines). The hydroxyl groups on the sugar moiety also had an impact on phosphorylation, with the $k_{cat}$ decreasing in the following order: ribonucleoside, deoxynucleoside, and dideoxynucleoside analog diphosphates. L-Deoxynucleoside analog diphosphates, despite the absence of 2'-hydroxyl groups were better phosphorylated than the corresponding d-nucleoside analogs. Since the catalytic action of PGK is reversible, it was possible that the reverse reaction in the phosphorylation of nucleoside analog triphosphates could be a rate-limiting factor. The kinetics of dephosphorylation of d- and L-nucleoside analog triphosphates were evaluated. Although the structure itself had no impact on the $K_m$ value, the presence of γ-phosphate in nucleoside analog triphosphates resulted in a 5–8-fold decrease in $K_m$ as compared with the respective diphosphates. In contrast to the forward reactions, the configuration of the sugar and the presence or absence of hydroxyl groups on the sugar moiety did not impact the $k_{cat}$. For ribonucleoside and L-deoxynucleoside analog diphosphates, the efficiency of the forward reaction in the phosphorylation of diphosphates was favored at least 100-fold over dephosphorylation of the respective triphosphates. Interestingly, for 2'-deoxycytidine and β-2',3'-dideoxycytidine, the $k_{cat}$ value for dephosphorylation of their triphosphates was better than the $V_{max}$ value for phosphorylation of the respective diphosphates. This accounts for the
Phosphorylation of l-Nucleoside Analog Diphosphates by PGK

The $K_m$ and $k_{cat}$ values of the nucleoside analog diphosphates for the mutant enzymes were evaluated using 1,3-biphosphoglycerate as a phosphate donor. The values in parentheses represent $k_{cat}$ of mutant enzymes as compared with the values for wild-type PGK.

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ADP      | 1150.0 | 553.3     |
| dADP     | 569.6  | 0.3       |
| TDP      | 227.5  | 0.03      |
| d-FMAUDP | 253.6  | 0.008     |
| L-FMAUDP | 872.7  | 0.2       |

The $k_{cat}$ lower than 0.04 min$^{-1}$, which is the limit for detection by HPLC.

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ADP      | 68.3   | 56.0      |
| dADP     | 482.5  | 2.2       |
| L-FMAUDP | 267.4  | 0.02      |

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ATP      | 90.1   | 6.4       |
| dATP     | 92.8   | 9.4       |
| TTP      | 57.5   | 6.1       |
| d-FMAUTP | 63.1   | 8.4       |
| L-FMAUTP | 134.5  | 0.3       |
| CTP      | 126.3  | 2.0       |
| dCTP     | 82.5   | 1.7       |
| ddCTP    | 190.2  | 1.2       |

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ATP      | 79.3   | 10.6      |
| dATP     | 87.9   | 11.5      |
| TTP      | 59.9   | 7.1       |
| d-FMAUTP | 51.6   | 13.7      |
| L-FMAUTP | 67.7   | 0.6       |
| CTP      | 145.2  | 3.1       |
| dCTP     | 101.2  | 2.4       |
| ddCTP    | 162.4  | 1.1       |

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ATP      | 67.4   | 3.5       |
| dATP     | 88.6   | 4.2       |
| TTP      | 61.2   | 3.1       |
| d-FMAUTP | 111.6  | 0.3       |
| L-FMAUTP | 102.9  | 1.0       |
| CTP      | 89.3   | 1.3       |
| dCTP     | 600.4  | 2.7       |

In favorable phosphorylation of l-deoxynucleoside analog diphosphates (as compared with the corresponding d-nucleoside analogs) by PGK. Although l-deoxynucleoside analog diphosphates are efficiently phosphorylated by PGK, accumulation of the triphosphate form may limit its own synthesis.

Favorable phosphorylation of l-deoxynucleoside analog diphosphates over the d-nucleoside counterparts was solely attributed to differences in $k_{cat}$. This implied that both the base and the sugar induced changes in the orientation of the diphosphates in the catalytic site, thereby affecting the rate of phosphate transfer. To study varied configurations of l- and d-nucleoside analog diphosphates in the catalytic site, amino acids Glu$^{44}$, Lys$^{220}$, and Asn$^{337}$ were mutated.

The 5'- and 3'-hydroxyl groups on the sugar moiety form hydrogen bonds with the carboxylate of Glu$^{44}$. To assess the role of such hydrogen bonding on the $k_{cat}$ values of the nucleoside analog triphosphates for the mutant enzymes as compared with the values for wild-type PGK.

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ATP      | 90.1   | 6.4       |
| dATP     | 92.8   | 9.4       |
| TTP      | 57.5   | 6.1       |
| d-FMAUTP | 63.1   | 8.4       |
| L-FMAUTP | 134.5  | 0.3       |
| CTP      | 126.3  | 2.0       |
| dCTP     | 82.5   | 1.7       |
| ddCTP    | 190.2  | 1.2       |

|          | $K_m$  | $k_{cat}$ |
|----------|--------|-----------|
| ATP      | 79.3   | 10.6      |
| dATP     | 87.9   | 11.5      |
| TTP      | 59.9   | 7.1       |
| d-FMAUTP | 51.6   | 13.7      |
| L-FMAUTP | 67.7   | 0.6       |
| CTP      | 145.2  | 3.1       |
| dCTP     | 101.2  | 2.4       |
| ddCTP    | 162.4  | 1.1       |

The $k_{cat}$ lower than 0.04 min$^{-1}$, which is the limit for detection by HPLC.

Favorable phosphorylation of l-deoxynucleoside analog diphosphates over the d-nucleoside counterparts was solely attributed to differences in $k_{cat}$. This implied that both the base and the sugar induced changes in the orientation of the diphosphates in the catalytic site, thereby affecting the rate of phosphate transfer. To study varied configurations of l- and d-nucleoside analog diphosphates in the catalytic site, amino acids Glu$^{44}$, Lys$^{220}$, and Asn$^{337}$ were mutated.

The 5'- and 3'-hydroxyl groups on the sugar moiety form hydrogen bonds with the carboxylate of Glu$^{44}$. To assess the role of such hydrogen bonding on the $k_{cat}$ values of the nucleoside analog triphosphates for the mutant enzymes as compared with the values for wild-type PGK.
ATP (NTP) + 3-phosphoglycerate $\rightarrow$ ADP (NDP) + 1,3-biphosphoglycerate

**Fig. 1. Catalytic activity of human PGK.** The figure is a schematic representation of the catalytic activity of human PGK, a modified version of the model published by May et al. (29) for pig muscle PGK. \( ED \) represents a complex between NDP and PGK; \( EP \) represents a complex between NTP and PGK; the reaction goes through a transition complex: \( ED^* \) or \( EP^* \). It is generally assumed that \( ED^* = EP^* \). Only the amino acids interacting with the sugar and phosphates of nucleotides are shown in this figure.
the catalytic site were independent of their interactions with Glu\textsuperscript{344}. Mutation of Glu\textsuperscript{344} did not affect the reversible reaction in the dephosphorylation of nucleoside analog triphosphates (data not shown). This supported the observation that hydroxyl groups on the sugar moiety had no impact on the dephosphorylation of nucleoside analog triphosphates.

Lys\textsuperscript{220} and Asn\textsuperscript{337} interact with the phosphate side chain of nucleotides during the catalytic reaction. To study the importance of hydrogen bonding, Lys\textsuperscript{220} and Asn\textsuperscript{337} were substituted with Ala and Asp, respectively. The steric impact of increasing the length of amino acid side chain by K220R and N337Q mutations was also evaluated. The results showed that hydrogen bonding with Lys\textsuperscript{220} and Asn\textsuperscript{337} were essential for phosphorylation of all of the nucleoside analog diphosphates. Phosphorylation by K220R and N337Q was slightly better, although the increased side chain length conferred by Arg and Gln seemed to cause a steric hindrance. The varied impact of mutations on nucleoside analog diphosphates as substrates indicated that the orientation of the diphosphates in the catalytic site were probably different.

The effects of mutation of Asn\textsuperscript{337} and Lys\textsuperscript{220} on dephosphorylation of nucleoside analog triphosphates were also evaluated. In contrast to the forward reactions, the $k_{cat}$ value for dephosphorylation of all $\alpha$-nucleoside analog triphosphates increased upon mutation of Lys\textsuperscript{220} and Asn\textsuperscript{337} without affecting the binding. ddCTP was an exception; Lys\textsuperscript{220} mutation improved its $K_m$ value, whereas Asn\textsuperscript{337} mutation improved the $k_{cat}$ value, which implied that the interactions of $\beta$-ribonucleoside and $\beta$-deoxyribonucleoside analog triphosphates with Lys\textsuperscript{220} and Asn\textsuperscript{337} mutants were different from those of $\beta$-deoxyribonucleoside analog triphosphates. Increases in the $k_{cat}$ value upon K220R and N337Q mutation suggested that the increase in the length of the side chain allowed for a more favorable hydrogen bond formation between Arg or Gln and nucleoside analog triphosphates. Since both Ala and Asp are not capable of hydrogen bonding with the oxygen of $\alpha$-phosphate and the bridge oxygen between $\beta$- and $\gamma$-phosphate, respectively, it is proposed that these oxygen molecules interact with Ala and Asp through hydrogen bonds via ordered water molecules. For the $\alpha$-dideoxyribonucleoside analog diphosphates and its triphosphate are different despite both being substrates for PGK. The general assumption that the transitional state of enzyme is likely to be the same for the forward and reverse reactions of a reversible enzymatic process is not true for all enzymes.

In conclusion, favorable phosphorylation of pyrimidine $\beta$-deoxyribonucleoside analog diaphosphates as compared with the corresponding $\beta$-deoxyribonucleoside analogs by PGK is attributed to differences in the $k_{cat}$ value. These differences are consequences of different orientations of sugar and diaphosphate of $\alpha$- and $\beta$-nucleoside analogs during the transition state of PGK. The varied interactions of nucleoside analog diaphosphates and triphosphates with amino acids in the catalytic cleft indicated that the configuration of the transition state for the forward and reverse reactions are different, which is a property unique to PGK. The intracellular role of PGK in the phosphorylation of pyrimidine $\beta$-deoxyribonucleoside analogs is currently under investigation. Although anticancer and antiviral $\beta$-deoxy- and $\beta$-dideoxy nucleoside analogs like $\beta$-arabinofuranosylcytosine, gemcitabine, AXT, ddC, 2',3'-didehydro-2',3'-dideoxymethidine, etc., are unlikely to be phosphorylated by PGK, their accumulation in the cells as triphosphate metabolites may have an inhibitory effect on the enzyme. The impact of $\alpha$- and $\beta$-deoxyribonucleoside analogs and their phosphorylated metabolites in the regulation of the multiple cellular functions of PGK will be addressed in the future.

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