Stereoisomeric Selectivity of Human Deoxyribonucleoside Kinases

Jianghai Wang, Devapriya Choudhury, Jyoti Chattopadhyaya, and Staffan Eriksson

Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, Box 575, S-751 23 Uppsala, Sweden, Department of Molecular Biology, Swedish University of Agricultural Sciences, The Biomedical Centre, Box 590, S-751 24 Uppsala, Sweden, and Department of Bioorganic Chemistry, Uppsala University, The Biomedical Centre, Box 581, S-751 23 Uppsala, Sweden

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Abstract: Deoxynucleoside kinases catalyze the 5′-phosphorylation of 2′-deoxyribonucleosides with nucleoside triphosphates as phosphate donors. One of the cellular kinases, deoxycytidine kinase (dCK), has been shown to phosphorylate several L-nucleosides that are efficient antiviral agents. In this study we investigated the potentials of stereoisomers of the natural deoxyribonucleoside to serve as substrates for the recombinant cellular deoxynucleoside kinases. The cytosolic thymidine kinase exhibited a strict selectivity and phosphorylated only β-D-Thd, while the mitochondrial thymidine kinase (TK2) and deoxyguanosine kinase (dGK) as well as dCK all had broad substrate specificities. TK2 phosphorylated Thd and dCyd stereoisomers in the order: β-D- ≥ β-L- ≥ α-D- ≥ α-L-isomer. dCK activated both enantiomers of β-dCyd, β-dGuo, and β-dAdo with similar efficiencies, and α-D-dCyd also served as a substrate. dGK phosphorylated the β-dGuo enantiomers with no preference for the ribose configuration; α-L-dGuo was also phosphorylated, and β-L-dAdo and β-L-dCyd were substrates but showed reduced efficiencies. The anomer of the 2′,3′-dideoxy-d-nucleosides (ddNs) were tested, and TK2 and dCK retained their low selectivities. Unexpectedly, α-dideoxycytidine (ddC) was a 3-fold better substrate for dCK than β-ddC. Similarly, α-dideoxymethidine (ddT) was a better substrate for TK2 than β-ddT. dGK did not accept any D-ddNs. Thus, TK2, dCK, and dGK, similar to herpes simplex virus type 1 thymidine kinase (HSV-1 TK), showed relaxed stereoselectivities, and these results substantiate the functional similarities within this enzyme family. Docking simulations with the Thd isomers and the active site of HSV-1 TK showed that the viral enzyme may in some respects serve as a model for studying the substrate specificities of the cellular enzymes.

The precursors for DNA synthesis are mainly provided by ribonucleotide reduction, but in many organisms there exists a salvage system which makes direct use of nucleosides, either from DNA degradation or from uptake from extracellular sources, to form nucleotides. In mammalian cells, four kinases are involved in the salvage pathway, two of which, the cytosolic thymidine kinase (TK1) and the deoxycytidine kinase (dCK), function in the cytosol, and the other two, the mitochondrial thymidine kinase (TK2) and the deoxyguanosine kinase (dGK), are predominantly located in mitochondria (1−2). All four human kinases have been cloned and expressed in bacteria (4−9). Phylogenetic analysis indicated that dCK, TK2, and dGK share high similarities in their primary sequences, and form an enzyme family together with thymidine kinases (TKs) from several herpes viruses, e.g., herpes simplex virus type 1 (HSV-1). The structure for HSV-1 TK has been determined (10−13), and this is still the only solved structure of a deoxynucleoside kinase. TK1, on the other hand, diverges from this group, and its amino acid sequence is related to TKs from E. coli and Pox viruses (14, 15).

Nucleoside kinases play important roles in treatment of cancer and viral diseases because they are the enzymes which activate therapeutic nucleoside analogues. There are also higher levels of the kinases in rapidly proliferating cells such as cancer cells, as compared to resting cells (1). Nucleoside kinases of the herpesvirus family have broad substrate specificities, phosphorylating acyclic and unnatural stereo-isomeric forms of nucleoside analogues (16−18). Lately, a new field of antiviral drug development emerged with the synthesis of 2′,3′-dideoxy-3′-thiacytidine (BCH-189) and the evaluation of its biological activity (19). An important feature of this analogue is that it is a racemic mixture of β-DL-(±)-enantiomers, and further studies revealed that both isomers executed anti-human immunodeficiency virus (HIV) activity (20, 21), and they were both taken up and converted to monophosphates by cultured CEM cells. The cytoplasmic

1 Abbreviations: TK1, cytosolic thymidine kinase; dCK, deoxycytidine kinase; TK2, mitochondrial thymidine kinase; dGK, deoxyguanosine kinase; TK, thymidine kinase; HSV-1, herpes simplex virus type 1; HIV, human immunodeficiency virus; 3TC, 2′,3′-dideoxy-β-L-3′-thiacytidine; ddNs, 2′,3′-dideoxyribonucleosides; ddT, 2′,3′-dideoxythymidine; ddC, 2′,3′-dideoxycytidine; RTase, reverse transcriptase.
enzyme responsible for their initial activation was dCK (22, 23). The component with the unnatural β-L-configuration [3TC or L(−)-SddC] showed better antiviral effect and lower cytotoxicity than D(+) SddC (20, 21), and 3TC is now one of the most used anti-HIV drugs. A number of L-nucleoside analogues have since been synthesized and studied for their potential as antiviral drugs (16, 18, 24).

Nucleosides may exist in one of four stereoisomeric forms, i.e., β-D, β-L, α-D, and α-L isoforms. β-D-Nucleosides are selected by nature as the building blocks of nucleic acids, and the chemical forces responsible for this structural preference have been investigated (25). The observations where several L-nucleoside analogues were active with viral and cellular nucleoside kinases raised a question about the general stereospecificities of the cellular nucleoside kinases. Initiated by studies with 3TC, cellular dCK has been examined with other pairs of nucleoside enantiomers, and by now it has been established that dCK does not discriminate between D- and L- dCyd as its substrate (23, 26–28). A recent report showed that dCK can even use L-ATP as its phosphate donor (29). HSV-1 TK has been shown to be able to phosphorylate L-Thd, but cellular TK1 could not use this substrate (30). On the other hand, a relaxed enantioselectivity toward Thd and dCyd was observed with TK2 (31).

In this report, we examined the phosphorylation of the stereoisomers of the common nucleosides with defined preparations of the four cellular deoxynucleoside kinases, and the kinetic parameters were determined. An abstract of the most used anti-HIV drugs. A number of L-nucleoside analogues have since been synthesized and studied for their potential as antiviral drugs (16, 18, 24).

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In this report, we examined the phosphorylation of the stereoisomers of the common nucleosides with defined preparations of the four cellular deoxynucleoside kinases, and the kinetic parameters were determined. An abstract of part of this work has been published (32). The anomers of the 2′,3′-dideoxy-d-ribonucleosides (ddNs) have now been tested as substrates for the kinases, and computer docking experiments with the four Thd stereoisomers and the active site of HSV-1 TK are reported here. The viral TK appears to be a relatively good model for some of the cellular kinases and may help in the design of new pharmacologically active nucleoside analogues.

MATERIALS AND METHODS

Chemicals. β-D-deoxyribonucleosides were purchased from Sigma, and [γ-32P]ATP was from Amersham-Pharmacia.

Synthesis and Purification of the Nucleoside Stereoisomers and 2′, 3′-Dideoxy-d-ribonucleosides. The β-L-, α-D-, and α-L-stereoisomers of the nucleosides were prepared according to procedures described elsewhere and in the references therein (25). The purity of the nucleosides were higher than 99.5% as determined by NMR spectroscopy.

Purification of the Recombinant Human Deoxyribonucleoside Kinases. Recombinant TK2, dCK, and dGK were purified as described earlier (7, 9, 33). In the case of TK1, the coding sequence was PCR-amplified using plasmid pTK11 as the template (4). The 5′ sense primer, 5′CCATATGAGCTGACTAACCCTG, contained an Ndel site (underlined), and the 3′ reverse complement primer, 5′CGGGATCCCTCGATTGGGCAG, contained a BamHI site (underlined). The PCR product and the expression vector pET14b (Novagen) were digested with Ndel and BamHI before they were ligated to each other to yield pETK83, and the recombinant plasmid was transfected into E. coli host BL21(DE3) pLys (34). To express human TK1, an overnight culture of the recombinant bacteria was diluted 1:200 (in volume) into a fresh media, and the culture was grown with shaking at 37 °C until the optical absorbance at 600 nm reached 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 1 mM, and the culturing was continued for an additional 3 h. The bacteria were collected by centrifuging at 5000g for 20 min and resuspended in buffer A (20 mM Tris, pH 8.0, 500 mM KCl, 5 mM MgCl2, 10% glycerol, 0.5 mM phenylmethylsulfon fluoride, and 20 mM imidazole). Bacterial lysis was carried out with freezing/thawing 3 times in dry ice/ethanol. After being centrifuged at 100000g for 1.5 h, the crude extract was chromatographed on His-Bind® resin (Novagen), and the elution of the recombinant protein was performed with 500 mM imidazole. SDS–PAGE showed that the purity of the preparation is above 95%.

For the purpose of this study, the histidine-tags on all four kinases were not removed, since kinetic characterization had shown that the presence of the tag had no significant effect on the catalytic properties of the kinases (7, 9, 33, and unpublished experiments).

Enzyme Assays. Phosphorylation of nucleosides with the kinases was determined with the phosphoryl transfer assay as described earlier (35). The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 15 mM NaF, 125 mM KCl, 0.5 mg/mL bovine serum albumin, 10 mM dithiothreitol, 100 μM [γ-32P]ATP (10 μCi/μL), and substrate. The reaction was initiated by adding 50 ng of enzyme, proceeded by incubating at 37 °C for 15–30 min, and stopped by heating to 95 °C for 5 min. The separation and quantitation of the monophosphate products by polyethylene-imine–cellulose TLC was performed as described previously (35). The values obtained for the physiological substrate of each kinase, i.e., β-D-Thd for TK1 and TK2, β-D-dCyd for dCK, and β-D-dGuo for dGK, at saturating concentrations were set as 1, and the phosphorylations of other substrates with the corresponding enzyme were expressed relative to this value. For determination of the apparent Km and Vmax values, five to seven concentrations of a compound were assayed. The data for each concentration were transformed into relative phosphorylation rates and analyzed with the software KaleidaGraph 3.0.8 (Synergy), and curves were plotted using the Michaelis–Menten equation. The Km and Vmax values and their standard deviations were calculated by the program.

Computer-Assisted Molecular Modeling. The substrate docking simulations were carried out using the Monte Carlo simulated annealing procedure as implemented in the AutoDock suite of programs (36). The protein coordinates used in this study are the X-ray coordinates of HSV-1 TK determined at 2.75 Å resolution (12). Initial coordinates of the substrate molecules were generated manually from standard bond lengths, angles, and torsion angles. Polar hydrogens were explicitly added for both the substrates and the protein. Partial charges for the protein atoms were taken from an earlier study (37) while the charges for the substrate atoms were calculated using the AM1 procedure as implemented in the MOPAC suite of programs (38).

The docking procedure starts with the generation of a grid of affinity potentials representing both the shape as well as the electrostatic properties of the active site cavity in the protein. The substrates were then placed on this grid and allowed to perform a random walk within this grid. At each
step, the substrate was translated and rotated around its center, and the value of its flexible torsion angles was changed by a random amount, while its interaction energy with the protein was evaluated. During the simulation course, the glycosidic torsion angle (C2=N1=C1′−O4′, $\chi$) and the exocyclic torsion angle (C3′−C4′−C5′−O5′, $\gamma$) were allowed to vary around the sugar ring. Because of the technical limitations of the program, however, the sugar pucker had to be kept fixed. We attempted to partially alleviate the problem by designing 16 conformations to sample the entire accessible pseudorotational space. Coordinates for each of the Thd stereoisomers varying in pseudorotational phase ($P$) as well as the maximum torsion angle ($\varphi_{\text{max}}$) of the ribose sugar were generated according to the procedure described previously (39). Prior to the docking simulations, the geometry was further optimized by energy minimization using the MOPAC suite of programs (38). The conformation corresponding to the lowest interaction energy for each of the four nucleosides was used for further analysis. Parallel simulations were carried out with each starting model. The acceptance or rejection of a state depended on its interaction energy as well as the temperature factor. To ensure proper sampling of the state space, the initial temperature was set to a very high value and gradually reduced as the simulation proceeded. For each substrate, at least 100 simulated annealing runs were performed with 5000 intermediate states in each run. For the convenience of discussion, only the final structure of each conformation with the lowest interaction energy is presented.

RESULTS

Kinetics of Phosphorylation of the Deoxyribonucleoside Stereoisomers with the Human Deoxynucleoside Kinases. All four stereoisomers ($\beta$-$D$-, $\beta$-$L$-, $\alpha$-$D$-, and $\alpha$-$L$-) of the four deoxyribonucleosides, i.e., Thd, dCyd, dGuo, and dAdo (except $\alpha$-$D$-dGuo), were initially examined at a fixed concentration (100 $\mu$M) with the four human nucleoside kinases completely purified from bacterial expression of the recombinant enzymes. The results have been briefly presented previously (32), and in summary they showed that TK1 had a strict stereoselectivity, whereas dCK, TK2, and dCK displayed poor stereodiscrimination. Among the 15 nucleosides, only $\beta$-$D$-Thd was accepted by TK1 as a good substrate, while $\beta$-$L$- and $\alpha$-$D$-Thd were phosphorylated at a rate only 2–5% of that for $\beta$-$D$-Thd. TK2, on the other hand, showed a relaxed selectivity and phosphorylated $\beta$-$L$-Thd and both enantiomers of $\alpha$-Thd relatively well. It was known that TK2 can accept dCyd as substrate (35), and our results revealed that $\beta$-$L$-dCyd was an even better substrate for TK2 than $\beta$-$D$-dCyd (90% versus 56% in relative phosphorylation). Detectable activities with TK2 were also found for both enantiomers of $\alpha$-dCyd. dCK was known to have low enantioselectivity (26, 28). Compared to $\beta$-$D$-dCyd, the relative phosphorylation of $\beta$-$L$-, $\alpha$-$D$-, and $\alpha$-$L$-dCyd with dCK was 42%, 43%, and 11%, respectively. With purine $\beta$-nucleosides, the $L$-isomers were poorer substrates for dCK than the $D$-isomers. $\alpha$-$L$-dGuo and the enantiomers of $\alpha$-dAdo all showed <10% relative activities with dCK. Surprisingly, $\beta$-$L$-Thd was phosphorylated by dCK. dGK phosphorylated $\beta$-$D$- and $\beta$-$L$-dGuo equally well, while the $\alpha$-$L$-isomer was activated at a level roughly one-third of that for its $\beta$-counterparts. Unexpectedly, $\beta$-$L$-dCyd was phosphorylated to a high extent by dGK.

The apparent $K_m$ and $V_{\text{max}}$ values were determined for the active nucleosides by the phosphoryl transfer assay, using 100 $\mu$M ATP as the phosphate donor, and the apparent relative efficiencies ($V_{\text{max}}/K_m$) of the various substrates were calculated. With TK2, all four isomers of Thd and dCyd were examined. $\beta$-$L$-Thd showed a 2.5-fold higher $K_m$ than $\beta$-$D$-Thd (Table 1). $\alpha$-$D$- and $\alpha$-$L$-Thd exhibited 1 order of magnitude higher $K_m$ values, and the relative efficiencies of $\beta$-$L$-, $\alpha$-$D$-, and $\alpha$-$L$-Thd with TK2, in relation to that for $\beta$-$D$-Thd, were 60%, 2.4%, and 1.5%, respectively. Similarly, $\beta$-$D$- and $\beta$-$L$-dCyd showed close $K_m$ and $V_{\text{max}}$ values, while both enantiomers of $\alpha$-dCyd had low $V_{\text{max}}$ and high $K_m$ values (Table 1).

The kinetic parameters for various isoforms of the four nucleosides with dCK are shown in Table 2. The $K_m$ value obtained for $\beta$-$D$-dCyd was 6.3 $\mu$M, which is considerably higher than that reported in earlier studies (40, 41), and this value probably represents the high $K_m$ value ($K_m^a$) in the bimodal kinetics described for dCK (33, 41). $\beta$-$D$-dCyd and $\beta$-$L$-dCyd showed similar $K_m$ values, but the $V_{\text{max}}$ for $\beta$-$L$-dCyd was 2.5-fold lower. The $\beta$-$L$-isomers of the purine nucleosides showed $K_m$ values several times lower than their $\beta$-$D$-counterparts, but in both cases the $V_{\text{max}}$ values for the former isomers were also severalfold lower than the latter. The net result of these is that $D$- and $L$-isomers of $\beta$-$D$-dGuo and $\beta$-$D$-dAdo exhibited similar apparent efficiencies, and they are of the same order of magnitude as for $\beta$-$D$-dCyd isomers (Table 2). Surprisingly, $\beta$-$L$-Thd was a relatively good substrate for dCK, although $\beta$-$D$-Thd, as shown earlier (35), was not.

Three stereoisomers of dGuo ($\beta$-$D$-, $\beta$-$L$-, and $\alpha$-$L$-) and both isomers of $\beta$-$D$-dCyd and $\beta$-$D$-dAdo were examined with

![Table 1: Kinetic Parameters for the Thd and dCyd Stereoisomers with Human TK2](image)

<table>
<thead>
<tr>
<th>substrate</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{\text{max}}$</th>
<th>relative efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$D$</td>
<td>9.6 ± 2.1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>$\beta$-$L$</td>
<td>24 ± 0.8</td>
<td>1.5 ± 0.11</td>
<td>60</td>
</tr>
<tr>
<td>$\alpha$-$D$</td>
<td>300 ± 95</td>
<td>0.75 ± 0.06</td>
<td>2.4</td>
</tr>
<tr>
<td>$\alpha$-$L$</td>
<td>318 ± 102</td>
<td>0.51 ± 0.05</td>
<td>1.5</td>
</tr>
<tr>
<td>dCyd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$D$</td>
<td>6.9 ± 0.2</td>
<td>0.67 ± 0.04</td>
<td>93</td>
</tr>
<tr>
<td>$\beta$-$L$</td>
<td>15 ± 3.6</td>
<td>1.09 ± 0.02</td>
<td>70</td>
</tr>
<tr>
<td>$\alpha$-$D$</td>
<td>161 ± 57</td>
<td>0.31 ± 0.12</td>
<td>1.8</td>
</tr>
<tr>
<td>$\alpha$-$L$</td>
<td>143 ± 98</td>
<td>0.06 ± 0.01</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The relative efficiency was obtained by comparing the $V_{\text{max}}/K_m$ value of the substrates to that of $\beta$-$D$-thymidine.

![Table 2: Kinetic Parameters for the Deoxynucleoside Stereoisomers with Human dCK](image)

<table>
<thead>
<tr>
<th>substrate</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{\text{max}}$</th>
<th>relative efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCyd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$D$</td>
<td>6.3 ± 2.5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>$\beta$-$L$</td>
<td>6.2 ± 1.3</td>
<td>0.39 ± 0.05</td>
<td>40</td>
</tr>
<tr>
<td>$\alpha$-$D$</td>
<td>364 ± 79</td>
<td>1.04 ± 0.15</td>
<td>1.8</td>
</tr>
<tr>
<td>Thd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$L$</td>
<td>272 ± 88</td>
<td>0.36 ± 0.08</td>
<td>0.8</td>
</tr>
<tr>
<td>dGuo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$D$</td>
<td>93 ± 42</td>
<td>4.74 ± 0.28</td>
<td>32</td>
</tr>
<tr>
<td>$\beta$-$L$</td>
<td>9.8 ± 3.2</td>
<td>0.45 ± 0.05</td>
<td>29</td>
</tr>
<tr>
<td>dAdo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-$D$</td>
<td>80 ± 23</td>
<td>4.98 ± 0.79</td>
<td>39</td>
</tr>
<tr>
<td>$\beta$-$L$</td>
<td>26 ± 4.2</td>
<td>1.69 ± 0.11</td>
<td>41</td>
</tr>
</tbody>
</table>

The relative efficiency was obtained by comparing the $V_{\text{max}}/K_m$ value of the substrates to that of $\beta$-$D$-deoxyctydine.
dGK (Table 3). β-L-dGuo displayed a $K_m$ and relative $V_{max}$ value similar to the natural isomer. The rest of the nucleosides all showed high $K_m$ values, ranging from 32 to over 300 μM. As a result, although the $V_{max}$ values were generally much higher than that for β-d-Guo, the relative efficiencies of these nucleosides varied between 1.6% and 17%. It is worth noting that β-L-dAdo showed a comparatively low $K_m$ value, resulting in an enzymatic efficiency with dGK 7-fold higher than that for β-d-dAdo.

Activity of 2′,3′-Dideoxy-d-ribonucleoside Anomers with the Cellular Kinases. The capacities of the nucleoside kinases to phosphorylate anomer of D-ddNs were initially examined with the substrate at a fixed concentration of 100 μM. TK1 phosphorylated 2′,3′-dideoxy-β-d-thymidine (d-ddT) at a relative rate of 48% compared to β-d-Thd, consistent with the earlier observation (35), and this compound was the only nucleoside among the eight d-ddNs tested here that could be recognized by TK1. TK2, on the other hand, phosphorylated β- and α-ddT to 7% and 29%, respectively, in relation to β-d-Thd, and showed minimal activity with α-ddC and none with β-ddC, thus exhibiting a preference for α-dideoxy-pyrimidine over the β-anomer. With dCK, however, α-ddC was phosphorylated at a relative rate of 59%, while its β-anomer was activated 97% as much as β-d-dCyd. dGK showed no activity with any of the d-ddNs, and none of the d-dideoxypurine nucleoside anomers could be phosphorylated by any of the nucleoside kinases.

The $K_m$ and $V_{max}$ values for β-ddT with TK1, for β- and α-ddT with TK2, and for the d-ddC anomers with dCK are listed in Table 4. The $K_m$ for β-ddT with TK1 was more than 30-fold higher than that for the natural substrate, resulting in a relative efficiency around 4% of that for β-d-Thd. The $V_{max}$ values for both anomers of d-ddT were low with TK2, but their $K_m$ values were only 2–3-fold higher than that for β-d-Thd, and, surprisingly, α-ddT exhibited an enzymatic efficiency 3 times as high as β-ddT. This unexpected preference for α-anomer was also observed with dCK, with which β-ddC showed a $V_{max}$ about 3-fold higher, and a $K_m$ value 11-fold higher than α-ddC, resulting in the apparent enzymatic efficiency of the latter isomer 4 times that of β-ddC (Table 4).

Computerized Docking of Thymidine Stereoisomers to the Active Site of HSV-1 TK. TK2, dCK, and dGK share sequence similarities with HSV-1 TK (15). Since the 3-D structure of HSV-1 TK is known (11, 12), a series of docking simulations were performed with the active site of HSV-1 TK and the Thd stereoisomers. The superimpositions of the docked substrate stereoisomers with the lowest interaction energy are shown in Figure 1, together with the three amino acid residues of HSV-1 TK within 3 Å radius of the substrate, residue Glu83 being close to the 5′-OH group, Glu225 interacting with the 3′-OH group, and Gln125 being adjacent to the pyrimidine ring (12, 42). It is clear that both β-d- and α-d-Thd make similar interactions with the protein, after aligning the base and the 5′-OH moieties of the two nucleosides by mean of adjustments in the glycosidic torsion angle (C2′-N1-C1′-O4′, γ) and the exocyclic torsion angle (C3′-C4′-C5′-O5′, γ) (Figure 1A). In α-d-Thd, these two angles were found to be trans while in β-d-Thd the γ torsion angle became gauche+. The net effect of this is that the mean plane of the sugar ring is roughly perpendicular to the plane of the base in α-d-Thd, whereby the 5′-OH can be placed in the same position as in β-d-Thd.

When the substrate is β-L-Thd, a much smaller conformational change was required to align the base and the 5′-OH group to the conformation of β-d-Thd (Figure 1B). These two moieties could be positioned in relatively similar conformations without any drastic rearrangement, but the O4′ atom of the sugar ring in β-L-Thd now points in the opposite direction as compared to that in β-d-Thd. From the docking simulations, it appears that all four stereoisomers of Thd can bind to the enzyme with comparable efficiencies. The interaction energies varied slightly with different sugar puckers. However, this effect was largely neutralized by the
free rotation of the sugar plane about the exocyclic single bonds. Notwithstanding the above observation, there may be a quantitative influence of the sugar pucker on the binding strengths. Further computational studies are underway to elucidate this point.

The protein coordinates used in this study were from the HSV-1 TK structure (1VTK.pdb) determined crystallographically at 2.75 Å resolution (12). This structure was chosen because it was complexed with dTMP and ATP, the ligands most similar to the substrates discussed here. To ascertain the effect of the relatively low-resolution structure on the coordinates, this structure used here was compared with another HSV-1 TK structure (2KI5.pdb) solved at 1.9 Å resolution (43). It was found that the root-mean-square (RMS) deviation between the two structures was only 0.689 Å, indicating that they are very similar and the results of the docking simulation should be valid.

**DISCUSSION**

This work describes the capacity of defined and highly active preparations of the four cellular nucleoside kinases to phosphorylate pure stereoisomers of 2'-deoxynucleosides and 2',3'-dideoxy-d-ribonucleosides. For technical reasons, we used the method of phosphoryl transfer assay with 100 μM ATP as the phosphate donor. This is obviously not physiological condition since the cellular ATP level is 20–40-fold higher in most cells, and for some of the cellular kinases UTP may serve as the in vivo physiological phosphate donor (1, and references cited therein). The kinetic parameters reported here are therefore apparent Kₘ and Vₘₐₓ values and are only valid for this specified assay condition.

TK1 is genetically and biochemically very different from other members of the cellular deoxynucleoside kinase family exemplified by dCK (15). Although minor modifications in either the base or the sugar moieties were acceptable (35), no configuration other than the β-D-form appeared to be recognized by this enzyme. TK2 phosphorylated Thd and dCyd, and, only to a very minor extent, purine nucleosides and both enantiomers of β-Thd had similar Kₘ and Vₘₐₓ values. TK2 was also able to use α-Thd and β-dCyd enantiomers as its substrates, indicating that TK2 has minimal selectivity for the sugar configuration. The activity of β-L-Thd with TK2 has been reported earlier (31), and our results are in agreement with that study, although the sources and preparations of the enzymes are different. The 3'-hydroxyl group seems to be important for the phosphorylation of nucleosides by TK2, since both α-ddT anomer showed low Vₘₐₓ values with this enzyme. But the relatively low Kₘ values of this pair of anomers indicated that the 3'-OH group might not interfere substantially with the binding to TK2.

The stereoselectivity of dCK has already received much attention, but our results provide a general comparison among the complete set of nucleoside stereoisomers. This enzyme seemed to have a higher affinity for β-L-isomers than for the β-D-isomers, although the catalytic velocity is higher with substrates in the natural configuration. Overall, no significant difference in kinetic efficiency was found between β-D- and β-L-substrates, and the same conclusion was also reached earlier (26, 28, 44). We describe here for the first time the capability of dCK to phosphorylate α-D- as well as α-L-dCyd, which further exemplifies the remarkable capacity of this enzyme to accommodate various nucleosides in the active site. The fact that dCK can phosphorylate β-L-Thd is in line with the recent observation that dCK could activate the antiviral analogue 2'-fluoro-5-methyl-β-L-arabinofuranosyl-uracil (L-FMAU) (45), and this may significantly extend the possibility to discover new pharmacologically active pyrimidine analogues.

The stereoselectivity of dGK has not been much studied, but enantiomers of the carbocyclic analogue of β-dGuo have been tested with dCK and dGK, and the L-isomer was shown to have lower Kₘ and higher Vₘₐₓ values compared to its d-isomer (46). We disclosed here that dGK, just like TK2 and dCK, does not distinguish efficiently between d- and L-isomers of β-nucleosides. Interestingly, dGK unexpectedly displayed a high Vₘₐₓ level with β-L-dCyd. A recent complementary study using different assay conditions but the same preparations of recombinant dGK and dCK as in this work has led to similar conclusions for some of the analogues (44).

The results presented here unambiguously demonstrate that there is no discrimination against L-nucleosides by dCK, TK2, and dGK, and, in some cases, they apparently are preferred substrates. In this study only recombinant enzymes were used. A parallel study performed with TK1, TK2, and dCK purified from cells has shown some results significantly different from ours (47). For instance, purified lymphoblast TK1 showed a relatively high activity with α-Thd. However, the overall conclusions regarding the stereoselectivities of the cellular kinases agree between the two investigations.

We also described in this report that pyrimidine α-ddNs are relatively good substrates for dCK and TK2 compared to the β-counterparts. This has not been recognized earlier, even though α-Cyd was shown to be an inhibitor for calf thymus dCK (48), and L-(-)-dioxolane-cytidine (L-OddC) showed high cytostatic activity and was a substrate of dCK (49). The relatively low Kₘ value for α-ddC indicates that dCK has high affinity for this analogue. Earlier study has established that β-d-nucleosides have more flexible conformations than the corresponding α-anomers (25), and that α-dideoxynucleosides are often driven into the N-conformation (C2'-exo-C3'-endo), while β-ddNs are predominantly found in the S-conformation (C2'-endo-C3'-exo). Therefore, we may conclude that the S type of α-ddC serves as a better substrate for dCK than the N-type counterpart. The preference for the S-conformation in the anabolism of active anti-HIV nucleosides has been reported earlier (50). On the other hand, it has been shown recently that HIV reverse transcriptase (RTase) interacts selectively with the N-type conformation of deoxyribonucleoside triphosphate analogues (51). The fact that the cellular kinases have different specificities compared to HIV-RTase, with regard to the sugar conformation, is one of the complications in the design of more efficient anti-HIV nucleosides.

The phosphorylation of α-nucleosides by cellular kinases demonstrated here may be involved in the cytotoxicity observed with several analogues with anti-HIV activity, e.g., α-L-oxathiolane-5-methylcytosine and α-L-oxathiolane-5-fluorocytosine (52), as well as α-L-dioxolanyl-5-fluorocytosine (53). The results also emphasize the central role of dCK in the anabolism of chemotherapeutic nucleosides. It is yet to be determined if TK2 also contributes significantly
to the cytotoxic or antiviral effects of nucleoside analogues in vivo.

The similarity of TK2, dCK, and dGK in their substrate specificities is likely to be a reflection of the fact that these three enzymes show substantial sequence identity and belong to the same family (15). These proteins probably have similar 3-D structures which also most likely resemble that of HSV-1 TK, especially in the catalytic cavity. By applying the coordinates of HSV-1 TK to a molecular docking experiment with some of these substrates, we attempted to evaluate which of the Thd stereoisomers best fitted into the active site of the protein, β-D- and β-L-Thd could easily be docked in with almost identical positions of the base and the 5′-OH group, provided that the stereocchemical orientation of the sugar ring was reversed with regard to the O4′ atom. The docking experiments implied that no obvious preference for Thd with S- or N-type sugar pucker was exhibited by HSV-1 TK. This indicates that the primary requirement for the binding is a proper alignment of the base and the 5′-OH moieties, and there seems to be many ways to achieve this by concerted variations of the sugar pucker and by rotations around the glycosidic bond and the C4′−C5′ bond. Further analysis of the influence of the sugar conformation on the interaction with the active site of kinases is presently under investigation. In general, the modeling results and the calculated energies are consistent with the kinetic data presented above with the cellular kinases, and with earlier studies with viral TK (17, 30). However, the molecular details of the cellular enzymes remain to be determined, since there are dramatic differences in the primary sequence between the cellular and viral kinases. Nevertheless, this report should encourage further attempts to develop chemotherapeutically active nucleoside analogues of different stereoisomeric configurations.

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