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Chiral Discrimination of 2′-Deoxy-L-cytidine and L-Nucleotides by Mouse Deoxycytidine Kinase: Low Stereospecificities for Substrates and Effectors[1]

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The effects of four kinds of 2′-deoxy-L-nucleoside 5′-triphosphates and L-ATP, which are enantiomers of natural D-dNTPs and D-ATP, on deoxycytidine kinase (dCK) partially purified from mouse leukemic P388 cells were investigated. Only L-dCTP did not act as a phosphate donor while other L-dNTPs and L-ATP showed 15-30% of the activity of the corresponding D-dNTP or D-ATP. L-dCTP inhibited dCK non-competitively with 2′-deoxycytidine (D-dCyd) and competitively with phosphate donor D-ATP. These inhibitory effects of L-dCTP on dCK were similar to the results of earlier studies using D-dCTP. Thus, L-dCTP was shown to be capable of serving as a feedback inhibitor for dCK instead of D-dCTP. Mouse dCK was also able to phosphorylate L-dCyd, as demonstrated in the case of human dCK. The present results suggest that the chirality of not only dCyd as the substrate but also nucleotides as the substrate or effector is not strictly discriminated by dCK. © 1997 Academic Press

Enzymes are constructed from only L-enantiomers of amino acids, and are thought to exhibit high substrate specificity both in binding chiral substrates and in catalyzing their reactions, since their active sites can form asymmetric and geometrically complementary shapes for their chiral substrates. However, in recent years, it has been reported that several L-enantiomers of nucleoside analogs (Figure 1) exert notable biological activities, such as anti-HIV-1 (1-5), anti-hepatitis B virus (6), and anticancer activities (7). In order to exert their biological activities, these nucleoside analogs need to be converted to their respective 5′-monophosphate derivatives by deoxynucleoside kinase(s) (7,8), especially cellular deoxycytidine kinase (dCK), an enzyme related to the salvage pathway (9,10). Additional phosphorylations of the 5′-monophosphates yield 5′-triphosphate derivatives, which then inhibit viral or cellular DNA polymerase(s) (7,11). Thus, dCK appears to be an enzyme with low enantiospecificity. More recently, Verri *et al.* demonstrated that an L-enantiomer of 2′-deoxycytidine (dCyd) (Fig. 1) is phosphorylated as efficiently as the natural substrate, D-dCyd, by human dCK (12). This finding prompted us to investigate in detail the chiral discrimination of nucleotides by dCK. dCK is the rate-limiting enzyme in the activation for several clinically important antitumor agents. From early studies, dCK has been recognized as an enzyme with broad substrate specificity, phosphorylating the pyrimidines, dCyd and cytidine (Cyd), and the purines, 2′-deoxyguanosine (dGuo) and 2′-deoxyadenosine (dAdo), as well as many biologically active nucleoside analogs, such as 9-β-D-arabinofuranosylcytosine (araC), 2′,3′-dideoxycytidine (ddCyd), 2′,2′-diflurodeoxycytidine (dFdC) and 2-chloro-2′-deoxyadenosine (10,13-15). Additionally, dCK has a broad specificity with regard to phosphate donors (16-20). Of the naturally occurring D-NTPs and D-dNTPs, only D-dCTP is unable to donate a phosphate group in the reaction (16). dCTP, the end product of the salvage pathway initiated by dCK, was an efficient inhibitor of dCK (19-23) and dCTP probably serve as the physiological feedback regulator. We describe here the chiral specificity of mouse dCK in recognizing dCyd, ATP, dNTPs and dCTP as substrates or effectors.

MATERIALS AND METHODS

Inhibitors. L-dTTP was synthesized as described previously (24). L-Adenosine (25), N^4-Benzoyl-L-dCyd, 2′-deoxy-L-adenosine and 2′-

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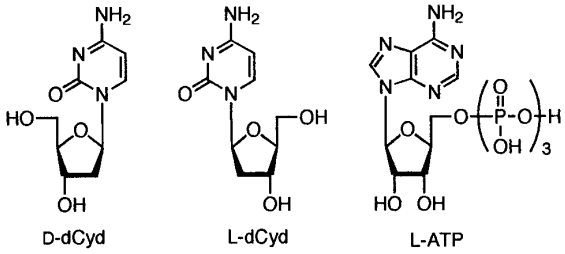

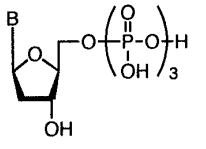

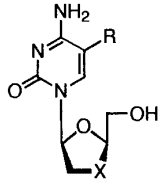

L-dNTP	B
L-dATP	Adenin-9-yl
L-dGTP	Guanin-9-yl
L-dCTP	Cytosin-1-yl
L-dTTP	Thymin-1-yl

	X	R
1: BCH-189	S	H
2: FTC	S	F
3: L-ddC	CH_2	H
4: L-FddC	CH_2	F
5: L-(−)-OddC	O	H

FIG. 1. Enantiomers of deoxycytidine (dCyd), L-enantiomers of deoxyribonucleoside 5′-triphosphates (dNTPs) and ATP, and biologically active L-deoxycytidine analogs (**1-5**).

deoxy-L-guanosine (26) were converted to L-ATP (27), N^4-benzoyl-L-dCTP, L-dATP and L-dGTP by selective phosphorylation with $POCl_3$ in triethylphosphate (28), followed by further phosphorylation using the phosphoroimidazolidate method (29). L-dCTP was obtained by treatment of N^4-benzoyl-L-dCTP with 1M NH_4OH. When analyzed by HPLC (TSK-GEL, DEAE-2SW column, 4.6 mm × 25 cm/ 0.21 M potassium phosphate buffer, pH 6.95, containing 20% CH_3CN), the retention times of the four kinds of L-dNTPs and L-ATP agreed with those of the corresponding D-enantiomers and the purities of these compounds measured by UV absorption at λ_{max} were confirmed to be greater than 95%.

Enzyme assays. dCK activity was measured using a radiochemical method described by Karlsson *et al.* (30). The reaction mixture (25 μl) comprised 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 500 μg/ml BSA, 15 mM NaF, 4 mM D-ATP as the phosphate donor, 5 μM [^{3}H]D-dCyd (0.4 μCi, 400 cpm/pmol) and the enzyme preparation (0.1 unit). Incubation was carried out for 10 or 20 min at 37°C, and 20 μl of the mixture was transferred to a Whatman DE81 filter disk. The filter was washed three times with 5 mM ammonium formate and twice with distilled water, then dried. Radioactivity of D-dCMP retained on the paper was measured (8). One unit was defined as the amount of enzyme catalyzing the formation of 1 nmol of D-dCMP in 60 min using the incubation mixture described.

[^{32}P]Phosphate transfer assay using γ-^{32}P-labeled D-ATP (Amersham) as the phosphate donor was performed as follows. The enzyme (0.2 unit) was incubated in the reaction mixture (10 μl) described above, except that 0.5 mM [γ-^{32}P]D-ATP in place of 4 mM D-ATP was used, and the concentration of D-dCyd or L-dCyd was varied to 200 μM. After incubation for 1 h at 37°C, each mixture was heated for 5 min at 95°C and a 1-μl aliquot of the mixture was spotted onto paper (Advantec, No 51A) which had been previously spotted with D-ATP and dCMP as markers. Radioactive ATP and the phosphorylated products were separated by paper electrophoresis in 50 mM sodium citrate buffer, pH 3.5, for 1 h at 400 V. The radioactivity of the products was determined by Cerenkov counting after excising the radioactive band from the paper.

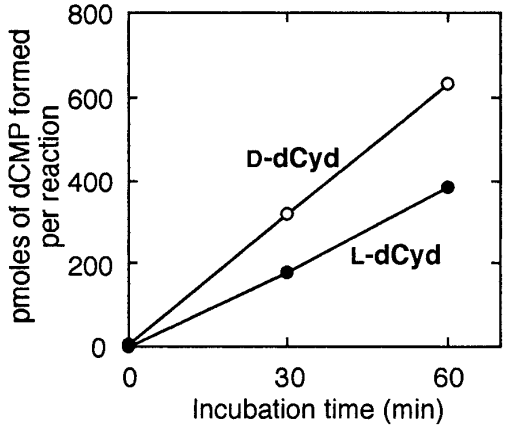

FIG. 2. Phosphorylation of D-dCyd and L-dCyd by dCK. The enzyme activity was measured by [^{32}P]phosphate transfer assay as described in 'Materials and Methods'.

Purification of dCK from mouse P388 cells. dCK was partially purified from crude extract of P388 cells (wet 4.0 g), by fractionation with ammonium sulfate, and by column chromatography on Sephadex G-150, DEAE-cellulose and dCTP-agarose, as described by Datta *et al.* (18,20). The final preparation had a specific enzyme activity of 700 units/mg protein.

RESULTS

A comparison of D-dCyd and L-dCyd as substrates for mouse dCK was performed by comparing the phosphate group transfer reaction from [γ-^{32}P]D-ATP to the substrate. The data presented in Figure 2 demonstrate that L-dCyd was convertible to the monophosphate derivative by dCK and that its phosphorylation efficiency was approximately 60% that of D-dCyd.

We investigated the effect of L-dCyd on the phosphorylation of [^{3}H]D-dCyd by mouse dCK in the presence of D-ATP as a phosphate donor (Figure 3). Under our

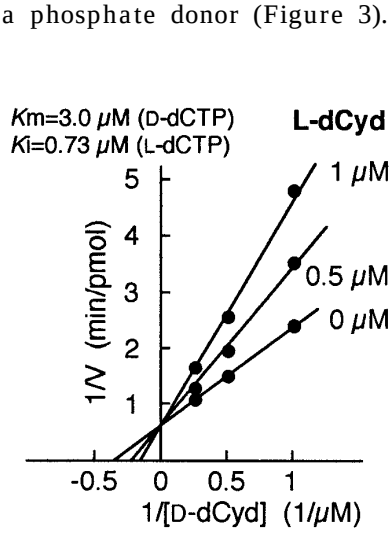

FIG. 3. Inhibitory effect of L-dCyd on the phosphorylation of [^{3}H]D-dCyd by mouse dCK. The figure presents a Lineweaver-Burk plot.

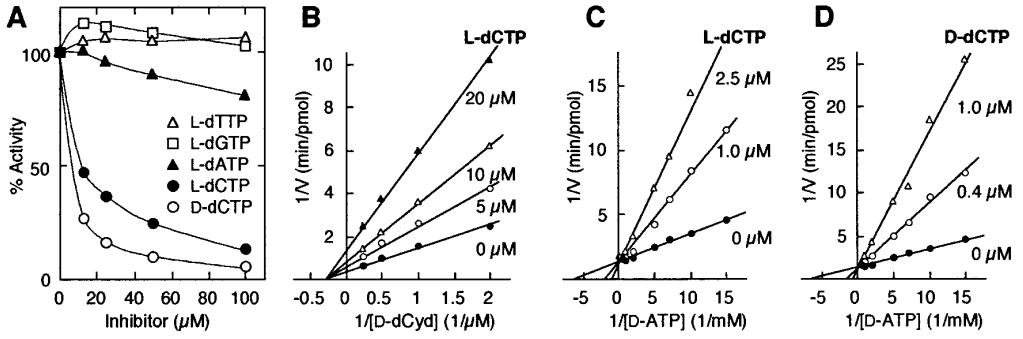

FIG. 4. Inhibitory effects of L-dNTPs and D-dCTP on the phosphorylation of [^{3}H]D-dCyd by mouse dCK (**A**). Activity without inhibitor was taken as 100%, and the remaining activities in the presence of various concentrations of inhibitors were shown. Lineweaver-Burk plot analyses of the inhibition by L-dCTP (**B** and **C**) and D-dCTP (**D**). Analyses were performed by changing the concentrations of both L-dCTP and [^{3}H]D-dCyd (**B**), L-dCTP and D-ATP (**C**) or D-dCTP and D-ATP (**D**).

assay conditions, L-dCyd showed potent inhibitory effects on dCK activity. The mode of the inhibitory action by L-dCyd was competitive with respect to D-dCyd. The apparent *K*i value (0.73 μM) for L-dCyd was 4 times lower than the *K*m for D-dCyd (3.0 μM).

The effect of the chirality of the feedback inhibitor, dCTP, on dCK was investigated. As shown in Figure 4, among L-enantiomers of natural D-dNTPs, only L-dCTP showed a significant inhibitory effect on D-dCyd phosphorylation activity (Figure 4A). However, this inhibition was slightly weaker than that of D-dCTP. The mode of the inhibitory action of L-dCTP was non-competitive with respect to D-dCyd (Figure 4B) and competitive with respect to D-ATP (Figure 4C). The *K*i values for L-dCTP and D-dCTP were 6.4 and 2.5 μM, respectively. Thus it was revealed that mouse dCK cannot discriminate strictly the chirality of dCTP as a feedback inhibitor at ATP binding site or a site very close to it.

The phosphate donor specificity of dCK was then assessed (Figure 5). When the concentration of the phosphate donor was 400 μM, L-ATP showed 30% activity in comparison with D-ATP. L-dCTP was unable to act as a phosphate donor like D-dCTP, while other L-dNTPs showed 15-26% the activity of the corresponding D-dNTP. Thus, it was demonstrated that mouse dCK cannot discriminate strictly the chirality of these enantiomers and recognize them as phosphate donors. To compare the ability of D-ATP and L-ATP as phosphate donors, the phosphate transfer reaction catalyzed by dCK was analyzed kinetically. As shown in Figure 6A, the apparent V_{max} values of dCMP production by dCK with D-ATP and L-ATP under the same assay conditions were 0.78 and 0.25 pmol/min, respectively. This reaction was inhibited by D-dCTP almost competitively at a L-ATP concentration higher than 0.25 mM (Figure 6B). Thus, it was clarified that the L-enantiomer of ATP can serve as the phosphate donor for dCK like D-ATP, although the apparent V_{max} value of L-ATP was 3 times smaller than that of D-ATP.

DISCUSSION

Enzymes are thought to be able to bind effectively to only one enantiomer of a chiral substrate at the active site in order to perform their functions. However, among enzymes involved in the synthesis of nucleotides and nucleic acids, some exceptions have recently been reported. HIV-1 reverse transcriptase utilized 2′,3′-dideoxy-L-thymidine 5′-triphosphate (L-ddTTP) and 2′,3′-dideoxy-2′,3′-didehydro-L-thymidine 5′-triphosphate (L-d4TTP) as substrates (31). L-dTTP has been shown to be an inhibitor of HIV-1 reverse transcriptase (24), and to be incorporated into the elongated primer as L-dTMP by DNA polymerase α and HIV-1 reverse transcriptase when poly(rA)/olligo(dT) or poly(dA)/oligo(dT) was used as the template-primer (32). L-dTTP was incorporated into the DNA chain by terminal transferase (33). It has been demonstrated that herpes

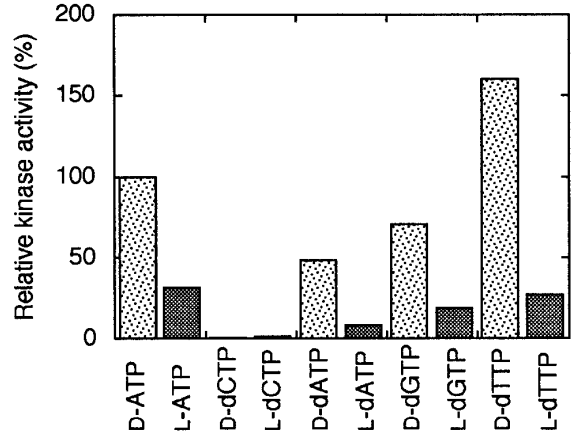

FIG. 5. Effect of phosphate donors on the phosphorylation of [^{3}H]D-dCyd by mouse dCK. The enzyme activity was measured under standard assay conditions, except that 0.4 mM nucleoside 5′-triphosphate indicated in the figure was used instead of 4 mM D-ATP. Activities are expressed as the percentage of the enzyme activity with 0.4 mM D-ATP.

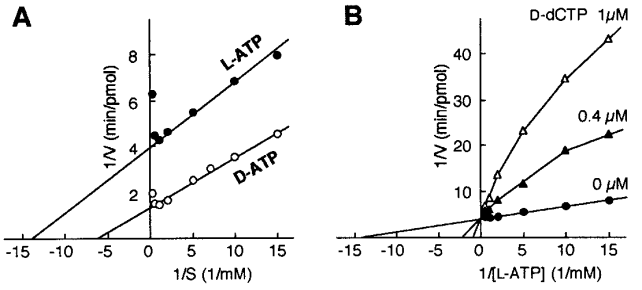

FIG. 6. Comparison of D-ATP and L-ATP as a phosphate donor (**A**), and inhibition of dCK by D-dCTP in the presence of L-ATP as a phosphate donor (**B**). The figures present Lineweaver-Burk plot analyses.

simplex virus type 1 thymidine kinase phosphorylates both enantiomers of thymidine and some thymidine analogs (34,35). More recently, Verri *et al.* demonstrated that L-dCyd was phosphorylated with the same efficiency as the natural substrate, D-dCyd, by human dCK (12). We studied here the chiral discrimination of dCyd as the phosphate acceptor, and dATP, dGTP, dTTP and ATP as the phosphate donor, by mouse dCK. It was found that dCK cannot discriminate strictly the chirality of dCyd and these nucleotides, and recognizes them as substrates. Only L-dCTP did not act as a phosphate donor (Figure 5). D-dCTP is thought to bind to another site different from the phosphate donor site, since dCTP is generally not simply competitive with D-ATP (10). However, Ikeda *et al.* and Jansson and Eriksson have indicated that dCTP binds to a site that overlaps both the phosphate donor and phosphate acceptor sites (36,37). As shown in Figure 4B, inhibition of L-dCTP was non-competitive with dCyd. L-dCTP, like D-dCTP, acted as a competitive inhibitor with respect to D-ATP for mouse dCK (Figure 4C and 4D). These inhibitory effects of L-dCTP on dCK were similar to the results of earlier studies using D-dCTP (22,38,39). This suggests that L-dCTP may bind to the ATP site itself, or a site very close to it and that dCK also cannot discriminate the chirality of dCTP.

L-dCyd was catalyzed by mouse dCK at a somewhat lower rate than the natural substrate D-dCyd (Fig. 2), although it has been demonstrated that the human enzyme catalyzed L-dCyd as efficiently as D-dCyd (12). Mouse dCK may be able to discriminate the sugar structure of the substrate more strictly than the human enzyme. Indeed, a significantly higher rate of 2′,3′-dideoxycytidine (ddCyd) phosphorylation has been observed for human dCK upon comparison of the human and mouse enzymes (14,30).

In contrast to human dCK, human cytidine deaminase has been demonstrated to be stereospecific (12). Cytidine deaminase catalyzes the deamination of cytidine, dCyd and several cytidine analogs. We have confirmed that L-dCyd was not susceptible to deamination by cytidine deaminase partially purified from mouse kidney (data not shown). It is of interest that cytidine deaminase can discriminate strictly the chirality of dCyd whereas dCK cannot do so, although both enzymes utilize chiral dCyd as the substrate.

With regard to substrate specificity, dCK recognizes the nucleic base, C-2′ and 5′-OH of the sugar moiety of the phosphate acceptor, and the nucleic base and triphosphate group of the phosphate donor. Therefore, it is likely that a stereospecific complex of dCK, phosphate acceptor and phosphate donor is formed. However, the complex formed during the reaction may not be so tight that the reaction occurs highly stereospecifically. The tertiary and quaternary structures of dCK, especially the structure of the active site, are of great interest and remain to be elucidated.

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