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Nucleotide Specificity of Human Deoxycytidine Kinase

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SUMMARY

The ability of deoxycytidine kinase (dCK) to phosphorylate 2′-deoxycytidine (dCyd) and its analogs in the presence of eight nucleoside triphosphates (NTPs), simulating the cellular milieu, was investigated. Using highly purified dCK from MOLT-4 T lymphoblasts, K_m and V_{max} values were determined for the phosphorylation of dCyd in the presence of cellular concentrations of the eight endogenous NTPs. The results demonstrated that the efficiency of dCyd phosphorylation was greatest in the presence of all eight nucleotides, relative to ATP alone, according to relative V_{max}/K_m values. UTP was a better phosphate donor than ATP but was less efficient than the NTP mixture. The greater efficacy of the NTP mixture, compared with ATP alone, was due in large part to the presence of UTP, although the results suggested that the presence of other nucleotide(s) also enhanced dCyd phosphorylation. Previous results demonstrated that dCTP was a potent competitive or noncompetitive (with respect to dCyd) inhibitor of dCK, with a K_i value of approximately 1 μM. In contrast, the results presented here demonstrated that, in the presence of either the NTP mixture or UTP, inhibition of dCK was uncompetitive with respect to dCyd, with a K_i value of approximately 60 μM. Furthermore, the results demonstrated that the clinically relevant nucleoside analogs 1-β-D-arabinofuranosylcytosine, 2′,2′-difluoro-2′-deoxycytidine (dFdC), and 9-β-D-arabinofuranosyl-2-fluoroadenine also preferred UTP or the NTP mixture, compared with ATP alone, as a phosphate donor. Of the three nucleoside analogs tested, dFdC was the most efficient dCK substrate. These data indicate that the preferred phosphate donor for dCK is UTP or a combination of UTP and another nucleotide. Furthermore, the dCTP concentration in intact cells, which is typically 10–20 μM, is not sufficient to cause substantial inhibition of dCK, due to the presence of UTP. Strategies to increase cellular dCK activity should focus on optimizing UTP concentrations.

dCK (EC 2.7.1.74) is a salvage pathway enzyme that converts dCyd to its respective 5′-monophosphate derivative, in the presence of a NTP as phosphate donor. This enzyme has been intensively investigated, due to its crucial role as the rate-limiting activation step for several nucleoside antitumor agents, including araC, araG, F-araA, and dFdC (1–5). In addition, the antiviral agent dideoxycytidine is phosphorylated initially by dCK (6). *In vitro* studies have demonstrated that leukemic cells can acquire resistance to araC and araG by depletion of dCK activity (7–10), and this mechanism may confer clinical resistance to araC as well (11). The prominent role of dCK in nucleoside analog chemotherapy has prompted investigations of the substrate specificity and regulation of this enzyme.

There are numerous reports in the literature describing the purification of dCK from a variety of tissues, with conflicting results regarding the molecular and biochemical characteristics of this enzyme (12–20). It has been suggested that these discrepancies were due to proteolysis of the enzyme during purification (21). We have purified dCK to near-homogeneity from cultured MOLT-4 T lymphoblasts, demonstrating that this enzyme exists in active form as a dimer with a subunit molecular mass of 30.5 kDa (22). The recent cloning of the cDNA sequence coding for this enzyme confirms that the true subunit molecular mass is, indeed, 30.5 kDa (23). Thus, dCK from this source is well characterized and ideally suited for kinetic investigations.

Kinetic studies with dCK have demonstrated that this enzyme can accept both purine and pyrimidine nucleoside substrates (1, 19, 22, 24). Although ATP is usually used as the phosphate donor for this enzyme, a variety of NTPs can serve as phosphate donors (12, 14, 25–27). In fact, it has been shown that phosphorylation of the dCyd analog araC by dCK is more efficient with UTP as the phosphate donor (14, 26, 28). The nucleotides ADP, UDP, dCMP, and dCTP have all been reported to inhibit dCK activity, with the most potent inhibition being exhibited by dCTP (K_i = 1 μM) (12–14, 24). Considering

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ABBREVIATIONS: dCK, deoxycytidine kinase; araC, 1-β-D-arabinofuranosylcytosine; araCTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; araG, 9-β-D-arabinofuranosylguanine; dCyd, 2′-deoxycytidine; dFdC, 2′,2′-difluoro-2′-deoxycytidine; dTHU, 3,4,5,6-tetrahydro-2′-deoxyuridine; F-araA, 9-β-D-arabinofuranosyl-2-fluoroadenine; NTP, nucleoside 5′-triphosphate; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; F-araATP, 9-β-D-arabinofuranosyl-2-fluoroadenine 5′-triphosphate.

that the cellular concentration of dCTP is usually at least 10-fold greater than this K_i value (29), it seemed likely that dCK in the intact cell would be strongly inhibited. However, the facile phosphorylation of dCK substrates in intact cells would argue against this hypothesis (30, 31). For example, araC is phosphorylated rapidly in leukemic cells at nanomolar concentrations, despite the fact that it exhibits an apparent K_m value for dCK of approximately 40 μM (4, 22, 32, 33). Although it is possible that purified preparations of dCK may lack chemical or enzymatic cofactors that alter the activity of this enzyme in intact cells, it also seemed likely that the enzyme kinetics with one phosphate donor would not reflect the effects of several regulatory nucleotides to which dCK is exposed intracellularly. Thus, in an effort to understand the nucleotide regulation of dCK in intact cells, we have studied the kinetic behavior of highly purified dCK from MOLT-4 T lymphoblasts in the presence of cellular concentrations of the eight major NTPs.

Experimental Procedures

Materials. [5-^{3}H]dCyd was obtained from either ICN Radiochemicals (Irvine, CA) or Moravek Biochemicals, Inc. (Brea, CA). Tritiated araC and F-araA were purchased from Moravek Biochemicals, Inc. [^{3}H]dFdC was synthesized by Lilly Research Laboratories (Indianapolis, IN). The purity of each lot of tritiated nucleoside was verified by reverse phase HPLC to be >90%, and the isotope was used within a 2–3-month period after receipt, to ensure that this high degree of purity was maintained. F-araA was a gift from Dr. William Plunkett of M. D. Anderson Cancer Center (Houston, TX). Nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO) and were >99% pure. All other chemicals were reagent grade.

Enzyme purification. dCK was purified from MOLT-4 cells that had been collected during logarithmic phase growth. The purification procedure was essentially as described previously (22), with minor modifications to increase the yield of enzyme. Briefly, 5–20 g of MOLT-4 cells were lysed by bomb cavitation and Dounce homogenization, and the lysate was then centrifuged at 48,000 × *g*. The supernatant was treated with ammonium sulfate at 35–60% saturation and then centrifuged at 16,000 × *g*. The precipitated dCK was then resuspended in buffer containing 50 mM imidazole, pH 6.5, 2 mM ATP, 2.4 mM $MgCl_2$, 200 mM KCl, 5 mM dithiothreitol, 5% glycerol, 100 μM *o*-phenanthroline, and 100 μM phenylmethylsulfonylfluoride and was subjected to HPLC-gel filtration on a Superose-12 column (Pharmacia, Piscataway, NJ) equilibrated with the same buffer. The resulting fractions containing dCK activity were then diluted 4-fold, loaded onto a column of Whatman DE-52 cellulose, and eluted with a gradient of 100–600 mM KCl. The dCK was then diluted 4-fold and applied to a dCTP-Sepharose affinity column that had been prepared as described previously (22). This column was washed with loading buffer (50 mM Tris, pH 7.4, 25 mM dithiothreitol, 5% glycerol), followed by a wash with loading buffer containing 2 mM ATP and 2 mM $MgCl_2$, and then dCK was eluted with this buffer plus 100 μM dCyd, 200 mM KCl, and 2 mM TTP. The resultant dCK was >80% pure, and the activity was stable for >1 year when stored with 1 mg/ml BSA and 20% glycerol at −70°.

Enzyme assay. Before assay, the purified dCK preparation was desalted using a fresh 1.7-ml Sephadex G-25 column (Isolab, Inc., Akron, OH) equilibrated in buffer containing 50 mM imidazole, pH 7.4, 25 mM dithiothreitol, 200 mM KCl, 5% glycerol, and 1 mg/ml BSA. Desalting efficiency was calculated to be >99.5%. The desalted enzyme was then diluted at least 10-fold, so that this preparation contributed <50 nM dCyd and <1 μM ATP and TTP to the dCK assay mixture.

For the assay, dCK was incubated in a mixture containing 50 mM imidazole, pH 7.4, 25 mM dithiothreitol, 2.5 mM $MgCl_2$, 5% glycerol, 1 mg/ml BSA, variable concentrations of tritiated nucleoside, and ATP or the NTP mixture, in a final volume of 30 μl. Higher concentrations of $MgCl_2$ resulted in lower reaction rates. For the K_m determinations, the dCyd concentration ranged from 0.1 to 10 μM when ATP alone was used as the phosphate donor and from 0.10 to 2.0 μM with the NTP mixture. The reaction mixture was incubated in a water bath at 37° for 10 min and then inactivated at 85° for 1 min. Aliquots of the reaction mixture were pipetted onto DE-81 filter discs, which were then washed with 5 mM ammonium formate to remove unreacted substrate. HPLC analysis verified that >99% of the radioactivity retained on the filter discs was [^{3}H]dCMP. No more than 12% (usually <6%) of the substrate was consumed during the incubation period. The reaction rate was linear for at least 20 min. For the assays with araC, F-araA, or dFdC, a 20-min incubation time was used, and these reactions were linear for at least 30 min. All assays were performed in duplicate or triplicate.

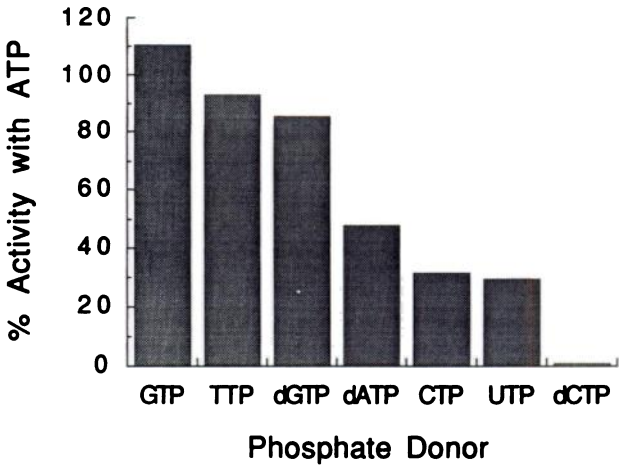

Fig. 1. Single nucleotides as phosphate donors for dCK. The concentration of dCyd was 10 μM, and each nucleotide was present at 2 mM.

Kinetic analysis. The kinetic data were fit to a hyperbola, by using a computer program (34, 35), from which the apparent K_m and V_{max} values were calculated. Lines describing the double-reciprocal plots were drawn using the computer-derived values. K_i values were calculated from replots of the corresponding intercept values versus inhibitor concentration, by using linear least squares regression analysis to estimate the best fit line describing the data points.

Determination of cellular NTP concentrations. MOLT-4 T lymphoblasts were maintained in logarithmic phase growth and harvested for nucleotide analysis at densities ranging from 4 × 10^5 to 6 × 10^5 cells/ml. At least 2 × 10^7 cells were collected by centrifugation, extracted with 0.4 N perchloric acid, and neutralized with potassium hydroxide. Ribonucleoside triphosphates were detected and quantitated by a previously described HPLC method (4). Deoxyribonucleoside triphosphates were first separated from the ribonucleotides by means of a boronate affinity column and then separated and quantitated by HPLC techniques.[1] Cell number and cell volume were determined with a Coulter electronic particle counter (Coulter Electronics, Hialeah, FL).

Results

Effect of single nucleotides as phosphate donors. The relative abilities of individual NTPs to act as phosphate donors, with a fixed concentration of dCyd, were assessed (Fig. 1). Under these conditions, dCK exhibited a strong preference for purine nucleotides, as previously reported (22). The reaction velocity was similar in the presence of ATP, GTP, and dGTP. Of the pyrimidine nucleotides tested, TTP yielded the highest rate of phosphorylation. The apparent K_m value for dCyd

[1] D. S. Shewach. Quantitation of deoxyribonucleoside 5′-triphosphates by a sequential boronate and anion exchange high pressure liquid chromatographic procedure. Submitted for publication.

phosphorylation by dCK, using ATP at its optimum concentration of 2 mM (22), was 0.83 μM (Table 1), similar to its true K_m value of 0.8 μM (24).

Effect of NTP mixture on dCyd phosphorylation. To determine the effect of the cellular nucleotides on the kinetic activity of dCK, the apparent K_m value for dCyd was measured using highly purified enzyme and a mixture of NTPs at their cellular concentrations (Table 2). This apparent K_m determination was compared with that obtained with ATP alone as the phosphate donor. A concentration of 6.78 mM ATP was used, so that the total phosphate concentration was identical in both the NTP and ATP-only mixtures. Fig. 2 illustrates that Lineweaver-Burk plots of these data were linear over the dCyd concentration range used (0.1–10 μM). The standard calculation of apparent V_{max}/K_m was used to compare the efficiencies of each phosphate donor mixture. The substrate efficiency was more than twice as high in the presence of the eight nucleotides (11.0) than with ATP alone at a concentration of either 6.78 mM (5.09) or 2 mM (4.89) (Table 1). Thus, the NTP mixture was more efficient than ATP as the phosphate donor. The cellular NTP concentrations were determined by assuming that the nucleotides were evenly distributed throughout the cell. Although the concentration of NTPs at the site of dCK may vary, the values listed in Table 2 are reasonable approximations.

TABLE 1

Effect of phosphate donors on the kinetic constants for dCyd phosphorylation by dCK

For each determination, dCyd was used as a substrate at concentrations below and above its K_m value. Double-reciprocal plots of the data were linear, and the kinetic constants were calculated using a computer program (34, 35).

Phosphate donor	K_m	V_{max}	V_{max}/K_m
	μM	nmol/ml-hr	
NTP mixture	0.35 ± 0.03	3.85 ± 0.13	11.00
2 mM ATP	0.83 ± 0.09[a]	4.06 ± 0.12	4.89
6.78 mM ATP	1.88 ± 0.17[a]	9.56 ± 0.39[a]	5.09
NTP mixture − UTP	1.54 ± 0.19[a]	2.54 ± 0.13[a]	1.65
1.51 mM UTP	0.23 ± 0.03[b]	1.82 ± 0.07[a]	7.91

[a] Significantly different from the corresponding NTP mixture value, $p \ll 0.001$.
[b] $0.02 < p < 0.05$.

TABLE 2

Concentrations of NTPs in MOLT-4 T lymphoblasts

Ribonucleotide and deoxyribonucleotide concentrations were measured using at least 2×10^8 cells or 1×10^7 cells, respectively. Values represent the means of triplicate determinations.

NTP	Concentration
	mM
CTP	0.57
UTP	1.51
ATP	3.78
GTP	0.87
dCTP	0.013
TTP	0.055
dATP	0.049
dGTP	0.022

Inhibition of dCK by dCTP. It was shown previously that, using ATP as the phosphate donor, dCTP was a competitive (with respect to dCyd) inhibitor of MOLT-4 dCK, with a K_i value of 1 μM (22). Hence, it might have been expected that the presence of this nucleotide at a concentration of 13 μM in the NTP mixture would result in a higher K_m value, relative to that determined with ATP alone. In contrast, the K_m value was lower with the NTP mixture, suggesting that dCTP did not inhibit the reaction. To determine whether higher concentrations of dCTP could inhibit dCK in the presence of the NTP mixture, the phosphorylation of dCyd was measured in the presence of 0, 75, 100, or 300 μM dCTP, with the other seven NTPs at the concentrations indicated in Table 2. The double-reciprocal plots of these data yielded a series of parallel lines (Fig. 3), demonstrating that dCTP acted as an uncompetitive inhibitor of dCK in the presence of the NTP mixture. A replot of the dCTP concentration versus the y-intercepts yielded a K_i value of 67.1 μM. Thus, dCTP inhibited dCK in the presence of the NTP mixture at a concentration >60 times greater than that observed with ATP alone.

Previous reports have demonstrated that dCTP-mediated inhibition of dCK can be reversed by millimolar concentrations of TTP (12, 13, 24) and, thus, the possibility that the presence of TTP or another nucleotide in the NTP mixture lessened the inhibitory effect of dCTP on dCK was considered. This hypothesis was evaluated using the optimal ATP concentration (2 mM), 20 μM dCTP, 10 μM dCyd, and 2 mM CTP, UTP, or GTP. Of the conditions tested, only TTP produced a substantial reversal of dCK inhibition (data not shown). Additional

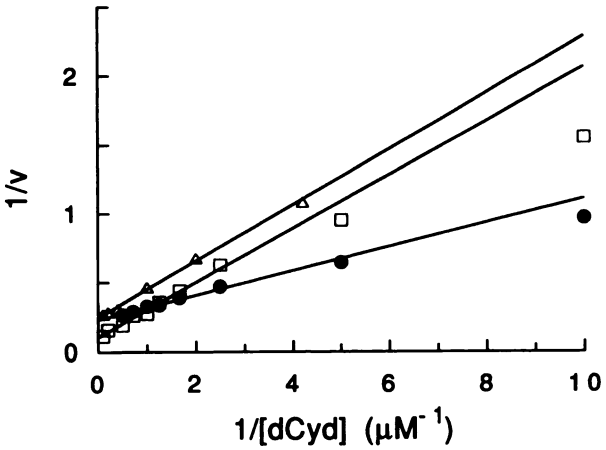

Fig. 2. Comparison of ATP and the NTP mixture as phosphate donors for phosphorylation of dCyd by dCK. ●, NTP mixture; □, 6.78 mM ATP; △, 2 mM ATP.

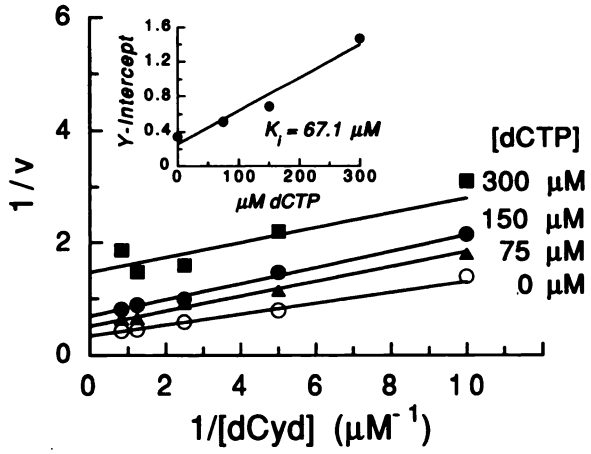

Fig. 3. Inhibition of dCK by dCTP in the presence of the NTP mixture. The *lines* in the double-reciprocal plot were derived from the K_m and V_{max} values calculated with a computer program (34, 35). The *line* in the slope replot (*inset*) was obtained by linear least squares regression analysis.

studies demonstrated that at least 0.5 mM TTP was necessary to reverse the dCTP-mediated inhibition by 50% (data not shown). Hence, these data suggest that the concentration of 55 μM TTP in the NTP mixture was too low to significantly antagonize dCTP in the reaction.

Contribution of UTP to the dCK reaction. Although the studies described in the preceding section demonstrated that the NTP mixture was preferable to ATP, it could not be determined whether one or several of the nucleotides in the NTP mixture were being utilized as phosphate donor(s). To determine whether there was a single most important nucleotide in the NTP mixture, the activity of dCK with 10 μM dCyd was measured using all possible combinations of seven of the eight nucleotides. As illustrated in Fig. 4, only the elimination of UTP significantly affected the velocity of this reaction ($0.01 < p < 0.02$, Student's t test), causing a decrease to approximately 50% of the control value. Deletion of ATP, GTP, or dGTP had no effect on the phosphorylation of dCyd. Elimination of dCTP had no substantial effect on the activity, consistent with the high K_i value for this nucleotide in the presence of the NTP mixture. Furthermore, the elimination of TTP had no effect on the reaction rate, directly demonstrating that the presence of this nucleotide does not reverse any potential inhibition by dCTP.

The data in Fig. 4 demonstrate that UTP is an important component of the NTP mixture. In view of other reports that UTP is a preferred phosphate donor for the phosphorylation of araC by dCK (25–28), the possibility was considered that only UTP in the NTP mixture was being utilized as the phosphate donor. The apparent K_m value was determined using dCyd and UTP as substrates, and the effect of dCTP on this reaction was evaluated (Fig. 5). Estimation of the K_m and V_{max} values for the uninhibited reaction indicated that UTP was less efficient than the NTP mixture, with an efficiency ratio of 7.91, approximately 70% of the efficiency of the NTP mixture (Table 1).

With UTP as the phosphate donor, dCTP was an uncompetitive inhibitor with respect to dCyd phosphorylation by dCK (Fig. 5). A replot of the dCTP concentration versus y-intercept yielded a K_i value of 57.2 μM, which was similar to the value of 67.1 μM determined with the NTP mixture but >50 times higher

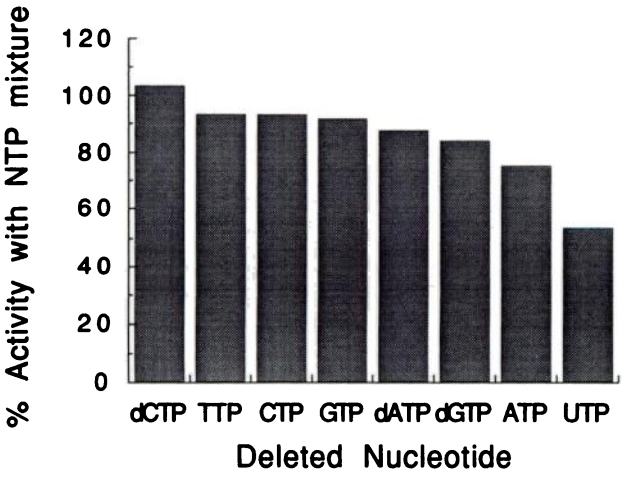

Fig. 4. Effect of the deletion of single NTPs from the NTP mixture on the phosphorylation of dCyd by dCK. The concentration of dCyd was 10 μM. The nucleotide concentrations used in this study are displayed in Table 2.

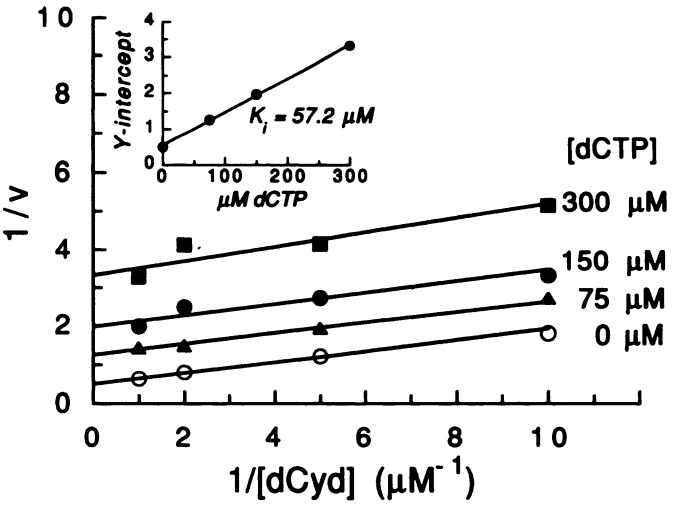

Fig. 5. Inhibition of dCK by dCTP in the presence of UTP as phosphate donor. The *lines* in the double-reciprocal plot were derived from the K_m and V_{max} values calculated using a computer program (34, 35). The *line* in the slope replot (*inset*) was obtained by linear regression analysis.

than the value obtained with ATP as the phosphate donor. In view of the similar K_i values for dCTP in the presence of either UTP alone or the NTP mixture, these data suggest that the lesser inhibition of dCK by dCTP with the NTP mixture, relative to ATP alone, may be dependent solely on the presence of UTP in the reaction.

To verify the importance of UTP in lowering the K_m value for dCyd phosphorylation, UTP was deleted from the NTP mixture and the apparent K_m value was determined for dCyd (Table 1). Under these conditions, the K_m value was more similar to that determined with ATP alone (1.54 μM), but the V_{max} value was substantially lower (2.54 nmol/ml-hr), yielding a relatively poor efficiency ratio of 1.65. At a concentration of 50 μM, dCTP inhibited the phosphorylation of 10 μM dCyd, in the presence of the seven nucleotides, by 80%. Thus, the presence of UTP can lower the K_m value for dCyd and alleviate the inhibition by dCTP. It is likely that the lower V_{max} value observed for dCyd in the presence of the seven-NTP mixture (without UTP) reflected the ability of the 13 μM dCTP in that mixture to inhibit the reaction significantly.

The phosphorylation of dCyd was examined in the presence of various concentrations of UTP and $MgCl_2$, to determine the optimum MgUTP concentration. These studies demonstrated that 2 mM UTP and a 1:1 Mg/UTP ratio was optimum for dCyd phosphorylation (data not shown). Excess $MgCl_2$ or excess UTP was inhibitory to the reaction, similar to the results with ATP (24).

The observation that dCTP was a competitive inhibitor of dCK in the presence of ATP but uncompetitive in the presence of UTP was used in refining the purification scheme for dCK. The final step in the procedure to purify this enzyme to apparent homogeneity from T lymphoblast cells uses a dCTP-Sepharose affinity column to bind the dCK, followed by specific elution with a dCyd/ATP/TTP/$MgCl_2$/KCl mixture (22). In view of the uncompetitive inhibition by dCTP in the presence of UTP, we reasoned that eluting dCK from the dCTP-Sepharose column with a mixture of dCyd/UTP/$MgCl_2$/KCl would improve the recovery of enzyme at this step. Whereas the yield of dCK from the dCTP-Sepharose column was typically 25% or less in the presence of the ATP mixture (22), the recovery

with the UTP mixture was approximately 100% on two separate occasions. This provides further proof that the interaction of dCK with dCTP is substantially less potent in the presence of UTP, compared with ATP.

Kinetic determinations with nucleoside analogs. In view of the more efficient phosphorylation of dCyd in the presence of the NTP mixture or UTP, it was of interest to determine whether these alternate phosphate donors would improve the phosphorylation by dCK of several nucleoside analog substrates. Table 3 compares the kinetic constants for the clinically relevant nucleoside analogs araC, F-araA, and dFdC. For araC and dFdC, the use of UTP increased the efficiency of phosphorylation by factors of 2 and 3, respectively. The NTP mixture increased phosphorylation efficiency for these analogs, but not as much as did UTP. For F-araA, UTP lowered the apparent K_m value by a factor of approximately 10, but a similar decrease in the V_{max} value resulted in an overall increase of only 36% in phosphorylation efficiency. It should be noted that F-araA phosphorylation was assayed at a maximum nucleoside concentration of 600 μM, due to its poor solubility, which is less than half the apparent K_m value with ATP as the phosphate donor. Thus, the kinetic constants for F-araA phosphorylation in the presence of ATP may be substantially different at concentrations of F-araA closer to the apparent K_m value. If the values listed in Table 3 for F-araA represent underestimations of the K_m value or overestimations of the V_{max} value, then the substrate efficiency with ATP would be considerably lower than observed and the effect of UTP greater. Alternatively, F-araA is a purine analog, and it is possible that purines interact differently with dCK than do pyrimidines.

Discussion

dCK has gained widespread attention due to its unique role as the rate-limiting phosphorylation step in the activation of several antitumor and antiviral agents currently in clinical use, such as araC, F-araA, and dFdC. It has been demonstrated previously that dCK is inhibited by micromolar concentrations of dCTP in the presence of ATP (12–14, 17, 19, 20, 24, 36). Because the concentration of dCTP in intact cells is typically in the range of 10–20 μM (29), it has been assumed that dCK in the intact cell is strongly inhibited. Here we present data supporting the hypothesis that dCK is not inhibited in intact cells, due to the presence of other NTPs that allow dCK to function more efficiently than with ATP alone. Furthermore, we have demonstrated that cellular levels of dCTP do not inhibit the purified enzyme in the presence of eight NTPs at their physiologic concentrations.

TABLE 3

Effect of phosphate donor on the kinetic constants for dCyd analog phosphorylation by dCK

Kinetic constants were determined using nucleoside substrates below and above their K_m values. Each value represents the average of two separate experiments. These studies used a more concentrated preparation of dCK than that used for Table 1.

Nucleoside	Phosphate donor	K_m	V_{max}	V_{max}/K_m
		μM	nmol/ml-hr	
AraC	ATP	14.8	62.4	4.2
	UTP	2.4	20.9	8.9
	NTP mixture	3.3	17.1	5.2
dFdC	ATP	9.3	180	19
	UTP	0.73	43	59
	NTP mixture	0.96	31	33
F-araA	ATP	1600	172	0.11
	UTP	177	26	0.15
	NTP mixture	238	21	0.09

Increased efficiency of dCK with the NTP mixture or UTP alone. Of the conditions tested, the phosphorylation of dCyd by dCK was most efficient when the complete NTP mixture was used, based on relative V_{max}/K_m values. Two lines of evidence indicated that UTP was an important component of the NTP mixture; 1) only the deletion of UTP significantly lowered the velocity of the dCK reaction at a fixed concentration of dCyd, and 2) the V_{max}/K_m value was >6-fold lower when UTP was eliminated from the NTP mixture. These data suggested that the increased efficiency of dCyd phosphorylation conferred by the NTP mixture, compared with ATP alone, may be due solely to the presence of UTP in the nucleotide mixture. However, there appeared to be an additional advantage to using the NTP mixture, because the substrate efficiency ratio was greater for the NTP mixture, compared with that obtained with UTP alone. Previous reports using partially purified preparations of dCK indicated that lower K_m values for dCyd or araC phosphorylation could be obtained by using UTP as the phosphate donor (12, 14, 26, 27). The results presented here, using highly purified dCK, confirm the previous observations and, furthermore, suggest that additional cellular NTPs are required for optimum dCK activity.

It has been demonstrated that most nucleotide-binding proteins contain an amino acid sequence corresponding to Gly-Xaa-Xaa-Gly-Xaa-Gly-Lys (37–39). Cloning of the cDNA sequence coding for dCK revealed a putative nucleotide binding site with the amino acid sequence Gly-Asn-Ile-Ala-Ala-Gly-Lys (23). In view of the data presented in this paper, it is tempting to speculate that the presence of alanine as the fourth amino acid in this sequence, instead of the more typical glycine, allows dCK to utilize UTP more efficiently than ATP as a phosphate donor. A recent report on adenylate kinase used site-directed mutagenesis to alter the fourth amino acid in the nucleotide binding site from glycine to valine; this resulted in a dramatic increase in the K_m value of this enzyme for the substrate AMP (40), highlighting the importance of this amino acid in determining substrate affinity. Similar techniques could be used with dCK, to determine whether changing the alanine to glycine would increase the affinity of this enzyme for ATP.

Inhibition of dCK activity by dCTP. Previous studies with dCK demonstrated that dCTP competitively or noncompetitively inhibited the phosphorylation of dCyd in the presence of ATP as the sole phosphate donor, with a K_i value of approximately 1 μM (12, 14, 15, 17, 20, 24). In the studies described here, inhibition of dCK activity by dCTP was strikingly different in the presence of alternate phosphate donors. Figs. 3 and 5 demonstrated that, when the NTP mixture or UTP was used as phosphate donor, dCTP was an uncompetitive inhibitor of dCyd phosphorylation by dCK, with K_i values of 67 μM and 57 μM, respectively. The similarity in these values suggests that the presence of UTP in the NTP mixture mediated the higher K_i value for dCTP, relative to that observed with ATP alone. In addition, deletion of UTP from the mixture resulted in potent inhibition of dCK by lower concentrations of dCTP, lending further support to this hypothesis. These results suggest that, in the cellular environment, in which dCTP is typi-

cally <20 μM and UTP is approximately 1–2 mM, dCK is not substantially inhibited by dCTP.

In view of earlier reports demonstrating potent inhibition of dCK by dCTP, using ATP as a phosphate donor, it was assumed that dCK under native conditions was substantially inhibited. Investigators then reasoned that araC phosphorylation by dCK might be increased by decreasing the endogenous dCTP level. There are numerous reports of attempts to biochemically modulate araC phosphorylation and subsequent cytotoxicity according to this rationale, using various antimetabolites such as pyrazofurin, deazauridine, phosphonoacetyl aspartate, thymidine, deoxyguanosine, methotrexate, or hydroxyurea. Although some investigators observed increased araCTP accumulation in response to the modulator and attributed it to a reduction in dCTP pools (41–45), others found a lack of correlation between effects on dCTP pools and araC phosphorylation (2, 46–49). In fact, in two studies dCTP pools decreased but araC phosphorylation was not enhanced (28, 49). These studies were complicated by the fact that the antimetabolite modulators can also alter deoxy- and ribonucleotide pools other than dCTP, thus making it difficult to attribute synergy to a single effect. In most of these studies only dCTP pool measurements were made, without assessment of effects on UTP pools, which, according to the data presented here, may have had a larger impact on araC phosphorylation. In addition, these agents can all induce partial synchrony in cells, which may alter the phosphorylation of araC (50). It has been suggested that the generation of dCyd in these studies is a more important determinant of araC phosphorylation than is dCTP pool reduction (49, 51). It should be noted that, although the data presented in this manuscript argue against enhancement of araC phosphorylation by decreasing dCTP pools, araC cytotoxicity could be increased by this mechanism, due to decreased competition for DNA polymerases.

In a recent report, a noncytotoxic agent, dTHU, was used to increase dCTP levels in cells through inhibition of dCMP deaminase (52). The authors established that no other deoxy- or ribonucleoside triphosphate pool levels were affected as a result of the dTHU treatment. Their results demonstrated that the phosphorylation of 0.1 or 100 μM araC was inhibited by 50% when the intracellular dCTP concentration was approximately 150 μM. These results are consistent with the data presented here, demonstrating that relatively high levels of dCTP are required to inhibit dCK substantially under cellular conditions.

Efficiency of phosphorylation of nucleoside analogs by dCK. For the three nucleoside analogs tested, the efficiency of phosphorylation was greatest in the presence of UTP only as the phosphate donor. Consistently, dFdC exhibited the lowest K_m and highest V_{max} values, and thus the greatest efficiency of phosphorylation, whether ATP, UTP, or the NTP mixture was used as the phosphate donor. Furthermore, the increase in phosphorylation efficiency conferred by UTP was greater for dFdC (3-fold) than for the natural substrate dCyd (<2-fold). Thus, the K_m values for araC and dFdC, when measured using UTP as a phosphate donor, are 2–60-fold lower than the plasma levels attained during infusion of these analogs (33, 53–55), indicating that these agents achieve concentrations *in vivo* that should allow maximal phosphorylation by dCK. Indeed, these data are consistent with reports that araC and dFdC phosphorylation by leukemic or mononuclear cells *in vivo* is saturated at plasma concentrations of >10 μM (53, 55). Although substitution of UTP for ATP as the phosphate donor reduced the K_m value for F-araA phosphorylation 10-fold, the apparent K_m value of 177 μM is >15 times higher than the reported plasma concentrations for this agent (56). Apparently the high V_{max} value for F-araA phosphorylation, combined with a slow half-life for the 5′-triphosphate *in vivo* (56), allows therapeutic levels of F-araATP to accumulate in patients with leukemia. Although F-araA has been reported to decrease dCTP pools through inhibition of ribonucleotide reductase (57, 58), this effect should not increase F-araA phosphorylation in intact cells, because the data presented here demonstrate that cellular dCTP concentrations do not inhibit dCK activity. Because the levels of F-araA attained *in vivo* are significantly below the K_m value for this compound (56), biochemical modulation of F-araA phosphorylation may produce substantially higher F-araATP levels.

There are several reports in the literature of synergistic cytotoxicity with araC and a uridine analog, such as deazauridine or fluorouracil, either *in vitro* or *in vivo* (59–61). It has been demonstrated that fluorouracil, fluorouridine, and fluorodeoxyuridine can augment araCTP accumulation and cytotoxicity by a mechanism independent of effects on dCTP pools (60). It is attractive to hypothesize that analogs that can be metabolized to uridine nucleotide analogs in cells may be acting as efficient phosphate donors, in a manner similar to that of UTP. We are currently investigating whether these nucleotide analogs can be utilized by dCK as efficiently as is UTP.

These studies demonstrated that UTP and the NTP mixture were the most efficient phosphate donors for the phosphorylation of dCyd, araC, F-araA, and dFdC by dCK. Because the optimum UTP concentration for dCyd phosphorylation by dCK was 2 mM, yet its cellular concentration is typically less than that, it may be possible to increase phosphorylation of these analogs in intact cells by increasing the UTP concentration. Such studies are currently underway in this laboratory.

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