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REGULATION OF PURINE DEOXYNUCLEOSIDE PHOSPHORYLATION BY DEOXYCYTIDINE KINASE FROM HUMAN LEUKEMIC BLAST CELLS

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Abstract—The kinetics and regulation of nucleoside phosphorylation by highly purified human deoxycytidine kinase from leukemic lymphoblasts were studied. The phosphorylation of purine nucleosides by this enzyme showed sensitivity to the endogenous inhibitors dCTP and UDP three times greater than the phosphorylation of dCyd. Examination of nucleotide pools in human T and B lymphoblasts disclosed that the levels of dCTP and UDP in these cells were sufficient to regulate kinase activity. The enhanced sensitivity of the kinase to dCTP and UDP was related to its reduced ability to interact with purine nucleosides. Comparison of the phosphorylation kinetics for pyrimidine and purine dideoxynucleosides used in antiviral therapy showed that the purine nucleosides were at least 50-fold less efficient as enzyme substrates. These results suggest that the phosphorylation of pharmacologically active purine nucleosides by deoxycytidine kinase is regulated by cellular nucleotide pools.

Cytosolic deoxycytidine kinase (ATP : deoxycytidine 5′-phosphotransferase, EC 2.7.1.74), found in human lymphoid cells [1, 2], serves as a key enzyme in the conversion of both purine and pyrimidine nucleosides to biologically active triphosphates. Enzymatic activation of nucleosides has particular relevance for the use of certain antileukemic (ara-C, ara-A)¶ and antiviral (ddCyd, ddAdo) agents [3–5]. Although dCyd kinase has been purified from a variety of sources and characterized, many aspects of the regulation of this enzyme remain unexamined [3, 6, 7]. The role of dCyd kinase in the cellular anabolism of various purine nucleosides, including ara-A and ddAdo, has been examined with human T-lymphoblast mutants (CEM) that lack dCyd kinase [8–10]. These studies have demonstrated alternate pathways for the generation of nucleotides from these compounds [8, 9]. Factors that regulate dCyd kinase would be expected to influence purine nucleoside drug metabolism and may favor alternate pathways.

In human immunodeficiency diseases in which the purine nucleoside catabolic enzymes adenosine deaminase and purine nucleoside phosphorylase are deficient, the function of dCyd kinase in lymphocytes in enhanced [1, 11]. In the absence of normal purine catabolism, dAdo and dGuo are phosphorylated more extensively by dCyd kinase, leading to elevated pools of dATP and dGTP. Both of these triphosphates are inhibitors of ribonucleotide reductase, a key enzyme in DNA synthesis. Severe T-cell dysfunction may be mediated by increased levels of dATP and dGTP [11].

Human dCyd kinase has been isolated as an homogenous protein from leukemic spleen and purified 6000-fold [7]. It has also been obtained from human leukemic T lymphoblasts in our laboratory and purified 2000-fold, yielding a protein that is 20% pure [3]. Both enzymes appear to have similar properties. dCyd kinase is a dimer with a subunit molecular weight of 30 kD. The nucleoside substrates dCyd, dAdo, and dGuo all show characteristic biphasic kinetics, indicating phosphorylation at two active sites with different substrate affinities. The preferred substrate is dCyd (apparent K_{m_1} of 1 μM, compared with apparent K_{m_1} values of approximately 100 μM for dAdo or dGuo). Preliminary studies with the B-cell enzyme have shown potent inhibition of purine nucleoside phosphorylation by dCTP (K_i of approximately 3 μM) and by dCyd (K_i of approximately 0.1 μM) [7]. These results suggest that endogenous nucleosides and nucleotides may have a role in regulating purine nucleoside metabolism via dCyd kinase.

The purpose of this study was to identify the endogenous nucleosides and nucleotides that inhibit dCyd kinase activity and to characterize mechanisms of this inhibition. The results show that natural nucleotides can have a profound effect on dCyd kinase activity and thus may regulate nucleoside drug metabolism in T lymphocytes.

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¶ Abbreviations: ara-C, 1-β-D-arabinofuranosylcystosine; ara-A, 9-β-D-arabinofuranosyladenine; dCyd, 2′-deoxycytidine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; ddCyd, 2′,3′.dideoxycytidine; ddAdo, 2′,3′-dideoxyadenosine; ddGuo, 2′,3′-dideoxyguanosine; ddIno, 2′,3′-dideoxyinosine; ddFCyd, 2′,3′-dideoxy-5-fluorocytidine; ddBrCyd, 2′,3′-didexoy-5-bromocytidine; DTT, dithiothreitol; BSA, bovine serum albumin; and Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Table 1. Phosphorylation of deoxy- and dideoxynucleosides by dCyd kinase

Substrate	K_m* (μM)	V_{max}* (nmol/hr/mg)	V_{max}/K_m	V_{max}/K_m (% dCyd)
dCyd	2.8	700	250	100
dAdo	780	47,000	60	27
dGuo	270	30,000	110	44
ddCyd	280	8,000	30	12
ddFCyd	210	6,000	30	12
ddBrCyd†	350	100	0.3	0.1
ddAdo†	300	180	0.6	0.2
ddGuo†	200	16	0.08	0.03

* Deoxycytidine kinase was purified 2000-fold from leukemic T-cells as described in Materials and Methods. Substrates exhibiting high K_m values (>100 μM) were assayed from 20 to 1000 μM. dCyd was assayed from 0.1 to 20 μM. Kinetic constants were calculated according to the method of Cleland [14] using computer analysis. Deoxynucleosides were assayed using 15–250 ng protein; dideoxynucleosides were assayed with 100–700 ng protein.

† The K_m and V_{max} for these substrates are not true Michaelis constants, but represent the substrate concentration and maximum velocity at one-half the maximum velocity determined from a sigmoidal substrate saturation curve.

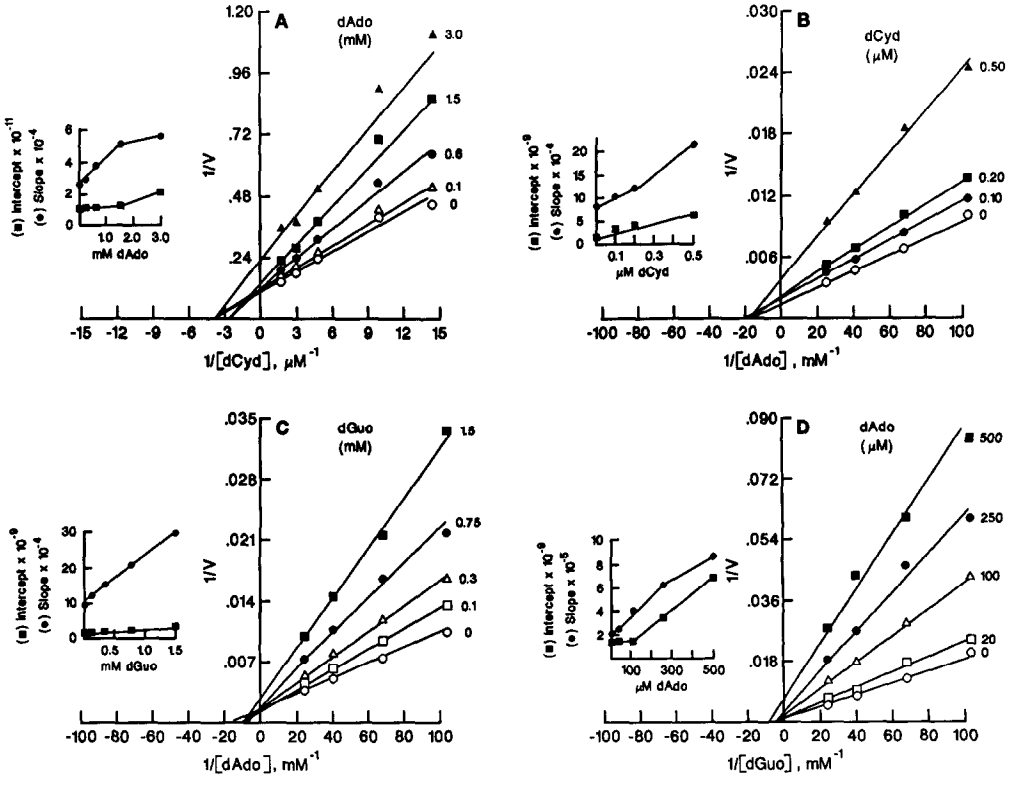

Fig. 1. Nucleoside substrate competition with double-reciprocal plots. The four panels show effects of (A) dAdo concentration on dCyd phosphorylation, (B) dCyd concentration on dAdo phosphorylation, (C) dGuo concentration on dAdo phosphorylation, and (D) dAdo concentration on dGuo phosphorylation. Mg · ATP was fixed at 5 mM. Values for inverse rates ($1/V$) are given in (nmol/hr/mg). Insets show replots of slopes and intercepts against nucleoside competitor concentration.

MATERIALS AND METHODS

Compounds. Naturally occurring nucleosides and nucleotides, DTT and BSA, were obtained from the Sigma Chemical Co. (St Louis, MO). ddCyd, ddAdo, and ddGuo were from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). ddFCyd, ddBrCyd, [5-^{3}H]ddCyd (6 Ci/mmol), [2,8-^{3}H]ddAdo (28 Ci/mmol), [^{3}H]ddFCYd (40 Ci/mmol), and [^{3}H]ddBrCyd (40 Ci/mmol) were all gifts of Dr. David Johns (National Cancer Institute, Bethesda, MD). [8-^{3}H]dGuo (16 Ci/mmol) and [8-^{3}H]ddGuo (3.5 Ci/mmol) were from Moravek Biochemicals (City Indus-

Table 2. Inhibition of dCyd kinase by alternative substrates

Substrate	Inhibitor (μM)	Type of inhibition	K_{is}† (μM)	K_{ii}† (μM)
dCyd	dAdo (25–625)	C	1000	
dCyd	dAdo (1500–3000)	NC	2000	2750
dCyd	dGuo (500–3000)	C	2350	
dCyd	dGuo (3000–5000)	NC	4130	9870
dAdo	dCyd (0.05–0.5)	NC	0.4	0.3
dAdo	dGuo (100–750)	C	540	
dGuo	dCyd (0.05–1.0)	NC	0.3	0.5
dGuo	dAdo (20–100)	C	72	
dGuo	dAdo (250–500)	NC	113	122

* C = competitive, NC = noncompetitive.

† K_{is} and K_{ii} are inhibited constants for slope and intercept effects, respectively. Experimental details are indicated in the legend to Table 1.

Table 3. Inhibition of dCyd kinase by selected nucleotides*

Nucleotide	Nucleotide concn (mM)	% Inhibition of nucleoside phosphorylation*			
		dCyd	dAdo	dGuo	ara-A
dCMP	0.1	10	80	70	90
	1.0	80	>99	80	>99
dAMP	0.1	<5	<5	7	<5
	1.0	6	30	35	20
dGMP	0.1	<5	<5	<5	<5
	1.0	6	10	20	<5
dCDP	0.1	49	96	81	97
	1.0	91	>99	85	>99
dADP	0.1	17	72	56	62
	1.0	56	>99	83	>99
dGDP	0.1	9	33	18	18
	1.0	13	67	61	62
ADP	0.1	11	10	25	<5
	1.0	16	31	32	15
CDP	0.1	<5	15	<5	<5
	1.0	11	43	20	34
UDP	0.1	59	57	48	57
	1.0	73	85	71	>99
CTP	0.1	15	32	5	19
	1.0	28	77	27	75
UTP	0.1	26	22	31	27
	1.0	24	54	33	75
dGTP	0.1	<5	<5	18	<5
	1.0	<5	38	51	59
dATP	0.1	<5	18	40	22
	1.0	<5	62	63	70
dCTP	0.01	14	65	68	68
	0.10	70	95	81	95

* Purified dCyd kinase was assayed with dCyd (0.2 μM), dAdo (20 μM), dGuo (20 μM) or ara-A (1.5 mM) at 10 mM Mg · ATP. Nucleotide inhibitors were added with equimolar $MgCl_2$. The results are those of a typical experiment which was repeated.

tries, CA). [5-^{3}H]dCyd (26 Ci/mmol) and [2,8-^{3}H]dAdo (20 Ci/mmol) were from ICN Biochemicals (Irvine, CA). All radioactive nucleosides were tested for purity by HPLC. The dideoxynucleosides were shown to be free from deoxynucleosides. All other chemicals were of reagent grade.

Cells. The origin and growth of the human T- and B-lymphoblast cell lines used in this study (CEM, Molt 4, PF 2S and WIL-2) have been described [10].

Intracellular nucleotide pools. Intracellular concentrations of nucleotides were determined by separation of nucleotides using the HPLC method of Garret and Santi [12]. Nucleotides were identified by comparing their retention times with those of authentic standards. Cell volumes were determined with an electronic particle counter (Coulter counter) using calibrated beads.

Enzyme assays. Deoxynucleoside kinase activity

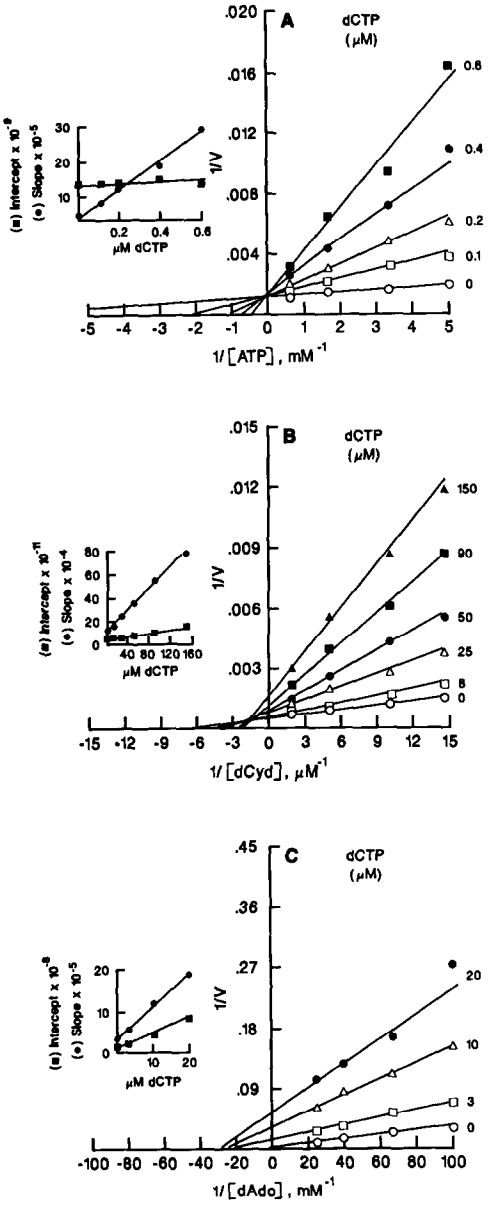

Fig. 2. dCTP inhibition patterns with dCyd kinase. The double-reciprocal plots indicate the effects of dCTP concentration on (A) dAdo phosphorylation (dAdo fixed at 600 μM with various ATP concentrations), (B) dCyd phosphorylation, and (C) dAdo phosphorylation. Mg · ATP was fixed at 5 mM. Values for inverse rates ($1/V$) are given in (nmol/hr/mg). Insets show replots of slopes and intercepts against dCTP concentration.

was assayed by a previously described radiochemical method [3]. The activity in purified preparations was assayed at 37° in a total volume of 50 μl, containing 0.1 M Hepes (pH 7.5), 3 mM DTT, 5 Mm $MgCl_2$, 5 mM ATP, 0.5 mg/ml BSA (used as a carrier with this highly purified enzyme) and radioactive nucleoside as indicated [3]. Protein was quantitatively measured by the Bradford procedure [13], with BSA as a standard. Deoxynucleosides were assayed with 15–250 ng protein per assay; dideoxynucleosides were assayed with 100–700 ng protein.

Source of enzyme. Human leukemic cells were obtained by leukophoresis from patients with T-cell leukemia and were provided by members of the Pathology Department at St Jude Children's Research Hospital. The supernatant fluid extracted from about 25 g of leukemic cells was used to purify dCyd kinase as previously described [3]. The purified enzyme (2000-fold) produced a single radiolabeled product from either [^{3}H]dCyd or [^{3}H]dAdo, as analyzed by HPLC. No radioactivity was found in either the di- or triphosphate region of the nucleotide profile, indicating the absence of diphosphokinases.

Analysis of kinetic data. In all kinetic studies, no more than 20% substrate was used. The kinetics were analyzed by plotting slopes and the ordinate axis intercepts of Lineweaver–Burk plots against the substrate and inhibitor concentrations as described by Cleland [14]. The data were analyzed with a modification of Cleland's program using an IBM/PC minicomputer.

RESULTS

Nucleoside substrates. 2′-Deoxynucleosides and 2′-aranucleosides, excluding thymidine analogues, serve as substrates for human T-lymphoblast dCyd kinase [3]. This observation combined with recent interest in the antiretroviral effects of 2′,3′-dideoxynucleosides led us to examine the kinetic properties of dCyd kinase interactions with these nucleoside analogues. The characteristics of phosphorylation of the natural substrates and selected dideoxynucleosides are compared in Table 1. The natural pyrimidine nucleoside, dCyd, was clearly the most efficient substrate for this enzyme, based on V_{max}/K_m determinations. Similarly, in the dideoxynucleoside series, both ddCyd and the 5-fluoro-substituted analogue ddFCyd were used most efficiently. The increase in both V_{max} and K_m for dAdo compared with dCyd phosphorylation was not observed for ddCyd and ddAdo. Rather, removal of the 3′-hydroxyl resulted in nucleoside analogues with minimal differences in binding affinity (i.e. 200–350 μM). It also caused large decreases in the efficiency of phosphorylation of these compounds, ranging from 8-fold for the small pyrimidine moiety of ddCyd to 1000-fold for the bulky purine groups of ddAdo and ddGuo or the bulky pyrimidine group of ddBrCyd. These findings indicate a key role for the 3′-hydroxyl in orienting nucleosides for normal phosphorylation. Without this group, substrates bind with similar affinity but are phosphorylated very slowly.

Kinetic interaction of deoxynucleosides. The phosphorylation of dAdo and dGuo by the human dCyd kinase was inhibited very strongly by dCyd. Figure 1 shows the inhibition of phosphorylation of the purine dAdo by dCyd and vice versa. At concentrations below 0.2 μM and 1.5 mM, respectively, dCyd and dAdo were mutually competitive. The slopes were linear, indicating that these nucleosides bind to the same catalytic site(s). At concentrations higher than 0.2 μM and 1.5 mM, respectively, both dCyd and dAdo acted as noncompetitive inhibitors. By contrast, the two purine nucleosides, dAdo and dGuo, were mutually competitive over a broad range

Table 4. Types of inhibition and inhibition constants for dCTP and UDP interaction with dCyd kinase

Variable substrate	Constant substrate	dCTP Type of inhibition*	dCTP K_{is}† (μM)	dCTP K_{ii}† (μM)	UDP Type of inhibition*	UDP K_{is}† (μM)	UDP K_{ii}† (μM)
Mg · ATP	dCyd (10 μM)	C	0.9		NC	7.0	17.0
Mg · ATP	dAdo (600 μM)	C	0.030		NC	1.2	15
dCyd	Mg · ATP (10 mM)	NC	13.0	99	UC		312
dCyd	Mg · ATP (2.0 mM)	NC	3.0	16	NC	668	178
dAdo	Mg · ATP (10 mM)	NC	4.0	0.3	NC	116	49
dAdo	Mg · ATP (2.0 mM)	NC	0.7	0.4	NC	90	12

* C = competitive, NC = noncompetitive, and UC = uncompetitive.
† K_{is} and K_{ii} are inhibition constants for slope and intercept effects respectively. Experimental details are indicated in the legend to Table 1.

of concentrations. Table 2 summarizes the pattern of inhibition and inhibitory constants determined for each of the deoxynucleosides at a constant ATP·$MgCl_2$ concentration (5 mM).

Effect of nucleotides on phosphorylation of deoxynucleosides by dCyd kinase. The activity of the human enzyme was strongly influenced by the presence of nucleotides (Table 3). The triphosphate dCTP was the most effective inhibitor of both purine and pyrimidine deoxynucleoside phosphorylation. At 10 μM, it inhibited dAdo and dGuo by 65% and dCyd by 14%. The purines dATP and dGTP inhibited phosphorylation only when their concentrations were 100-fold higher than that of dCTP and then only when the substrates were purine deoxynucleosides. Three other nucleotides—dCMP, dCDP, and UDP—produced greater than 50% inhibition of nucleoside phosphorylation at concentrations of 0.1 mM. Other nucleoside monophosphates and purine diphosphates were much less effective in blocking the activity of the kinase.

The end product, dCTP, produced a competitive-type inhibition against ATP·$MgCl_2$ at variable concentrations (Fig. 2; Table 4), with a fixed concentration of either dCyd or dAdo (Fig. 2). By contrast, with ATP·$MgCl_2$ at 2 mM and variable concentrations of dCyd, dAdo, or dGuo (data not shown), dCTP exhibited a noncompetitive-type inhibition. UDP produced noncompetitive inhibition when it was used with variable deoxynucleosides and fixed ATP·$MgCl_2$, or the reverse (Fig. 3).

Intracellular nucleotide pool sizes. Extracts of T and B lymphoblasts were analyzed for nucleotide concentrations to distinguish the potential physiological significance of their effects on dCyd kinase activity. ATP, ranging in concentration from 2 to 5 mM, was the most abundant nucleotide (Table 5). Concentrations of the ribonucleoside triphosphates were 40- to 100-fold greater than those of the deoxyribonucleoside triphosphates. In each cell type, the level of dCTP was about 4-fold lower than levels of the other three deoxyribonucleoside triphosphates.

Table 5. Nucleotide concentrations in human T and B cells*

Nucleotide	Nucleotide concentrations (μM) T lymphoblasts CEM	T lymphoblasts Molt 4	B lymphoblasts PF 2S	B lymphoblasts WIL-2
ATP	2500	2475	1975	4650
GTP	800	700	525	1525
CTP	200	225	175	550
UTP	525	975	775	1950
dATP	25	40	8	21
dGTP	20	29	5	10
dTTP	30	54	15	27
dCTP	5	8	2	4
ADP	1500	325	1300	2950
GDP	475	75	300	850
CDP	75	25	100	300
UDP	250	15	450	1150

* Nucleotide concentrations were determined by HPLC analysis of cell extracts and particle counter analysis of cell volumes, as described in Materials and Methods.

B and T cells did not differ appreciably in their nucleotide concentrations, except that in B cells dGTP levels were approximately 4-fold lower than in T cells and UDP levels were about 2- to 4-fold lower than in T cells.

DISCUSSION

Deoxycytidine kinase is a nucleoside salvage enzyme that phosphorylates dCyd as well as the purine nucleosides dAdo and dGuo during DNA synthesis. Although essentially one enzyme phosphorylates dCyd, multiple enzymes can salvage purine nucleosides. The relative importance of dCyd kinase in normal purine nucleoside anabolism remains unknown. Purine nucleoside activation by dCyd kinase appears important in the conversion of certain antileukemic or antiviral drugs to their

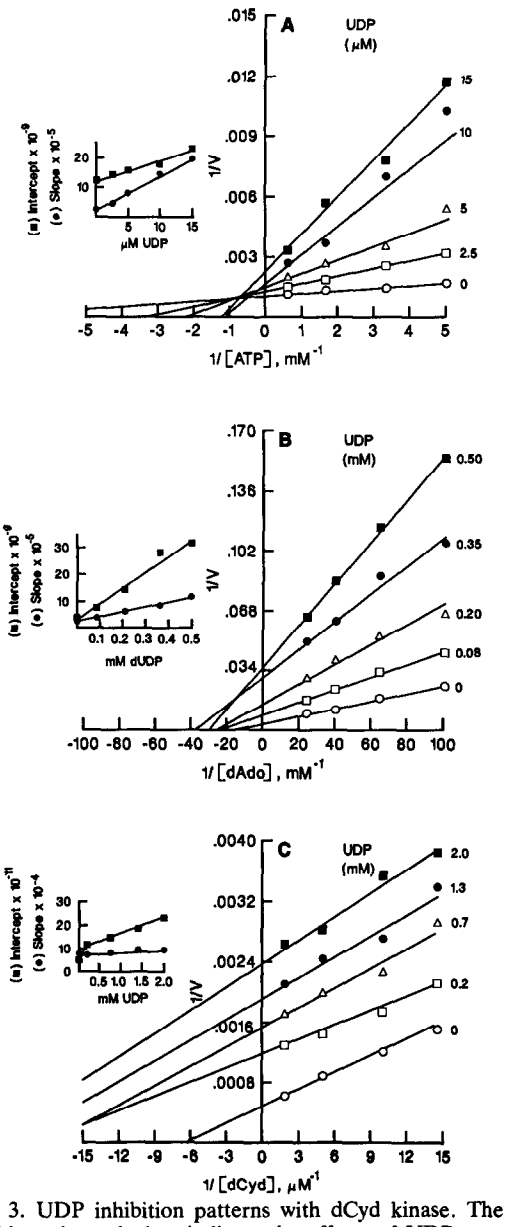

Fig. 3. UDP inhibition patterns with dCyd kinase. The double-reciprocal plots indicate the effects of UDP concentration on (A) dAdo phosphorylation (with dAdo fixed at 600 μM with various ATP concentrations), (B) dAdo phosphorylation and (C) dCyd phosphorylation. Mg · ATP was fixed at 5 mM. Values for inverse rates ($1/V$) are given in (nmol/hr/mg). Insets show replots of slopes and intercepts against UDP concentration.

respective triphosphates, because alternate salvage pathways usually require some catabolism of the nucleoside. The major catabolic pathways for drugs include deamination of analogues of dAdo by adenosine deaminase and deglycosylation of analogues of dIno and dGuo by purine nucleoside phosphorylase.

Because of the large difference in substrate binding affinities between the pyrimidine nucleoside dCyd and the purine nucleosides dAdo and dGuo (at least 100-fold), various cellular pyrimidine nucleosides might be expected to interfere with purine nucleoside phosphorylation by dCyd kinase. We examined this possibility by studying competition among the natural substrates for phosphorylation by this enzyme. The preferred substrate for this enzyme, dCyd, is an excellent noncompetitive inhibitor of dAdo and dGuo phosphorylation with K_i values of about 0.5 μM (Table 2). Since the plasma level of dCyd is near 1 μM [15], free dCyd could inhibit cellular purine nucleoside phosphorylation.

The triphosphate dCTP is a well-known effective inhibitor of dCyd kinase from both mammalian and bacterial sources [6, 16, 17]. It presumably acts as a negative feedback regulator of dCyd kinase activity [17]. Inhibition by dCTP was competitive with ATP and noncompetitive with dCyd or dAdo. Studies with a bacterial enzyme suggested that dCTP is a particularly effective inhibitor because it acts as a bisubstrate analog, overlapping both the nucleoside and ATP binding sites [18]. Comparison of dCyd kinase activity using dCyd or dAdo as substrates in the presence of dCTP disclosed that dAdo phosphorylation was inhibited three times more effectively by dCTP than was dCyd phosphorylation (Table 4). This result suggests that dCyd is more potent than dAdo in displacing dCTP from the enzyme, as would be expected from the differences in their K_m values. The competition of dCTP with ATP during purine nucleoside phosphorylation is consistent with a bisubstrate binding model [18].

The pyrimidine nucleotide UDP was also an effective inhibitor of dCyd or dAdo phosphorylation, showing mixed-type noncompetitive inhibition with ATP and noncompetitive inhibition with nucleoside substrates. It also showed a 3- to 6-fold lower K_i with dAdo than with dCyd (Table 4). Binding of UDP does not fit the bisubstrate model proposed for dCTP since dCyd kinase does not phosphorylate uridine or other ribonucleosides. The inhibitory effect of UDP has been reported previously for the calf thymus enzyme [19].

Concentrations of nucleotides in different T- and B-lymphoblast cell lines (Table 5) were consistent with a role for dCTP and UDP as potential endogenous regulators of purine nucleoside phosphorylation by dCyd kinase. The ranges of the pool sizes of both dCTP (2–8 μM) and UDP (15–1150 μM) were adequate to inhibit dCyd kinase activity significantly. Because of the greater sensitivity of dAdo phosphorylation to these inhibitors, one would predict a more substantial reduction in purine nucleoside phosphorylation.

Several of the antileukemic and antiviral drugs now in clinical use (ara-C, ara-A, ddCyd, and ddAdo) require phosphorylation by cellular kinases for activation within cells. Cytosolic dCyd kinase is a required enzyme in the activation of the pyrimidine analogues ara-C and ddCyd, and contributes to the activation of the purines ara-A and ddAdo [3–5, 8, 9]. As demonstrated in the present study and elsewhere, the pyrimidine analogues are much more efficiently phosphorylated by dCyd kinase than are the purines [3, 6, 7]. We also report that purine nucleoside phosphorylation inhibitors, namely dCyd, dCTP and UDP, are present in sufficient quantities to regulate dCyd kinase activity. Because

of the lower efficiency of phosphorylation of the purine nucleosides (ddAdo and ara-A), phosphorylation of these drugs by dCyd kinase should show greater sensitivity to endogenous nucleotide inhibitors than their pyrimidine nucleoside counterparts. This effect may hinder attempts to achieve inhibitory concentrations of active purine nucleotide analogues in T and B lymphocytes, but it may also stimulate the development of novel drug combinations that lower nucleotide pools.

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