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HUMAN DEOXYCYTIDINE KINASE

PURIFICATION AND CHARACTERIZATION OF THE CYTOPLASMIC AND MITOCHONDRIAL ISOZYMES DERIVED FROM BLAST CELLS OF ACUTE MYELOCYTIC LEUKEMIA PATIENTS

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Summary

A procedure for purifying human cytoplasmic and mitochondrial deoxycytidine kinase (NTP:deoxycytidine 5'-phosphotransferase, EC 2.7.1.74) was developed. Both purified isozymes have a similar molecular weight, activation energy and catalyze the reaction by a sequential mechanism.

These two isozymes differ with respect to their substrate specificities. With cytoplasmic deoxycytidine kinase, ATP, GTP and TTP have the highest reaction velocity. Pyrimidine nucleoside triphosphates have higher affinity but lower V than purine nucleoside triphosphates. Cytidine and arabinosylcytidine can serve as substrates.

With mitochondrial isozyme only ATP gives the highest reaction velocity. ATP and dATP have the same K_m but different V values. Besides deoxycytidine, also deoxythymidine but not cytidine or arabinosylcytidine can serve as substrates.

There are also differences between these two isozymes with respect to their sensitivity to inhibition. For cytoplasmic enzyme, Br^{5}dCyd and Iodo^{5}dCyd are not inhibitory. Both dCTP and UTP are competitive inhibitors (K_i 0.25 and 0.5 μM, respectively) with respect to ATP. For mitochondrial isozyme both Br^{5}dCyd and Iodo^{5}dCyd are inhibitory and dCTP and TTP are competitive inhibitors (K_i 2 and 10 μM, respectively) with respect to ATP.

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Abbreviation: Ara-Cyd, arabinosylcytidine.

Introduction

Deoxycytidine kinase (dCyd kinase) (NTP:deoxycytidine 5′-phosphotransferase EC 2.7.1.74) which catalyzes the phosphorylation of dCyd to its monophosphate nucleotide, has been isolated from many tissues and many of these preparations have been partially purified and characterized [1—7]. The properties of dCyd kinase seem to be different from one source to another. For instance, the enzyme from calf thymus seems to be able to use deoxyadenosine and deoxyguanosine as a substrate [4,5]. In contrast, dCyd kinase from human sources could use only dCyd as substrate [7]. Recently, the existence of two forms of dCyd kinase in mammalian cells was suggested [8]. This raises the question of which form of the enzyme was studied previously by various investigators and how pure their preparations were with respect to the presence of isozymes.

Studies in this laboratory are concerned with deoxyribonucleotide metabolism in human cells and with the use of pyrimidine analogs for cancer or viral chemotherapy. It is important to understand the properties of dCyd kinase since it is the key enzyme involved in the activation of dCyd analogs such as arabinosyl cytidine [1]. The information obtained will be important in understanding the metabolism of dCyd in human cells as well as in the design of new dCyd analogs for use in chemotherapy.

This communication describes a simple procedure for purifying both types of dCyd kinase and comparative studies on the general properties, substrate specificities, and kinetic properties of isozymes of this enzyme which are present in different cellular particulate fractions.

Experimental Procedure

Materials. [^{14}C]dThd, [^{14}C]dCyd, [^{14}C]Urd, [^{14}C]Cyd and [^{3}H]Ara-Cyd were obtained from Schwartz Mann. Vinyl^{5}dUrd was provided by Dr. M. Bobek of this department. All other nucleosides and nucleotides were purchased from Sigma Chemical Co. All reagents used were reagent grade. Calcium phosphate gel was kindly provided by Dr. David Baccanari of Burroughs Wellcome Co. Tetrahydrouridine was kindly provided by Dr. L.B. Mellet of Southern Research Institute. The affinity column matrix was essentially the same as described previously by Kowal and Markus [9] and modified by Lee and Cheng [10].

Assay procedures. The total volume of the enzyme assay mixtures was 0.1 ml and included 0.1 M Tris · HCl, pH 7.5, and 2 mM dithiothreitol. The reaction mixture for dThd kinase activity: 2 mM ATP, 2 mM $MgCl_2$, 0.1 mg bovine serum albumin, 1 unit of creatine kinase, 6 mM creatine phosphate, 7 mM NaF, 0.1 mM dThd, and 0.1 μCi of [^{14}C]dThd (46 Ci/mol).

The dCyd kinase reaction mixture contained 6 mM ATP, 6 mM $MgCl_2$, 0.2 mM tetrahydrouridine, 0.2% (w/v) bovine serum albumin, 1 unit of creatine kinase, 6 mM creatine phosphate, 7 mM NaF, 0.3 mM dCyd and 0.1 μCi of [^{14}C]dCyd (52 Ci/mol).

The Cyd kinase reaction mixture consisted of 3 mM ATP, 3 mM $MgCl_2$, 0.2 mM tetrahydrouridine, 0.15 mM Cyd and 0.02 μCi of [^{14}C]Cyd (40 Ci/mol).

The Urd kinase reaction mixture: 3 mM ATP, 3 mM $MgCl_2$, 0.15 mM Urd and 0.02 μCi of [^{14}C]Urd (51 Ci/mol). The Ara-Cyd kinase mixture contained 6 mM ATP, 6 mM $MgCl_2$, 0.2 mM tetrahydrouridine, 0.2% (w/v) bovine serum albumin, 7 mM NaF, 0.3 mM Ara-Cyd and 0.4 μCi of [^{3}H]Ara-Cyd (16 Ci/mmole).

The assays were performed as described previously [10,11] except for the washing procedures. Time of incubation of each assay was 1 h in general. The DE-81 discs for Cyd dCyd, and Ara-Cyd kinase activities were washed 3 times with 1 mM ammonium formate, once with water, and once with alcohol. The discs for the Urd kinase activities were washed three times with 2 mM ammonium formate, once with water, and once with alcohol. The unit of enzyme activity is defined as 1 nmol product formed per min at 37° C under our assay condition.

Electrophoresis and glycerol gradient centrifugation. Electrophoresis was essentially the same as that described previously [10,11]. Glycerol gradient centrifugation conditions were the same as described by Kit and coworkers [12], except that the centrifugation time was 36 h.

Results

Electrophoresis

The crude extract of acute myelocytic leukemia blast cells was loaded on the electrophoretic apparatus and the electrophoresis was performed as described previously [10]; at the end of the run, the gels were sliced and dThd and dCyd kinase activities were assayed; the electrophoretic profiles of the enzyme activities are presented in Fig. 1A. Two peaks of dCyd kinase activities were demonstrated. The one moving slower with an R_F value of 0.58 had the same mobility as dThd kinase isolated from mitochondria of the same type of cells. No activity was associated with the fraction containing cytoplasmic dThd kinase. The activities moving with the marker bromophenol blue yielded a single peak when a buffer with a pH of 7.5 was used for electrophoresis. This suggested that this enzyme activity is relatively homogeneous with regard to its electrophoretic behavior. The results shown in Figs. 1b, 1c and 1d were obtained when each of the soluble fractions of the subcellular fraction of acute myelocytic leukemia cells was electrophoresed. The nuclear and cytoplasmic fractions contain the fast moving isozyme; in contrast, mitochondrial dCyd kinase has an R_F value of 0.58. It should be noted that Urd kinase and Cyd kinase activity could only be found in the cytoplasmic fraction of the cells.

Enzyme purification

15 ml of the packed acute myelocytic leukemia blast cells were washed and subsequently disrupted by the same procedure as described previously [10]. The cell homogenate was centrifuged at 100 000 × *g* for 60 min and the supernatant (45 ml) was used as the starting material for further purification. The initial purification procedures (1% streptomycin sulfate and 20—55% $(NH_4)_2SO_4$ fractionation) were the same as those used previously in the purification of dThd kinase [10] up to the dThd kinase affinity column chromatography. The column was eluted with buffers indicated in Fig. 2. Three peaks of dCyd kinase

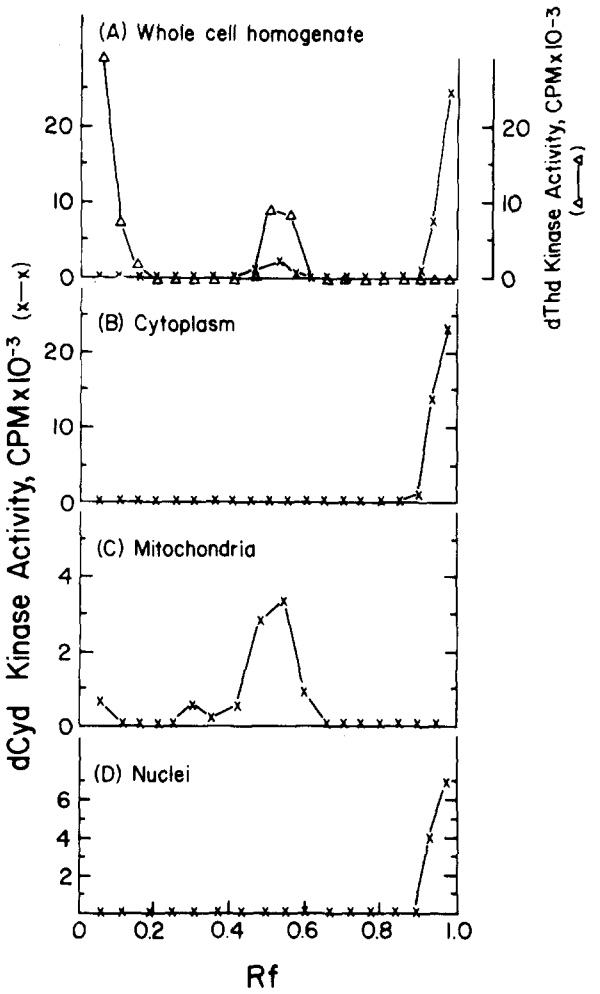

Fig. 1. Electrophoretic patterns of dCyd kinase and dThd kinase activities from cellular fractions of acute myelocytic leukemia blast cells. Cellular fractionation, electrophoretic techniques and enzyme assays were the same as described previously [10,11]. Samples of 50 μl (approx. 10 mg protein/ml) were applied to the gels. Electrophoretic mobilities (R_F values) were calculated with reference to the electrophoretic mobility of bromophenol blue.

activities were eluted from the column with a total recovery of 80%. The first peak came out slightly later than the void volume of the column. When this fraction of activity was reapplied to the column, then most of this dCyd kinase appeared at the position of the second peak which was eluted from the column by increasing the Tris · HCl concentration to 0.2 M. No Urd kinase activity was associated with this peak (Fig. 1d) nor were deoxyguanosine kinase and dThd kinase activities (data not shown). Cyd kinase activity was present in this peak. A third peak of dCyd kinase activity was observed only when the eluting buffer was changed to that used to elute our mitochondrial dThd kinase as described previously [10]. The activity in this fraction was not contaminated with Cyd, Urd or deoxyguanosine kinase activity. The degree of purification of the second and third activity peaks varied from sample to sample, depending

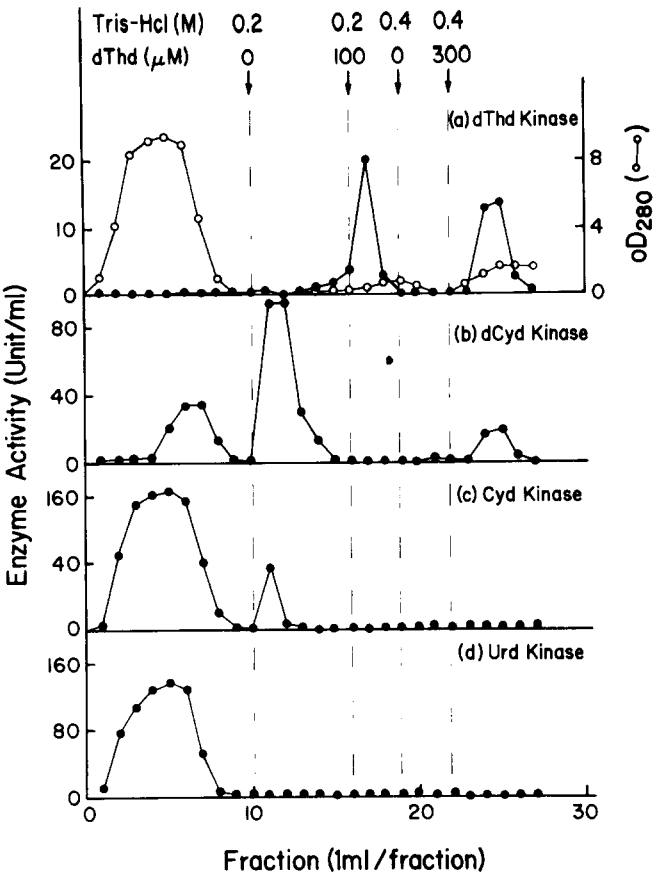

Fig. 2. Elution patterns of affinity column chromatography of dThd kinase, dCyd kinase, Cyd kinase and Urd kinase from acute myelocytic leukemic blast cells. The column (0.9 X 4 cm) was pre-equilibrated with Tris · HCl buffer (pH 7.5, 0.01 M) and the initial buffer used is the same as the pre-equilibrated column buffer. Glycerol (10%) and dithiothreitol (5 mM) were added as stabilizer. 65 mg of protein (after ammonium sulfate fractionation steps) were applied to a column which was packed with dThd-containing CH-Sepharose prepared as described previously [10]. The elution buffers are shown in the figure.

on the difference in levels of enzyme from the starting source of blast cells. It should be noted that the protein content in the fractions represented by the second and third dCyd kinase activity peaks was very small. The properties of the enzyme contained in the third peak (Fig. 2) were studied without further purification. The specific activity of this purified mitochondrial dCyd kinase was around 150 units/mg.

Enzyme contained in the second peak (Fig. 2) was further purified by chromatography on a calcium phosphate gel cellulose column (0.7 X 15 cm with gel/cellulose equal to 1.26 V) which was equilibrated with buffer containing 0.02 M Tris · HCl, pH 7.5, EDTA (0.4 mM), dithiothreitol (2 mM), 0.1 M NaCl and 10% glycerol. dCyd kinase activity was adsorbed on the column, and could be eluted by using a linear gradient of ATP-Mg^{2+} which is a substrate of the reaction; however, it could not be eluted with 0.2 mM dCyd which is also a substrate of the enzyme. Cyd kinase activity came out with dCyd kinase, and when Ara-Cyd was used as the substrate, the phosphorylation of this substrate

was also associated with this fraction (Fig. 3a). In order to rule out that the shoulder in Fig. 3a was due to the presence of another isozyme, the fractions under the shoulder were pooled and rechromatographed on an identical column. The elution profile (Fig. 3b) activity was identical to the original one (Fig. 3a). This suggested that the assymmetry of the peak was not due to the presence of a mixture of two or more non-interconvertible forms of the enzyme. The recovery in this step was more than 80%, the specificity of this final cytosol preparation was always around 80 units/mg independent of the starting material used.

In order to illustrate whether the enzyme preparation had reached electrophoretic homogeneity with respect to isozymes, enzymes from the third peak of the affinity column chromatography (Fig. 2b) and that from the peak I of calcium phosphate gel column chromatography (Fig. 3a) were subjected to electrophoresis. The results of this electrophoresis indicate that in the case of enzyme from calcium phosphate gel chromatography, the dCyd kinase activity moved as fast as that found in the cytoplasm or nuclei of blast cells. This enzyme also had Cyd kinase activity and will be designated in the further discussion as cytoplasmic dCyd kinase. The dCyd kinase activity from peak III of the affinity column moved the same distance as that from mitochondria and coincided with dThd kinase activity; it will be designated as mitochondrial

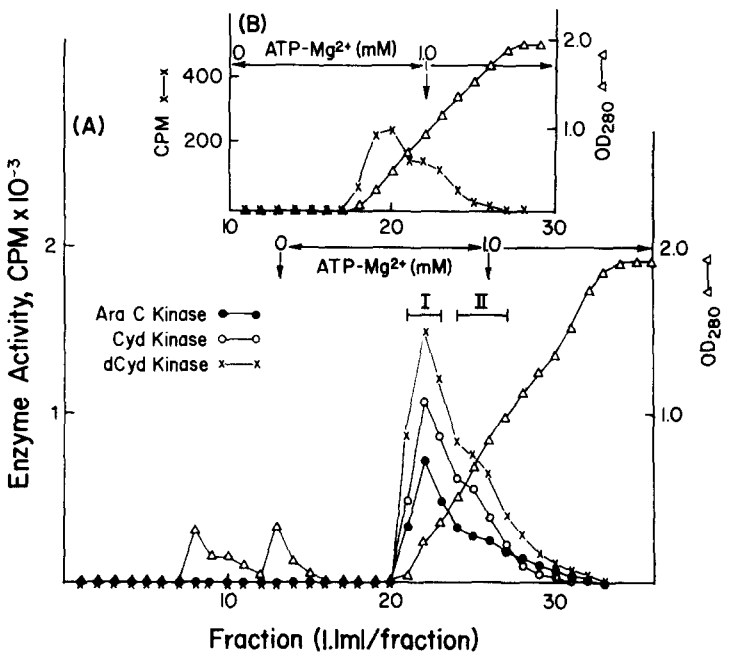

Fig. 3. Elution patterns of calcium phosphate gel cellulose chromatography of dCyd kinase, Cyd kinase and Ara-Cyd kinase from the pool of the second peak of dCyd kinase activity of affinity column chromatography (A) or re-chromatography of peak II from A (B). The calcium phosphate column (0.7 × 15 cm) was pre-equilibrated with Tris · HCl buffer as described in the text. 2 mg of protein were loaded on the column in A. A linear ATP-Mg^{2+} (0—1.0 mM) gradient elution buffer was used to elute enzyme out of the column. Fractions (24—27) of A were pooled. After dialysis for 4 h at 4°C against the column buffer, the pool was applied to the same size column pre-equilibrated with the column buffer. The elution profiles are shown in B. The rest of the details are described under Results.

dCyd kinase. Experiments performed for the following study were performed with these two purified preparations. It should be noted that both preparations were stable at 4°C for at least a week in the presence of 10% glycerol, 2 mM ATP-Mg^{2+} and 10 mM dithiothreitol.

Molecular weight and activation energy

Both cytoplasmic and mitochondrial dCyd kinase were subjected to glycerol gradient centrifugation and their migrations compared in the presence of 4 mM ATP-Mg^{2+} as described in the text. The results indicate that a single symmetrical peak of dCyd kinase activity was found for the enzyme from each source. The sedimentation rate of the two enzymes from the two sources was identical. dThd kinase activity coincided with mitochondrial dCyd kinase activity. No Cyd or Ara-Cyd kinase activity could be found. In contrast, Cyd and Ara-Cyd kinase activity was found to coincide with cytoplasmic dCyd kinase and no dThd kinase activity was observed. Both samples of dCyd kinase were estimated to have molecular weights of 70 000, which is in agreement with the molecular weight previously reported for mitochondrial dThd kinase [10].

When temperature dependence of the dCyd kinase reaction was studied with saturating amounts of either substrate in the regular assay mixture, a linear Arrhenius plot was obtained between 28 and 40°C for both isozymes. The activation energy calculated was 15 kcal/mol for both enzymes.

Initial velocity studies

Kinetic studies were performed for both isozymes, using non-saturating amounts of both substrates, and the results indicate that a cross-line pattern with the intersection near or on the axis of 1/dCyd was obtained. By replotting the intercept of $1/v$ vs. 1/fixed substrate, the K_m values of ATP for cytoplasmic and mitochondrial dCyd kinase were calculated to be 16 and 70 μM, respectively. The K_m values of dCyd were 3 μM for cytoplasmic and 25 μM for mitochondrial enzyme.

Specificity of phosphate donor

Various nucleoside triphosphates-Mg^{2+} were tested for their ability to act as a phosphate donor for the reaction catalyzed by either enzyme. The results are shown in Table I. These results do not tell us whether the differences in the enzyme velocities were due to a difference in K_m or in V; therefore, a few nucleoside triphosphates-Mg^{2+} were selected for further kinetic studies. Each of these compounds obeyed Michaelis-Menten kinetics. The K_m and relative V values are given in Table II. The relative V is almost the same as the one described in Table I, but the K_m values are different. The pyrimidine nucleoside triphosphates tested seem to have better apparent binding affinity than the purine analogs tested; this was the case for both enzymes.

Effects of various types of nucleosides

It was previously suggested that dCyd kinase could possibly serve as the kinase for phosphorylating other nucleosides [4,5]. In our purified preparations, the cytoplasmic enzyme phosphorylated Cyd and Ara-Cyd and the mitochondrial enzyme phosphorylated dThd. In view of these results, it was of

TABLE I

ACTIVITY OF VARIOUS NUCLEOSIDE TRIPHOSPHATES AS PHOSPHATE DONORS IN THE dCyd KINASE REACTION

The assays were performed in duplicate under conditions described in the text, except that ATP in the reaction mixture was substituted by various nucleoside triphosphates (4 mM). The results are expressed as percent of dCMP formed when ATP was used. 0.04 unit of the purified enzyme was used per assay.

Nucleoside triphosphate-Mg^{2+}	dCMP formed (percent of control)	
	Cytoplasm	Mitochondria
ATP	100	100
GTP	110	38
CTP	60	63
UTP	16	38
dATP	74	61
dGTP	100	31
dCTP	6	7
dTTP	94	2
dUTP	24	10
Iodo-dUTP	47	3
Ara-CTP	27	9

interest to determine whether various types of nucleosides would interfere with the dCyd kinase reaction. Inhibition of the reaction would suggest that the nucleoside was acting as an alternative substrate or as an inhibitor of the enzyme. The sensitivity of such an assay depends on the substrate concentration used [13]. The results of this study are presented in Table III. No marked inhibition by any of the nucleosides was obtained when the cytoplasmic enzyme was used. However, Iodo^{5}dCyd and Br^{5}dCyd exerted strong inhibition and dThd, dUrd and Urd were weaker inhibitors of the mitochondrial dCyd kinase catalyzed reaction.

The presence of Ara-Cyd kinase and Cyd kinase activities in our purified cytoplasmic enzyme preparation and the failure to demonstrate inhibition of dCyd phosphorylation under the above conditions by Ara-Cyd and Cyd posed the question whether this was due to the impurity of our enzyme preparation

TABLE II

KINETIC PARAMETERS OF NUCLEOSIDE TRIPHOSPHATES FOR dCyd KINASE ISOZYMES

The assays were performed as described in the text except that 200 μM of dCyd and various amounts of nucleoside triphosphate-Mg^{2+} were used. The K_m values were obtained by Lineweaver-Burk plots.

	Cytoplasm		Mitochondria	
	K_m (μM)	V (%) *	K_m (μM)	V (%) *
ATP	16	100	70	100
GTP	18	117	—	—
CTP	8	67	28	61
UTP	0.8	13	—	—
dATP	—	—	77	60
TTP	6	90	—	—

* The V values reported were the relative values with respect to that of ATP.

TABLE III

EFFECT OF VARIOUS NUCLEOSIDE ANALOGS ON THE dCyd KINASE REACTION

Reactions and assays were performed as described in the text with the exception that the amount of additives added was 500 μM and the amount of enzyme used was 0.02 unit per assay. The amount of dCyd and ATP-Mg^{2+} in the assay was 100 μM and 4 mM, respectively.

Additive	dCMP formed (percent of control)	
	Cytoplasm	Mitochondria
None	100	100
Iodo^{5}dCyd	105	5
Br^{5}dCyd	100	7
Ara-Cyd	94	98
Cyd	100	100
dThd	96	38
dUrd	100	65
Urd	100	80
dGuo	97	95
dAdo	100	99

or rather to the low sensitivity or our test. Kinetic experiments were performed by varying the amount of dCyd in the presence or absence of some of the nucleosides of interest. The results are shown in Table IV. All of the nucleosides studied acted as competitive inhibitors with respect to dCyd but not to ATP-Mg^{2+} for the reaction catalyzed by either isozyme (not shown). When Ara-Cyd or Cyd was used as the variable substrate for the cytoplasmic dCyd kinase-catalyzed reaction, dCyd was a competitive inhibitor. The K_m of Ara-Cyd and Cyd and the K_i of dCyd are also reported in Table IV. The reason for the discrepancy between the K_i and K_m values could be due to the difference in make-up of these two parameters by the different rate constants.

Effect of nucleoside triphosphate-Mg^{2+} on dCyd kinase-catalyzed reaction

The inhibitory effects of 4 mM of a nucleoside triphosphate-Mg^{2+} added to

TABLE IV

KINETIC PARAMETERS OF VARIOUS NUCLEOSIDES

K_m values were determined by varying the concentration of labeled nucleoside while keeping the ATP-Mg^{2+} concentration at 4 mM. K_i values were determined by varying the concentration of dCyd at two fixed concentrations of nucleoside additive and 4 mM ATP-Mg^{2+}. The slopes of these lines on double reciprocal plots were plotted against the additive concentration in order to determine the K_i.

	Cytoplasm		Mitochondria	
	K_m (μM)	K_i (μM)	K_m (μM)	K_i (μM)
dCyd	3	0.8 *	22	—
Br^{5}dCyd	—	360	—	5
Ara-Cyd	40	150	—	3000
Cyd	750	3500	—	—
dThd	—	—	5 **	5

* Either Ara-Cyd or Cyd was used as the variable substrate, otherwise the conditions were the same as above.

** Taken from the manuscript by Lee and Cheng [17].

TABLE V

EFFECT OF ADDITIONAL NUCLEOSIDE TRIPHOSPHATES ON THE dCyc KINASE REACTION IN THE PRESENCE OF ATP-Mg^{2+}

The assays were performed under conditions described in the text except that 4 mM of nucleoside triphosphate-Mg^{2+} were added to the reaction mixture. The results are expressed as percent of dCMP formed when no additional nucleoside triphosphate was included. 0.04 unit of the purified enzyme was used per assay.

Additional nucleoside triphosphate-Mg^{2+}	dCMP formed (percent of control)	
	Cytoplasm	Mitochondria
None	100	100
ATP	100	100
GTP	105	92
CTP	74	83
UTP	21	57
dATP	79	82
dGTP	105	70
dCTP	9	8
TTP	83	9
dUTP	32	31
Iodo dUTP	47	10
Ara CTP	56	30

a reaction mixture containing 4 mM of ATP-Mg^{2+} and 400 μM dCyd, on the reactions catalyzed by either isozyme are shown in Table V. dCTP and UTP showed the strongest inhibition of the cytoplasmic isozyme, whereas dCTP, dTTP and iodo-dUTP showed the strongest inhibitory effect on the reaction catalyzed by the mitochondrial isozyme. The kinetic study revealed that for cytoplasmic dCyd kinase, dCTP and UTP acted as competitive inhibitors with respect to ATP but ont dCyd. There was curvature of lines on a $1/v$ vs. 1/ATP-Mg^{2+} plot when UTP-Mg^{2+} was the inhibitor; this may be due to the fact that UTP-Mg^{2+} serves as a poor phosphate donor for the reaction, as indicated previously (Table II). The K_i values obtained were 0.25 μM for dCTP-Mg^{2+} and 0.5 μM for UTP-Mg^{2+}. In the case of mitochondrial dCyd kinase, dCTP-Mg^{2+} and dTTP-Mg^{2+} were also competitive inhibitors with respect to ATP-Mg^{2+}, but not with dCyd; the K_i values obtained were 2 μM for dCTP and 10 μM for dTTP.

Discussion

The subcellular localization of dCyd kinase isozymes demonstrated in this communication together with our previous observations concerning the subcellular localization of dThd kinase isozyme [10] seems not only to fit the idea of cellular compartmentation of dThd phosphorylation as suggested by other investigators [14], but also that of dCyd phosphorylation [15]. In contrast to the deoxypyrimidine nucleoside kinases, kinase activity for Cyd and Urd could be found only in cytoplasm and not in mitochondria (unpublished results). This is not agreeable to the observations made by other investigators; they have demonstrated the phosphorylating activity of Urd in the isolated mouse liver mitochondria [16]. Whether the difference is due to the species specificity or

the nature of acute myelocytic leukemia blast cells is not understood at the moment.

dCyd kinase activities of the homogenate of blast cells from difference patients with acute myelocytic leukemia were different. This has been shown by other investigators as well [7]. Our unpublished results also indicated that blast cells from even a single individual could be different if the samples were taken on different days. In addition, the specific activity of each isozyme was not known since the dCyd kinase assay could not distinguish each isozyme activity. Therefore, it is difficult to construct a purification table based on the differences in the specific activity of the initial starting material and final purified preparation. In general, the specific activity of the crude extract used for our enzyme purification was around 0.2 unit/mg, so both isozymes in our studies were purified extensively. In our purification step most proteins came out in the void volume of the dThd kinase affinity column. Furthermore, cytoplasmic enzymes were specifically de-adsorbed from calcium phosphate gels by their substrate, ATP, and contamination with some enzymes such as dCyd deaminase and dCMP phosphatase did not occur in our purified preparations. However, it is unclear at the moment whether we have a homogeneous enzyme preparation or not.

The co-purification of dThd kinase activities with mitochondrial dCyd kinase from the dThd kinase affinity column, the co-sedimentation of both activities on the glycerol gradient and the same mobility on electrophoresis support the idea that both dCyd and dThd kinase activities may reside in the same protein molecule as suggested by Leung et al. [8]. This was substantiated by the studies of substrate and inhibitor specificity. dTTP and dCTP which could inhibit both enzyme activities were the final products of the reaction pathways; dThd acted as a competitive inhibitor with respect to dCyd for the dCyd kinase reaction, and dCyd acted as an inhibitor with respect to dThd for dThd kinase [17]. Also, Iodo^{5}dCyd and Br^{5}dCyd which have structural similarities to both dCyd and dThd exerted inhibitory effects on both dCyd and dThd kinase activity in this preparation. Vinyl^{5}dUrd which has properties similar to Iodo^{5}dCyd also acted as inhibitor. It should be noted that although Ara-Cyd acted as a poor inhibitor for dCyd kinase, no Ara-Cyd activity could be detected in the purified preparation. This suggests that Ara-Cyd is acting only as a weak inhibitor of the enzyme, and not as an alternative substrate. In addition, this enzyme could not phosphorylate Cyd and deoxyguanosine. When initial velocity studies were performed, interesting results were found. The dCyd kinase reaction catalyzed by this enzyme seems to follow a sequential mechanism. In contrast, we previously indicated that the dThd kinase reaction catalyzed by this same preparation seems to follow a ping-pong mechanism, although the enzyme binds dThd in the absence of ATP-Mg^{2+} [17]. This may indicate that although this enzyme follows Michaelis-Menten type kinetics in the concentration range we have used, it may have a complex structure, e.g. more than one binding site for either dCyd or dThd but only one type of catalytic site for either substrate phosphorylation is present on the enzyme molecule: the reactions for dCyd and dThd phosphorylation are mutually exclusive.

In the cytoplasm it is clear that there are two distinct enzyme entities responsible for the phosphorylation of dCyd and dThd. The amino acid sequence

and structure of the active sites of cytoplasmic dThd and dCyd kinase must be different from each other and must also differ from that of the mitochondrial isozyme. Furthermore, cytoplasmic dCyd kinase has the ability to phosphorylate Ara-Cyd, but not deoxyguanosine or Urd. It is of interest that among the nucleoside triphosphates-Mg^{2+} tested, pyrimidine nucleoside triphosphates have a lower K_m than the purine nucleoside triphosphates. On the other hand, GTP and ATP have a higher V for these reactions. dCTP and UTP could serve as strong competitive inhibitors with respect to ATP, but not to dCyd. This is in contrast to the results reported by others [7] who claimed that for kinase from human acute myelocytic leukemia blast cells there is a competitive relationship between dCTP and Ara-Cyd. Since UTP acts as a strong inhibitor of dCyd kinase and is present inside the cells at higher concentration than the dCTP inside the cell, it is possible that it plays an important physiological role in regulating deoxyribonucleotide metabolism.

In summary, two dCyd kinase isozymes were isolated and purified from blast cells derived from acute myelocytic leukemic patients. One is associated with cytoplasm and the other with mitochondria. These enzymes differ in specificity. dCTP and UTP are good inhibitors for cytoplasmic dCyd kinase; in contrast, dCTP and TTP are good inhibitors of mitochondrial dCyd kinase. Ara-Cyd could only be phosphorylated by the cytoplasmic dCyd kinase.

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