Human Deoxythymidine Kinase II: Substrate Specificity and Kinetic Behavior of the Cytoplasmic and Mitochondrial Isozymes Derived from Blast Cells of Acute Myelocytic Leukemia†

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ABSTRACT: Cytoplasmic and mitochondrial deoxythymidine kinase isozymes derived from the blast cells of acute myelocytic leukemia differ in their substrate specificity and kinetic behavior. These enzymes require divalent cations for their activity. The data suggest that the major role of divalent cations is to chelate with ATP; the complex thus formed serves as the phosphate donor for the reaction. The activity of various triphosphate nucleosides as a phosphate donor for cytoplasmic deoxythymidine kinase is as follows: ATP = dATP > *ara*-ATP > GTP > CTP > dGTP = dCTP > dUTP, whereas for mitochondrial deoxythymidine kinase, the order of activity is ATP > CTP > UTP = dATP > GTP > *ara*-ATP > dGTP = dCTP > dUTP. Neither IdUTP nor dTTP could serve as a phosphate donor in the reaction catalyzed by either isozyme. From the many pyrimidine analogues tested for their binding affinity to each of these isozymes, I-dUrd and Br-dUrd had high good affinity which was equivalent to that of deoxythymidine. 5-Allyl-dUrd, 5-ethyl-dUrd, and 5-propyl-dUrd were only weakly bound to each isozyme. 5-I-dCyd, 5-Br-dCyd, dCyd, and 5-vinyl-dUrd were tightly bound to mitochondrial deoxythymidine kinase but not to the cytoplasmic isozyme. dTTP and I-dUTP are potent inhibitors of the reaction catalyzed by both isozymes. In contrast, dCTP and *ara*-CTP are potent inhibitors only of the mitochondrial isozyme, but not of the cytoplasmic isozyme. ATP-Mg^{2+} acts as a sigmoidal substrate of the cytoplasmic isozyme with a "K_m" of 0.22 mM, and as a regular substrate of the mitochondrial isozyme with a K_m of 0.1 mM. Deoxythymidine acts as a regular substrate for both cytoplasmic and mitochondrial isozyme with a K_m of 2.6 and 5.2 μM, respectively. Initial velocity as well as product inhibition studies suggest that the cytoplasmic isozyme catalyzes the reaction via a "sequential" mechanism. In contrast, mitochondrial deoxythymidine kinase catalyzes the reaction via a "ping-pong" mechanism.

The kinetic behavior of deoxythymidine (dThd)[1] kinases derived from various mammalian sources has been reported to differ depending on the source of the enzyme. For example, the inhibition of dThd-kinase by dTTP was found to be competitive with dThd for the enzyme derived from calf thymus (Her and Momparler, 1971), noncompetitive for the enzyme derived from Walker tumor (Bresnick and Thompson, 1965), and, depending on the pH, either competitive or complex for the Ehrlich ascites enzyme (Prusoff and Chang, 1969). For dThd-kinase derived from mouse ascites sarcoma 180, dTTP inhibition is competitive with ATP and noncompetitive with dThd (Cheng and Prusoff, 1974). Also there is evidence that dThd-kinase from Walker carcinoma or mouse ascites sarcoma 180 does not follow simple Michaelis-Menten type kinetics (Bresnick and Thompson, 1965; Cheng and Prusoff, 1974). In contrast, the calf thymus enzyme displayed strict Michaelis-Menten kinetics (Her and Momparler, 1971). In view of these differences, it appears warranted to study the kinetics of dThd-kinase derived from a human source. This will be useful in understanding the regulatory roles of dThd-kinase as well as the development of new agents in the viral or cancer chemotherapy of human diseases.

The deoxythymidine kinases from the blast cells of patients with acute myelocytic leukemia were purified and shown to exist in two molecular forms, one present in the cytoplasm and the other in the mitochondria. These isozymes differ in their physical properties such as activation energy, sensitivity to salt inhibition, and electrophoretic mobility (Lee and Cheng, 1976). The study of substrate specificity and kinetic behavior of both isozymes is the subject of this communication.

Materials and Methods

The method used for the purification of deoxythymidine kinase has been previously described (Lee and Cheng, 1976). The nucleotides and nucleosides were purchased from the Sigma Chemical Company or P-L Biochemical Company. All reagents were of reagent grade, and all of the solutions were freshly prepared for the kinetic studies.

The purified preparations of cytoplasmic and mitochondrial dThd-kinases were used for the kinetic studies within 2–24 h after they were obtained. Therefore, we minimized the possibility of changes in the kinetic properties of these enzymes as a function of time. Enzymes purified by affinity column chromatography were stored in solutions containing dThd (200 μM). For kinetic studies, the enzyme was passed through a column of Sephadex G-25 equilibrated with 0.2 M Tris-HCl (pH 7.5), 10% glycerol, 5 mM dithiothreitol, 2 mM $MgCl_2$, and 2 mM ATP. In some cases, albumin was added (1 mg/ml) to stabilize the enzyme. Enzyme assay procedures were similar to those described in the preceding paper (Lee and Cheng, 1976). One unit of dThd-kinase is defined as the amount of the enzyme that catalyzes the conversion of 1 nmol of dThd to dTMP per min under the assay conditions described.

Results

Effect of the Ratio of ATP to Mg^{2+} on dThd-Kinase Activity. Both the cytoplasmic and mitochondrial dThd-kinases

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[1] Abbreviations used: dThd, deoxythymidine; *ara*-ATP, arabinoadenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane.

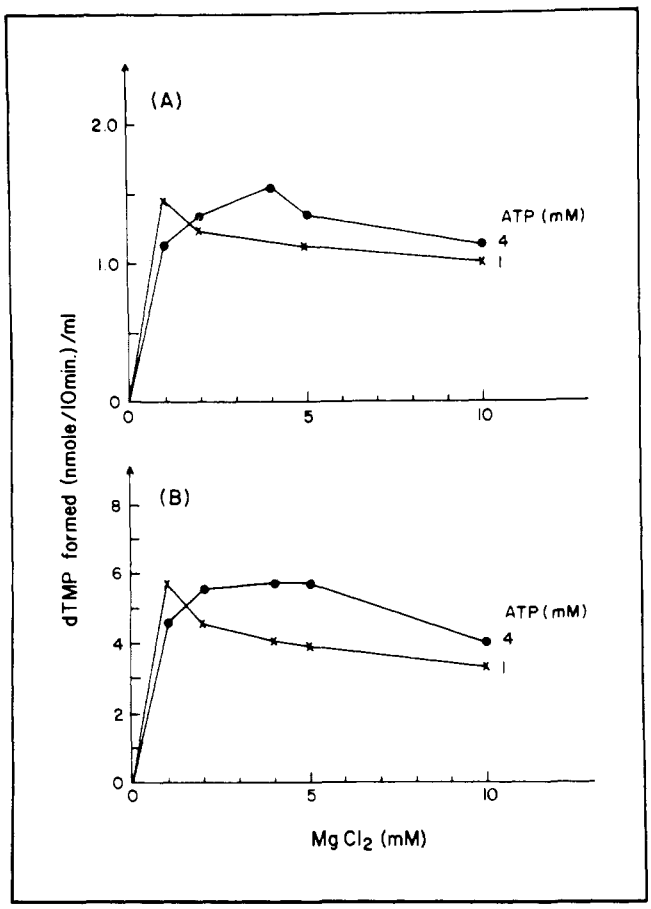


FIGURE 1: Effect of the molar ratio of ATP to Mg^{2+} on dThd kinases activities. (A) Mitochondrial dThd kinase; (B) cytoplasmic dThd kinase. The assay is as described in the preceding paper (Lee and Cheng, 1976) except for the concentrations of ATP and Mg^{2+}: (●) with 4 mM ATP; (X) with 1 mM ATP.

TABLE I: Product Inhibition Studies of the Reaction of dThd Kinases.

Product	Variable Substrate	Inhibition Pattern[a] Cytoplasmic Enzyme	Inhibition Pattern[a] Mitochondrial Enzyme
ADP	dThd	U	C
ADP	ATP	N	N
dTMP	dThd	C	N
dTMP	ATP	NI	C

[a] U, uncompetitive; C, competitive; N, noncompetitive; NI, no inhibition.

TABLE II: Effect of Various Triphosphate Nucleosides as Phosphate Donors for the dThd Kinases.[a]

Donor (2 mM)	% of Activity Cytoplasm	% of Activity Mitochondria
ATP	100	100
GTP	36	46
CTP	21	79
UTP	15	58
dATP	108	56
dGTP	17	26
dCTP	15	21
dUTP	6	10
dTTP	1	3
IdUTP	1	9
ara-CTP	21	7
ara-ATP	67	33

[a] The assay was performed under the conditions as described in the previous paper (Lee and Cheng, 1976). Various triphosphate nucleosides were tested at concentration of 2 mM in place of ATP.

responded similarly to changes in the concentration of Mg^{2+} and ATP. As shown in Figure 1, when the concentration of ATP was greater than that of Mg^{2+}, an inhibitory effect is observed. Maximal activity occurs when equal amounts of ATP and Mg^{2+} are included in the assay medium. An excess of Mg^{2+} relative to ATP produced little inhibitory effect on enzyme activity.

Initial Velocity and Product Inhibition Studies. In these experiments, the concentrations of the nucleoside triphosphate solutions were equal to those of $MgCl_2$.

Initial velocity studies were performed by keeping one substrate at a fixed concentration and varying the concentration of the other substrate. For cytoplasmic dThd-kinase, at a fixed concentration of dThd, linear plots are obtained when $1/v$ is plotted against $1/(Mg^{2+}\text{-ATP})^2$, whereas for mitochondrial dThd-kinase, $1/v$ plotted against $1/(Mg^{2+}\text{-ATP})$ results in linear plots. When the intercepts of these plots on the $(1/v)$ axis are plotted against 1/(dThd), the K_m of dThd was calculated to be 2.6 μM for cytoplasmic dThd-kinase and 5.2 μM for mitochondrial dThd-kinase. When ATP-Mg^{2+} was used at a fixed concentration, Lineweaver-Burk plots of $1/v$ vs. 1/dThd for both dThd-kinase are linear. By replotting the intercept of each line, the K_m of ATP-Mg^{2+} was determined to be 0.22 mM for cytoplasmic dThd-kinase and 0.10 mM for mitochondrial dThd-kinase. All K_m values obtained from these plots are consistent with values obtained at the saturation levels of the other substrate. Initial velocity studies indicated an intersecting line pattern for cytoplasmic dThd-kinase and a parallel line pattern for mitochondrial dThd-kinase, suggesting that these two isozymes might catalyze the reaction via different mechanisms.

Product inhibition studies with both enzymes were studied by including either ADP-Mg^{2+} or 5′-dTMP in the reaction mixture, and in the absence of an ATP-regenerating system when ADP-Mg^{2+} inhibition was studied. The highest concentration of ADP-Mg^{2+} used for this study was 4.3 mM and that of dTMP was 0.6 mM. The results are summarized in Table I.

Phosphate Donor Specificity of dThd-Kinases. In this study, the concentration of the triphosphates was equimolar (2 mM) to that of $MgCl_2$. Their efficiency as phosphate donors is compared in Table II. For cytoplasmic dThd-kinase, the maximal rate of reaction was obtained with ATP and dATP, whereas *ara*-ATP had about 67% the activity of these nucleotides. Mitochondrial dThd-kinase activity was highest when ATP and CTP were used as substrates; the other triphosphate nucleosides tested were poor phosphate donors. For the catalysis of cytoplasmic dThd-kinase, purine triphosphate nucleosides appear to donate phosphate better, whereas triphosphate ribonucleosides are better phosphate donors in the catalysis of mitochondrial dThd-kinases (Table II). The kinetics of ATP, dATP, and CTP were studied and their K_m and V_{max} values are reported in Table III. All three triphosphate nucleosides conform to Lineweaver-Burk kinetics when assayed with mitochondrial dThd-kinase. However, with cyto-

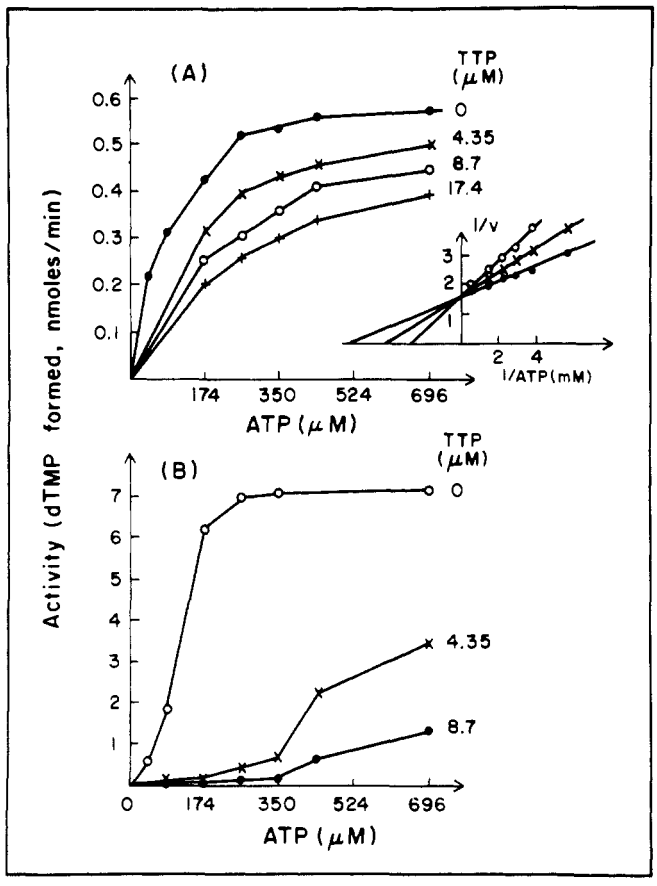


FIGURE 2. Effect of dTTP-Mg^{2+} on mitochondrial (A) and cytoplasmic (B) dThd kinase with varying amounts of ATP and a fixed concentration of dThd (81 μM).

TABLE III: K_m and V_{max} of Phosphate Donors.

	Cytoplasm		Mitochondria	
NTP-Mg^{2+}	K_m (mM)	V_{max}[c]	K_m (mM)	V_{max}[c]
ATP	0.22[a]	100	0.10[b]	100
dATP	0.20[a]	108	0.11[b]	56
CTP	1.00[b]	21	0.07[b]	79

[a] K_m was obtained from the plot of $1/v$ vs. $1/(NTP)^2$. The square root of the intercept at the abscissa of $1/(NTP)^2$ is taken as K_m^{-1}. [b] K_m was obtained from usual Lineweaver-Burk plot. [c] V_{max} are expressed as percent activities with respect to ATP.

TABLE IV: Effect of Various Triphosphate Nucleosides on dThd Kinase Catalysis with ATP and dThd as Substrates.[a]

	% TMP Formed	
Nucleotides (2 mM)	Cytoplasmic Enzyme	Mitochondrial Enzyme
ATP	100	100
GTP	81	79
CTP	92	71
UTP	98	85
dATP	87	64
dGTP	77	74
dCTP	85	18
dUTP	15	43
dTTP	1	6
IdUTP	0	4
ara-CTP	77	17
ara-ATP	86	54

[a] The assay was performed under the conditions described in the previous paper (Lee and Cheng, 1976) with the exception that assay mixture contained ATP-Mg^{2+} and various additives (with equimolar amounts of $MgCl_2$) at 2 mM each.

plasmic dThd-kinase, CTP showed Lineweaver-Burk kinetics, whereas ATP or dATP showed linear plots only with $1/v$ vs. $1/(ATP\text{-}Mg^{2+})^2$ or $1/(dATP\text{-}Mg^{2+})^2$ plots, respectively, suggesting a sigmoidal curve and possibly a cooperative effect. Thus, it seems that ATP and dATP are comparable phosphate donors for the cytoplasmic dThd-kinase because of similarity in apparent K_m and V_{max} values. CTP is a poor phosphate donor for cytoplasmic dThd-kinase and compared with ATP has a high K_m and a low V_{max} value, possibly due to its non-cooperative substrate nature. ATP and dATP also showed similar K_m values for mitochondrial dThd-kinase; however, V_{max} for dATP was only two-thirds that of ATP. CTP binds to mitochondrial dThd-kinase better than ATP, but its V_{max} is only four-fifths that of ATP.

Effect of Various Triphosphate Nucleosides (NTP) on the Catalysis of dThd-Kinase with ATP and dThd as Substrates. The assay mixtures contained 2 mM each of NTP-Mg^{2+} and 190 μM dThd. The results are shown in Table IV. Under these conditions, cytoplasmic dThd-kinase was more sensitive to inhibition by dUTP, dTTP, and IdUTP than the mitochondrial dThd-kinase. On the other hand, mitochondrial dThd-kinase was inhibited by dCTP and ara-CTP, whereas cytoplasmic dThd-kinase was only moderately inhibited by these compounds. Also we found that dUTP is inhibitory to both isozymes, but that UTP did not inhibit either enzyme. However, dGTP and GTP exert similar effects with each enzyme. Although both dATP and *ara*-ATP are effective phosphate donors, they compete with ATP when used as a substrate for both enzyme.

At saturating levels of ATP, and with dThd as a variable substrate, dTTP and dUTP were found to inhibit mitochondrial dThd-kinase competitively with K_i values of 5.7 and 76 μM, respectively. dCTP was found to inhibit this isozyme in a complicated way and its K_i value could not be determined. For cytoplasmic dThd-kinase, dTTP and dCTP were found to inhibit ATP competitively with K_i values of 0.6 and 45 μM, respectively. Under the same conditions, dUTP exhibits a complex inhibition pattern.

With ATP as a variable substrate and dThd at a saturating level, dTTP, dCTP, and dUTP were found to behave similarly toward the mitochondrial dThd-kinase, while they behaved in a complex manner toward the cytoplasmic dThd-kinase. For instance, dTTP tended to increase the sigmoid character of the curve in a plot of the rate of reaction vs. substrate (ATP-Mg^{2+}) concentration. A comparison of the effect of dTTP on the reaction catalyzed by mitochondrial and cytoplasmic dThd-kinase using ATP as a variable substrate is shown in Figure 2.

The effect of dCTP on the reaction catalyzed by the cytoplasmic dThd-kinase, using ATP as a variable substrate, showed a complicated pattern. Thus, at ATP concentrations less than 0.5 mM, the degree of inhibition decreases with increasing concentrations of dCTP; while at higher concentrations of ATP, the reverse situation was observed. This was also observed with the mitochondrial dThd-kinase catalyzed reactions, but to a lesser extent (Figure 3). This may be due, in part, to the fact that dCTP can act as a phosphate donor, al-

TABLE V: Effect of Various Pyrimidine Nucleoside Analogues on [^{14}C]dThd Phosphorylation Catalyzed by dThd Kinases.[a]

Compound (0.19 mM)	% of Inhibition	
	Cytoplasm	Mitochondria
5-I-dUrd	56	31
5-Br-dUrd	61	39
5-F-dUrd	9	3
dUrd	0	0
5-Ethyl-dUrd	3	0
5-Vinyl-dUrd	7	74
α-5-Vinyl-dUrd	6	0
5-Allyl-dUrd	0	0
5-Propyl-dUrd	14	22
5-Amino-dUrd	4	12
5-Diazo-dUrd	10	1
5-IUrd	1	0
5-Vinyl-Urd	10	0
Thymidine arabinoside	2	5
dCyd	0	31
Cytosine arabinoside	2	0
5-I-dCyd	0	64
5-Br-dCyd	0	61

[a] The assays were carried out with 0.19 mM [^{14}C]dThd and 0.19 mM nucleoside analogues in the table. ATP-Mg^{2+} was kept at a saturating level. Other experimental details are described in the previous paper (Lee and Cheng, 1976).

TABLE VI: K_m or K_i (μM) Values of Various dThd Analogues with dThd Kinases.

	Cytoplasmic Enzyme	Mitchondrial Enzyme
dThd	2.6	5.2
5-I-dUrd	2.4	8.2
5-Br-dUrd	1.8	7
5-F-dUrd	26	145
dUrd	273	315
5-Ethyl-dUrd	82	305
5-Vinyl-dUrd	35	1.7
5-Allyl-dUrd	315	303
5-Propyl-dUrd	21	18

though it has only little activity in this regard when compared with ATP. dUTP was found to inhibit cytoplasmic dThd-kinase competitively with respect to (ATP-Mg^{2+})^2 (K_i = 52 μM).

Effect of Various Pyrimidine Nucleoside Analogues on dThd-Kinase Reactions. Various pyrimidine nucleoside analogues were tested as inhibitors of mitochondrial and cytoplasmic dThd-kinase isozymes and the results are shown in Table V. Both enzymes are inhibited by 5-I-dUrd and 5-Br-dUrd, with cytoplasmic dThd-kinase being more sensitive than the mitochondrial enzyme. In contrast, 5-F-dUrd and dUrd were relatively weak inhibitors of these isozymes. It is of interest that 5-vinyl-dUrd markedly inhibited the mitochondrial dThd-kinase but not cytoplasmic dThd-kinase, while 5-ethyl-dUrd, 5-vinyl-dUrd, and 5-allyl-dUrd were essentially inactive against either enzyme. 5-Propyl-dUrd was found to inhibit both enzymes only moderately. Both 5-amino-dUrd and 5-diazo-dUrd are weak inhibitors of either enzyme, the former being a better inhibitor of mitochondrial dThd-kinase and the latter more effective against cytoplasmic dThd-kinase. Analogues modified at the 2′-position such as 5-I-Urd, 5-vinyl-Urd, and thymine arabinoside showed no significant inhibitory effect. This suggests that 2′-position of dThd is important for the binding of an analogue to these enzymes.

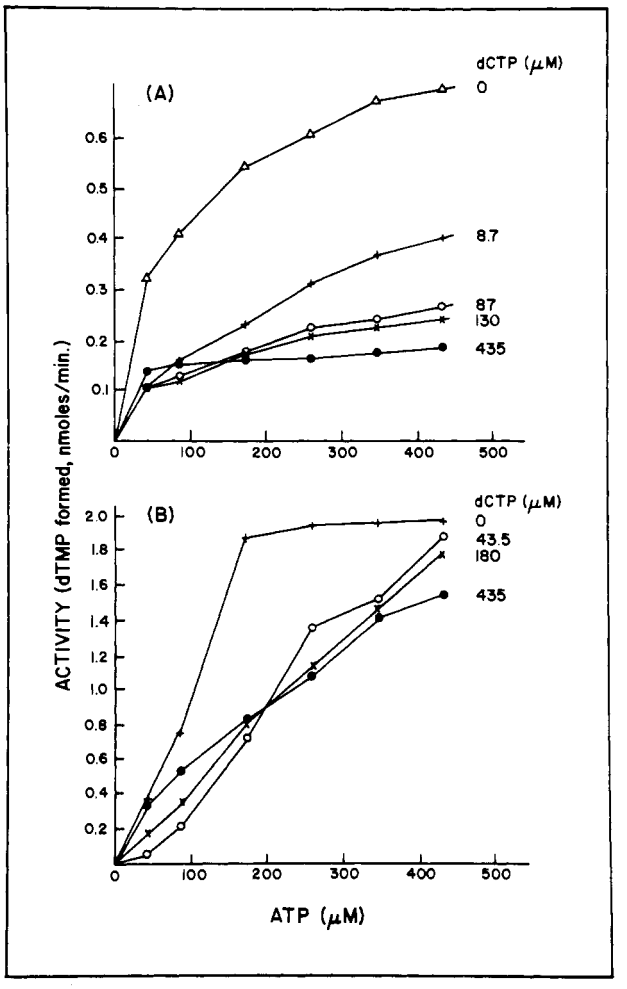


FIGURE 3: Effect of dCTP-Mg^{2+} on mitochondrial (A) and cytoplasmic (B) dThd kinase with varying amounts of dThd (81 μM).

None of the dCyd analogues tested showed any significant effect on the cytoplasmic dThd-kinase catalyzed reaction. In contrast, dCyd, 5-I-dCyd, and 5-Br-dCyd were all potent inhibitors of mitochondrial dThd-kinase. The K_i values for some of these analogues are shown in Table VI. They are all competitive in nature with dThd in both enzymatic systems.

Discussion

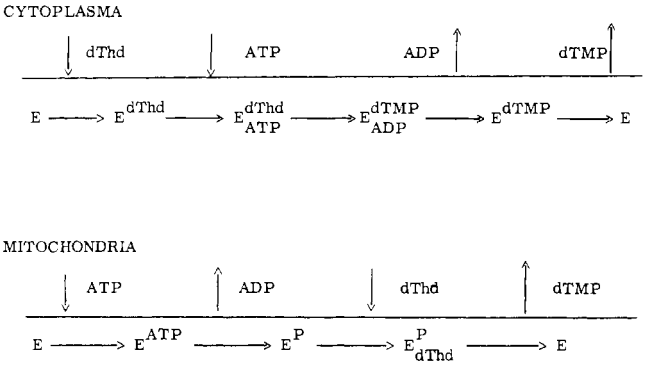
The fact that the dThd-kinase isozymes derived from blast cells of acute myelocytic leukemia cells require Mg^{2+} for optimal activity suggested that ATP-Mg^{2+} instead of ATP was the active substrate. This finding was in agreement with that for dThd-kinase derived from other sources (Cheng and Prusoff, 1974; Cheng, 1976).

It was found that Michaelis-Menten type kinetics was not always applicable to both cytoplasmic and mitochondrial dThd-kinases. In the case of cytoplasmic isozyme, when ATP-Mg^{2+} was used as variable substrate, a linear plot was obtained only by plotting $1/v$ vs. $1/(\text{ATP-Mg}^{2+})^2$. It should be noted that the "K_m" value obtained in this plot is different from the conventional K_m. This type of sigmoidal behavior of the kinetics of ATP with dThd-kinase has also been demonstrated for the enzyme derived from rat Walker carcinoma 256 tumor (Bresnick and Thompson, 1965) and mouse Sarcoma 180 (Cheng and Prusoff, 1974). The molecular explanation

for the sigmoidal character of ATP on cytoplasmic dThd-kinase could be due to a cooperative effect or a mechanistic effect, or both.

Initial velocity and product inhibition studies suggested that the major mechanism for the reaction catalyzed by cytoplasmic dThd-kinase was sequential, whereas for the mitochondrial enzyme, the "ping-pong" mechanism seems to be operative (Scheme I). This does not rule out the possibility that dThd

Scheme I



could still be able to bind to the mitochondrial dThd-kinase before the enzyme is phosphorylated by ATP-Mg^{2+}. Indeed, the mitochondrial dThd-kinase can be purified by affinity gel chromatography with dThd linked to the matrix. Further studies to gain support for the proposed mechanism of the reaction catalyzed by the mitochondrial enzyme are in progress.

The specificity of various nucleoside triphosphates as phosphate donors for the cytoplasmic and mitochondrial dThd-kinases is different. The adenine moiety of NTP-Mg^{2+} seems to play a more important role for the cytoplasmic dThd-kinase, while the ribose moiety of NTP-Mg^{2+} seems to be more important for the mitochondrial dThd-kinase. The difference in the effectiveness of various NTP-Mg^{2+} analogues as phosphate donors could be attributed to their different K_m, V_{max}, or the number of binding sites of the enzyme for the substrate. The binding sites on the enzyme can be reactive sites, catalytic sites or cooperative sites, which may or may not induce changes in the conformation of the enzyme when it was occupied. For example, ATP-Mg^{2+} and dATP-Mg^{2+} have the same K_m, V_{max}, and product sigmoid kinetics of the reaction catalyzed by cytoplasmic dThd-kinase; when the reaction medium contained both ATP-Mg^{2+} and dATP-Mg^{2+}, the activity was reduced to 80% of the control value. This could be explained by a change in the cooperative interaction, when the enzyme binds both ATP and dATP.

dTTP and dCTP have been found to act as feedback inhibitors of enzymes involved in DNA synthesis (Breitman, 1963; Bukovsky and Roth, 1964; Ives et al., 1963; Maley and Maley, 1962). Our data supported that dTTP can exert feedback inhibition on human deoxythymine kinases. Moreover, we have shown that dCTP could also exert feedback inhibition on mitochondrial dThd kinase since mitochondrial enzyme is more sensitive to dCTP inhibition than the cytoplasmic isozymes.

Of all the pyrimidine nucleoside analogues tested, only 5-I- and 5-Br-dUrd showed comparable K_i's with respect to K_m of the human cytoplasmic dThd-kinase; in contrast, human mitochondrial dThd-kinase has high affinity toward 5-I-dUrd, 5-Br-dUrd, 5-vinyl-dUrd, dCyd, 5-I-dCyd, and 5-Br-dCyd. It is of interest that 5-ethyl-, 5-allyl-, and 5-propyl-dUrd have K_i values, which are at least ten times higher than the K_m of dThd for either human isozyme, of less than 1 μM for HSV-I and HSV-II dThd-kinases (Cheng, 1976). The steric tolerance of human dThd-kinase at the 5-position of dThd seems to be less than that for the viral dThd-kinase.

It has been suggested that mitochondrial dThd-kinase could also act as dCyd-kinase (Leung et al., 1975). The substrate specificity shown by the nucleoside studies as well as triphosphate nucleoside studies as reported herein, together with our findings on the inhibition of the human dThd-kinases by dTTP and dCTP, are consistent with this concept. The properties and purification of dCyd-kinase from human myelocytic leukemia blast cells will be presented in a subsequent communication.

The differences in the nucleoside specificity of cytoplasmic and mitochondrial dThd-kinase may help in the design of analogues which inhibit selectively mitochondrial, but not nuclear DNA synthesis, or the converse selectivity, in human cells. Also, compounds with this type of selective action may provide a means for studying the role of mitochondrial DNA synthesis in the growth of human cells.

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