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DEOXYTHYMIDINE KINASE INDUCED IN HELA TK⁻ CELLS BY HERPES SIMPLEX VIRUS TYPE I AND TYPE II

SUBSTRATE SPECIFICITY AND KINETIC BEHAVIOR

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Summary

Deoxythymidine kinases (EC 2.7.1.—) induced in HeLa TK⁻ cells by Herpes simplex Type I and Type II viruses both had a requirement for divalent cations. The enzymes had the highest activities in the presence of Mg^{2+}, followed by Mn^{2+}, Ca^{2+}, Fe^{2+}, and in that order, whereas they were inactive in the presence of Zn^{2+} and Cu^{2+}. The amount of Mg^{2+} required for optimal activity was dependent on the amount of ATP present, so that optimal activities were found when the concentration of Mg^{2+} was equal to that of ATP; an excess of Mg^{2+} inhibited the reaction.

The activities of various nucleoside triphosphates as phosphate donors for Herpes simplex virus Type I deoxythymidine kinase were in the order: ATP = dATP = ara ATP > CTP > dCTP > UTP > dUTP > GTP > dGTP. Those for Herpes simplex virus Type II deoxythymidine kinase were in the order: CTP > dCTP = ara CTP > dATP > ATP > UTP > GTP > dUTP = dGTP. For both deoxythymidine kinases induced by Herpes simplex virus, the nucleoside triphosphates tested exerted cooperative effects. The K_m values of ATP and CTP for the Herpes simplex virus Type I enzyme were 30 and 70 μM respectively; whereas those for the Herpes simplex virus Type II enzyme were 140 and 450 μM.

Studies on binding of various thymidine analogs with free 5′-OH to these deoxythymidine kinases indicated that 5-substituted ethyl-, vinyl-, allyl-, propyl-, iodo- and bromo-dUrd as well ad iodo[5] dCyd and bromo[5] dCyd had good affinity to both enzymes. In contrast, vinyl[5] Urd, iodo[5] Urd and arabinosylthymidine had good affinity only to the Herpes simplex virus Type I enzyme but not to the Herpes simplex virus Type II deoxythymidine kinase. All of

Abbreviation used: NTP, triphosphate nucleoside.

these thymidine analogs were competitive inhibitors, with K_I values in the range of 0.25 to 1.5 μM. Herpes simplex virus Type I deoxythymidine kinase was less sensitive to either dTTP or iodo dUTP inhibition than Herpes simplex virus Type II.

Both dThd and dCyd could serve as substrates and competed with each other for Herpes simplex viruses Type I and Type II induced kinases, but they differed in their K_m values for these enzymes. The K_m values of dThd and dCyd were 0.59 μM and 25 μM for Herpes simplex virus Type I deoxythymidine kinase; while they were 0.36 μM and 88 μM respectively for ther Herpes simplex virus Type II enzyme.

Introduction

Herpes simplex virus Type II (HSV-I and HSV-II), each has been shown to induce its specific deoxythymidine (dThd) kinase (EC 2.7.1.—) in the infected host cell [1—3]. These newly synthesized dThd kinases are specific for the virus type and have properties distinctly different from those of the host cell dThd kinases. They are different with respect to immunogenicity [4,5], heat stability [6], and substrate as well as regulator specificities [7,8]. Unlike the host enzymes, the virus specific dThd kinases were found to phosphorylate deoxycytidine (dCyd) as well [4,9—11]. However, all of the studies mentioned above have been performed using either crude or partially purified enzyme preparations. Detailed kinetic and substrate specificity studies have not been performed.

By using differential affinity column chromatography, this laboratory has recently developed a procedure for purifying and separating dThd kinases from blast cells of patients with acute myelocytic leukemia [12] by using similar affinity gel preparation developed by Kowal and Marcus [13]. The dThd kinase from the cytosol fraction of HeLa TK^- cells infected with either HSV-I or HSV-II was also purified by the same method [11]. At present, the absolute purity of the various dThd kinase preparations is uncertain due to their low protein content, and to the difficulties encountered in attempts to concentrate them. However, dThd phosphorylases, dTMP phosphatase, dTMP kinase and NDP kinase were found to be absent in these dThd kinase preparations. This allowed us to study the substrate specificities and the other kinetic properties of both isozymes. It is of hope that through these studies, an analog of dThd which could act as an alternative substrate of viral but not human dThd kinases could be designed. Such an analog may be useful in anti-herpes virus chemotherapy.

Some general properties of the purified dThd kinases were reported in a previous paper from this laboratory [11]. The present report deals with further properties of these enzymes such as substrate specificities, requirement for divalent cations, regulator specificities and some of their kinetic behaviors.

Materials and Methods

Cells and viruses. The stock viruses of HSV-I (KOS strain) and HSV-II (333 strain) were obtained from Dr. W. Munyon. They were passaged at low multi-

plicity and assayed in a CV-1 monolayer culture. HeLa TK^- cells (BU 25) were also provided by Dr. W. Munyon and grown in 150 cm^2 Falcon flasks under conditions described previously [11].

Preparation of HSV-I- and HSV-II-specific dThd kinases. Both dThd kinases were extracted from the cytosol fraction of the infected cells, and then purified by the procedure previously reported [11].

Thymidine kinase assay. The assay procedure for either dThd kinase or dCyd kinase was the same as that published previously [11,13].

Material. The nucleoside triphosphates were purchased from either Sigma Chemical Co. or P-L Biochemicals, Inc. Ethyl^5 dUrd, vinyl^5 dUrd, allyl^5 dUrd, propyl^5 dUrd, α-vinyl^5 dUrd and vinyl^5 Urd were kindly provided by Drs. R.A. Sharma and M. Bobek of this department. Iodo^5 dUrd, Iodo^5 Urd and arabinosylthymine were obtained from Dr. W.H. Prusoff. The other nucleosides used were purchased from Sigma Chemical Co.

Results

Requirement for divalent cations

Neither HSV-I nor HSV-II specific dThd kinase activity was demonstrable in the absence of divalent cations (Table I). The catalytic activity of the divalent cations tested was in the order of $Mg^{2+} > Mn^{2+} > Ca^{2+} > Fe^{2+}$ for both enzymes while Zn^{2+} and Cu^{2+} were inactive. Fig. 1 shows how the optimal concentration of Mg^{2+} depends on the concentration of ATP. When the concentrations of ATP and Mg^{2+} were equal, optimal condition resulted. An excess of Mg^{2+} over that of ATP caused a slight inhibition. When the ATP was in excess, the reaction proceeded faster at lower that at higher levels of ATP. This seems to suggest that the excess free ATP may bind to the enzyme and act as a dead end inhibitor, whereas the ATP chelated with Mg^{2+} is likely to be the actual substrate.

Specificity for phosphate donor

The activity of the nucleoside triphosphates as phosphate donors for both dThd kinases is shown in Table II. At 3 mM of NTP-Mg^{2+}, the activities of nu-

TABLE I

REQUIREMENT FOR A DIVALENT CATION BY dThd KINASE

The assay was performed under the conditions described previously [11] with the exception that the amount of the divalent cation (2.8 mM) was varied. All the cations were added as sulfate salts. The values are the mean of 2 separate assays done in duplicate. 0.02 unit of the purified enzyme was used per assay.

	% Activity	
Cation	HSV-I	HSV-II
None	0	0
Mg^{2+}	100	100
Ca^{2+}	43	27
Mn^{2+}	63	67
Fe^{2+}	22	8
Zn^{2+}	0	0
Cu^{2+}	0	0

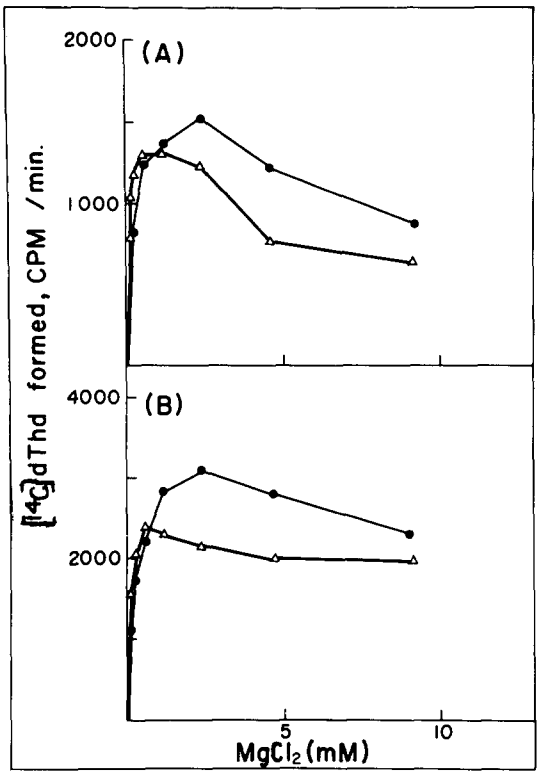

Fig. 1. Effect of Mg^{2+} and ATP on the activity of dThd kinases specific for HSV-I (A) and HSV-II (B). The assay conditions were as described previously [11]. ●———●, 2.5 mM ATP; △———△, 0.5 mM ATP. The concentration of dThd used was 100 μM.

cleoside triphosphates were in the order of ATP = dATP = ara ATP > CTP > dGTP > UTP > dUTP > GTP > dGTP > for the HSV-I-specific dThd kinase; and they were in the order of CTP > dCTP = ara CTP > dATP > ATP > ATP > UTP > GTP > dUTP = dGTP for the HSV-II enzyme. None of the dThd kinases could use dTTP or IdUTP as their substrate. At lower concentration (2 mM) of NTP-Mg^{2+}, the activities of NTP as phosphate donors were in the same order and similar magnitude as those at 4 mM.

Since the difference in NTP activities observed above could have resulted from the difference in the kinetic parameters of each enzyme, detailed kinetic studies were performed by using CTP · Mg^{2+} or ATP · Mg^{2+} as the substrate. It was found that neither CTP · Mg^{2+} nor ATP · Mg^{2+} could act as a Michaelis-Menten kinetics type substrate. However when using either one as the variable substrate at saturation concentration of dThd, linear lines were obtained by employing double reciprocal plots of velocity vs. square of the concentration of the substrate for the HSV-I and HSV-II enzymes as illustrated in Fig. 2A, and 2B, respectively. So both CTP · Mg^{2+} and ATP · Mg^{2+} acted as sigmoidal type substrates with a Hill constant of 2. For the HSV-I specific dThd kinase, K_m was 30 μM for ATP · Mg^{2+} and 70 μM for CTP · Mg^{2+}, and V for ATP · Mg^{2+} was twice as high as that for CTP · Mg^{2+}. For the HSV-II enzyme, K_m was 140 μM for ATP · Mg^{2+} and 450 μM for CTP · Mg^{2+}, and the V for ATP · Mg^{2+} was 0.4 of that for CTP · Mg^{2+}.

TABLE II

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

The assays were performed under conditions described previously [11], except that NTP (4 mM) was varied. The results are expressed as % of dTMP formed when ATP was used as phosphate donor. 0.02 unit of the purified enzyme was used per assay.

	dTMP formed (% of control)	
NTP · Mg^{2+}	HSV-I	HSV-II
ATP	100	100
GTP	31	40
CTP	65	250
UTP	50	87
dATP	100	130
dGTP	20	16
dCTP	61	203
dUTP	33	19
dTTP	0	0
IdUTP	0	2
Ara-CTP	36	210
Ara-ATP	100	—

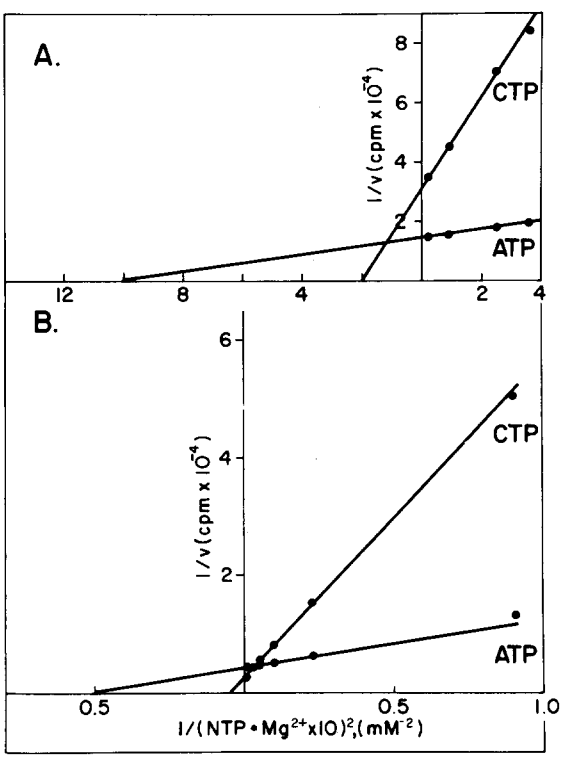

Fig. 2. Double-reciprocal plot of the reaction velocity vs. the square of the concentration of ATP · Mg^{2+} or CTP · Mg^{2+} for dThd kinase specific for HSV-I (A) and HSV-II (B). The assay mixture was the same as described previously except that the concentrations of ATP-Mg^{2+} or CTP-Mg^{2+} were varied. The concentration of dThd used was 100 μM in all assays.

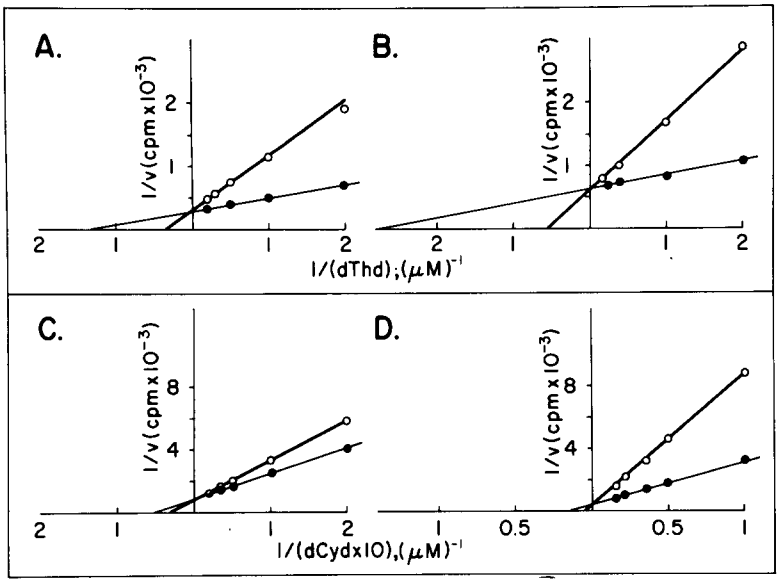

Fig. 3. Double reciprocal plots of the reaction velocity vs. the concentration of dThd (A and B) or dCyd (C and D) for dThd kinase specific for HSV-I (A and C) and HSV-II (B and D). The assay conditions were the same as described previously [11]. In A and B, the reactions were performed in the absence (●——●) or presence (○——○) of 1 mM dCyd and in C and D, in the absence (●——●) or presence (○——○) of 2 μM dThd. The concentration of ATP · Mg^{2+} used was 2 mM.

Specificity of nucleosides as phosphate receptors

The Lineweaver-Burk plots for both HSV-I and HSV-II specific dThd kinases are shown in Fig. 3 using dThd (Fig. 3A and 3B) or dCyd (3C and 3D) as the variable substrate and fixed saturable concentration (2 mM) of ATP · Mg^{2+}. K_m value of dThd was 0.59 μM for the HSV-I enzyme and 0.35 μM for the HSV-II enzyme, whereas K_m of dCyd was 25 μM for the HSV-I enzyme and 88 μM for the HSV-II enzyme. When dThd was the variable substrate, the addition of 1 mM dCyd inhibited dThd phosphorylation by both enzymes in a competitive manner. Similarly, 2 μM dThd was a competitive inhibitor when dCyd was used as the variable substrate.

When initial velocity studies were performed by using either dThd or dCyd as the variable substrate at fixed nonsaturable concentration of ATP · Mg^{2+} for both isozymes, a family of lines was yielded and intersected on double reciprocal plots. This suggested that both isozymes catalyzed the reaction following a sequential mechanism.

Pyrimifine nucleoside studies

Various pyrimidine nucleosides with free 5′-OH were tested for their binding affinity to the virus specific dThd kinases. The analogs were added to the reaction mixture using either [^{14}C]dThd or [^{14}c]dCyd as the substrate. The analogs were applied in a certain molar proportion with respect to the radioactive substrate, i.e., ([^{14}C]dThd)/(analog) = 0.47 for [^{14}C]dThd phosphorylation and ([^{14}C]dCyd)/(analog) = 6.4 for [^{14}C]dCyd phosphorylation. When an analog competes with [^{14}C]dThd or [^{14}C]dCyd for the phosphorylation, inhibition of the labeled nucleoside phosphorylation could be detected. In our conditions,

TABLE III

EFFECT OF 5'-OH PYRIMIDINE NUCLEOSIDES ON THE PHOSPHORYLATION OF dThd AND dCyd BY THE PARTIALLY PURIFIED KINASES

Each assay was performed with 0.04 unit of each purified enzyme.

5'-OH Analog	% Inhibition			
	HSV-I		HSV-II	
	dThd * Kinase	dCyd ** Kinase	dThd * Kinase	dCyd ** Kinase
dThd	66	91	71	92
Iodo^{5}dUrd	69	89	78	94
Br^{5}dUrd	67	84	73	92
F^{5}dUrd	51	74	5	65
dUrd	38	58	0	36
Vinyl^{5}dUrd	82	90	72	92
α-Vinyl^{5}dUrd	2	30	0	30
Ethyl^{5}dUrd	65	83	80	94
Allyl^{5}dUrd	69	84	34	82
Propyl^{5}dUrd	65	78	49	89
Iodo^{5}Urd	43	64	0	52
Vinyl^{5}Urd	51	55	1	42
Arabinosylthymidine	35	70	0	54
Arabinosylcytodine	9	23	9	4
dCyd	12	11	10	8
Iodo^{5}dCyd	66	78	35	80
Br dCyd	42	72	35	83

more than 10% inhibition resulted when $(K_m(\text{dThd}))/(K_I(\text{analog}))$ was greater than 0.05, or when $(K_m(\text{dCyd}))/(K_I(\text{analog}))$ was greater than 0.9 [14]. Table III shows how all the nucleoside analogs tested had different degrees of binding affinity to the virus specific dThd kinases. Since control experiments were performed by adding cold dThd or dCyd to the dThd kinase or dCyd kinase reaction, a dilution of radiospecific activity resulted and an apparent competitive inhibition was observed by the cold nucleoside for phosphorylation. When the enzymes were preincubated with the analogs, none of them cause inactivation

TABLE IV

INHIBITION CONSTANTS OF dThd ANALOGS FOR VIRUS SPECIFIC dThd KINASES

The K_I values were obtained by replotting the slopes of Lineweaver-Burk plots obtained in the presence of the varied concentrations of the analogs versus the concentration of the analogs [13].

Analog	K_I (μM)	
	HSV-I	HSV-II
dUrd	5	20
F^{5}dUrd	2	4
Br^{5}dUrd	0.6	0.5
Iodo^{5}dUrd	0.6	0.3
Vinyl^{5}dUrd	0.5	0.5
Ethyl dUrd	0.7	0.3
Allyl^{5}dUrd	0.7	0.9
Propyl^{5}dUrd	0.6	0.7

TABLE V

EFFECT OF ADDITIONAL NUCLEOTIDE TRIPHOSPHATE ON dThd OR dCyd KINASE REACTION IN THE PRESENCE OF ATP · Mg^{2+}

Additional NTP · Mg^{2+} (4 mM)	HSV-I ***		HSV-II ***	
	% dTMP formed *	% dCMP formed **	% dTMP formed *	% dCMP formed **
None	100	100	100	100
ATP	100	100	100	100
GTP	90	104	84	57
CTP	102	87	120	71
UTP	100	92	91	80
dATP	98	98	104	100
dGTP	87	98	91	39
dCTP	100	81	110	88
dUTP	93	45	45	20
dTTP	31	0	2	0
Iodo dUTP	21	0	1	0

* dThd kinase reactions were performed in the presence of 4 mM ATP · Mg^{2+} and 38 μM dThd.
** dCyd kinase reactions were performed in the presence of 4 mM ATP · Mg^{2+} and 380 μM dCyd.
*** The amount of the purified enzyme used in each assay was 0.04 unit.

of the enzymes. dUrd and its analogs substituted at 5-position with fluoro-bromo-, iodo-, vinyl-, ethyl-, allyl- and propyl- groups were examined for possible competition with dThd for these enzymes. The compound were added to the reaction mixture having dThd as the variable substrate and 2 mM ATP · Mg^{2+} as the fixed substrate, and all of these compounds were found to be compeititve inhibitors. Their K_I values listed in Table IV reveal that all of them except dUrd and FdUrd had relatively good binding affinity to the enzymes when compared with dThd.

Effect of varied NTP · Mg^{2+} on kinase reactions in the presence of ATP · Mg^{2+}

Various NTP · Mg^{2+} complexes were added at 4 mM concentration to the reaction mixtures already containing 4 mM ATP · Mg^{2+} and 28 μM dThd or 380 μM dCyd. The results as (Table V) indicated that dTTP · Mg^{2+} and iodo-dUTP · Mg^{2+}, which did not function as phosphate donors for dThd (Table II), inhibited the dThd or dCyd phosphorylation catalyzed by either enzyme, the HSV-II specific enzyme being the more sensitive one under these assay conditions. This is consistent with the observations made by others [6,7,9].

Most of the other NTP · Mg^{2+} tested showed no significant inhibition of dThd phosphorylation catalyzed by either HSV-I or HSV-II dThd kinase enzyme. Only dUTP · Mg^{2+} exerted 55% inhibition of dTMP formation catalyzed by the HSV-II enzyme. However when dCyd and ATP were used as the substrate, significant inhibition of dCMP formation was observed when the HSV-I enzyme was used with addition of dUTP · Mg^{2+}, or when the HSV-II enzyme was used with addition of either dUTP · Mg^{2+}, GTP · Mg^{2+}, or dGTP · Mg^{2+}. This seemed to suggest that although the enzymes appeared to be able to phosphorylate both dThd and dCyd, the active site for different enzymatic reaction might not be the same. The reciprocal plots of the velocity of HSV-I or HSV-II dThd kinase reaction vs. varied concentrations of dThd or dCyd at fixed amounts of

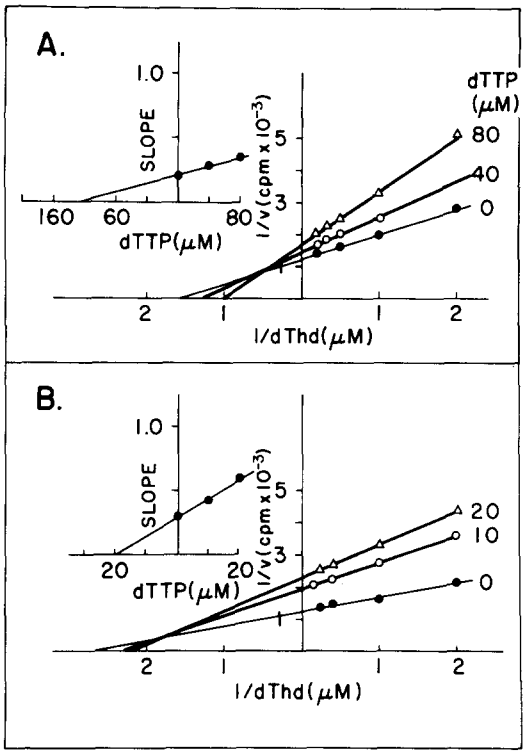

Fig. 4. Effect of dTTP on the reciprocal plot of the reaction velocity vs. dThd concentration for the kinases specific for HSV-I (A) and HSV-II (B). The assays were carried out in the presence of 2 mM ATP · Mg^{2+} and varied concentration of dThd and dTTP as indicated.

dTTP and saturating amount of ATP (2 mM) are shown in Figs. 4 and 5. They reveal that dTTP does not compete with dThd or dCyd for the reaction catalyzed by either enzyme. By replotting the slope versus dTTP concentration, the K_I values for dTTP could be determined; they were 120 μM (Fig. 4A) and 160 μM (Fig. 5A) for the HSV-I enzyme and 20 μM (Fig. 4B) and 22 μM (Fig. 5B) for HSV-II enzyme. Regardless of whether dThd or dCyd was used as the substrate, the K_I values were the same. When the studies were performed by using varing concentration of ATP · Mg^{2+}, fixed amount of dTTP and saturating amount of dThd (50 μM) together with the HSV-I or HSV-II enzyme, it was noted that ATP · Mg^{2+} could completely reverse the inhibition of enzymatic reaction exerted by dTTP.

Discussion

Both HSV-I and HSV-II specific dThd kinases require Mg^{2+} for activity. The concentration of Mg^{2+} required to give optimal activity of either enzyme depends on the concentration of ATP. Although ATP per se seems to be able to bind to the enzyme, the reaction could not proceed in the absence of a divalent cation. However, when ATP was chelated with Mg^{2+}, the resulting ATP · Mg^{2+} complex initiates the reaction with dThd. This observation is consistent with

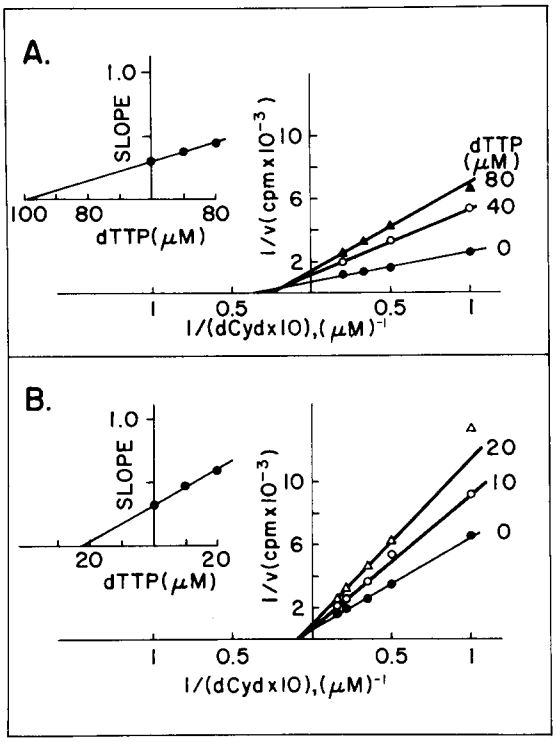

Fig. 5 Effect of dTTP on the reciprocal plot of the reaction velocity vs. dCyd concentration for the kinases specific for HSV-I (A) and HSV-II (B). The conditions were identical to those described for Fig. 4 except that dCyd was used as the variable substrate.

the finding by other investigators who have employed dThd kinase from other sources [14]. Since nucleoside triphosphate could bind to the enzyme and hence interfere with the binding of NTP · Mg^{2+} complex, it is important to keep the amount of Mg^{2+} equivalent to that of NTP.

The virus specific dThd kinases show dramatic differences with respect to the specificty for phosphate donors and acceptors. For instance, while ATP · Mg^{2+} is the best phosphate donor for HSV-I specific dThd kinase, CTP · Mg^{2+} is the best for HSV-II specific enzyme (Table II). It should be pointed out that the date presented in Table II are merely a comparison of V based on the assumption that a concentration of 4.0 mM NTP · Mg^{2+} is at least several times higher than the K_m value of the corresponding NTP · Mg^{2+}. When initial velocity studies were performed by varying either the concentration of ATP · Mg^{2+} or CTP · Mg^{2+}, at saturating levels of dThd for both dThd kinase, a cooperative type of binding of ATP · Mg^{2+} or CTP · Mg^{2+} to both enzymes with a Hill constant of 2 was observed. This seems to suggest that there are two binding sites for ATP · Mg^{2+} or CTP · Mg^{2+} on either isozymes. CTP · Mg^{2+} and ATP · Mg^{2+} are different with respect to the apparent K_m as well as apparent V values.

Either dThd or dCyd could be used as the phosphate acceptors for both dThd kinases and as expected they compete with each other for the phosphorylation. Interestingly, however, for both kinases, the K_m value for these two substrates were found to differ at least 50-fold, dThd being a much more favor-

able substrate than dCyd. Both types of dThd kinases were inhibited only by dTTP but not by dCTP. It is the author's speculation that under physiological conditions, both of these enzymes are mainly responsible for the phosphorylation of dThd and not of dCyd. They should still be called dThd kinases instead of deoxypyrimidine nucleoside kinases as other authors have suggested [9,10].

The studies on nucleoside analogs with free 5′-OH seem to suggest that: (1) the β-form of nucleoside is more favorable for binding than the α-form; (2) the variation at the 2-position of the nucleoside sugar moiety exerts a greater effect on its binding to HSV-II specific dThd kinase than to HSV-I dThd kinase; (3) the substituent at the 5-position of the nucleoside has a vital role in determining its binding affinity to both enzymes. Both enzymes have a greater steric tolerance for changes at the 5-position than dThd kinase of human mitochondria or cytoplasm [16]. Those analogs which were studied kinetically, were all competitive inhibitors of dThd phosphorylation for both kinases. Although it was not resolved whether those analogs acted as alternative substrates or dead end competitive inhibitors of the enzymes, our preliminary studies indicate that these competitive inhibitors can serve as the alternative phosphate acceptors for both enzymes [17].

dTTP · Mg^{2+}, with respect to ATP a competitive inhibitor, is a non-competitive inhibitor with respect to dThd or dCyd for either HSV-I or HSV-II specific dThd kinase. The K_I for HSV-I enzyme is higher than that for HSV-II enzyme. This is in agreement with other laboratory's observation that HSV-I specific dThd kinase is more resistant to dTTP-Mg^{2+} inhibition than HSV-II enzymes [6].

In conclusion, although HSV-I and HSV-II are two closely related viruses, and both induce a specific dThd kinase in HeLa TK^- cells after infection, the enzymes are different not only in physical properties [11] but also in substrate specificity. For both enzymes dCyd is a much poorer substrate than dThd. Both viral specific dThd kinases are also different from dThd kinases present in human cells in terms of physical or kinetic properties [16]. A comparative study of the substrate specificity of all four dThd kinases may lead us to finding a thymidine analog with free 5′-OH which could only be phosphorylated by viral specific dThd kinase but not by the correspondent human dThd kinases. This analog may exert a good antiviral activity through the selective phosphorylation by disturbing thymidine metabolism in viral infected cells without causing toxicity to human cells.

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