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KINETIC MECHANISM AND INHIBITION OF HUMAN LIVER THYMIDINE KINASE

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Thymidine kinase (ATP thymidine 5′-phosphotransferase, EC 2 7 1 21), purified to apparent homogeneity from human liver, was found to have Michaelis constants for thymidine and ATP of 5 and 90 μM, respectively Based on studies of initial velocity and product inhibition, the enzyme kinetic mechanism is compatible with an ordered sequential reaction with thymidine binding first and thymidine monophosphate released last The activity of various triphosphate nucleosides as phosphate donors for human liver thymidine kinase showed little specificity with ATP > CTP > UTP > GTP and the respective Michaelis constants ranged from 0 10 to 0 30 mM Among various purine and pyrimidine compounds, only TTP and dCTP were effective inhibitors of the enzyme Inhibition with TTP was competitive with respect to both thymidine and ATP with K_i values of 13 5 and 8 5 μM, respectively, while the inhibition produced by dCTP was complex Deoxycytidine was found to be an effective nucleoside substrate for human liver thymidine kinase with a Michaelis constant of 6 μM This finding suggests that human mitochondrial deoxycytidine and thymidine kinase activity is a single protein

Introduction

Thymidine kinase (ATP-thymidine 5′-phosphotransferase, EC 2 7 1 21) catalyzes the phosphorylation of thymidine to form thymidine 5-monophosphate [1,2] In mammalian tissue this activity occurs as distinct isoenzymes with peculiar subcellular organization and related cell cycle activity, increased cellular DNA synthesis is accompanied by increased cytosolic thymidine kinase activity, whereas that of the associated mitochondrial activity remains relatively constant [3–8] Despite these findings, the exact cellular role for thymidine kinase and, in particular, the mitochondrial enzyme, is poorly understood The kinetic studies of thymidine kinases, derived in varying degrees of purity from mammalian tissues [8–13], have all yielded divergent findings which warranted a study of the kinetic characteristics of thymidine kinase from a non-neoplastic human source We have recently purified thymidine kinase, from normal human liver [14], to apparent homogeneity, this is presumed to be the mitochondrial enzyme, and the substrate specificity, kinetic behaviour and mechanism of inhibition of this enzyme are the subject of this report

Materials and Methods

[5-^{3}H]Deoxycytidine (20 Ci/mmol) and [6-^{3}H]-thymidine (5 Ci/mmol) were obtained from The Radiochemical Center, Amersham All purine or pyrimidine bases, nucleosides and deoxynucleosides and the corresponding nucleotides or deoxynucleotides were obtained from Sigma These compounds were of the highest quality commercially available and used without further purification The concentration of all compounds used was based on the specification supplied by the manufacturer

Enzyme preparation Thymidine kinase activity used in this study was purified to apparent homogeneity from human liver [14] The enzyme preparation obtained has a specific activity of 22 units/mg

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protein, 1 unit enzyme activity is defined as the amount of thymidine kinase which converts 1 nmol dThd to thymidine monophosphate (TMP) per min The enzyme preparation does not exhibit thymidine phosphorylase or deoxycytidine deaminase activity All experiments were performed on the day of enzyme isolation, prior to the kinetic studies, the enzyme preparations were dialysed at 4°C for 4 h against 200 vol of 0 2 M Tris-HCl (pH 7 4)

Enzyme assay Thymidine kinase activity was assayed as described previously [15] The standard reaction mixture consisted of 0 1 M Tris-HCl, pH 7 4/ 2 mM ATP/4 mM $MgCl_2$/55 μM [6-^{3}H]thymidine (5 Ci/mmol)/bovine serum albumin 1 mg/ml, in a final volume of 0 1 ml After 5–10 min incubation at 37°C the reaction was terminated by placing in a boiling water bath for 1 min Aliquots (50 μl) were spotted on DE-81 paper squares (1 5 × 1 5 cm), which were processed and assayed for radioactivity in a liquid scintillation counter as described previously [15] 1–3 μg enzyme protein were used in each assay and under the described conditions the assay was linear with respect to time and protein concentration Lineweaver-Burk plots were drawn after using amounts of enzyme sufficient to convert 1–10% substrate to product under the standard assay conditions Secondary plots were used to calculate K_m and K_i values The points plotted in each figure are the actual data points, whereas the lines are those derived by weighted linear regression analysis

Protein concentrations were determined by the method of Lowry et al [16] using bovine serum albumin as standard

Results

Divalent cation requirements Human liver thymidine kinase has an absolute requirement for a divalent cation for catalytic activity Maximal activity was obtained with Mg^{2+}, and Mn^{2+} and Co^{2+} supported lesser degrees of activity With fixed ATP concentrations of 1 and 5 mM, $MgCl_2$ less than 2 mM produced an inhibitory effect on activity, comparable degrees of inhibition also occurred with concentrations of 1 mM ATP and 10 mM $MgCl_2$ Maximal enzyme activity was observed with equimolar excess of $MgCl_2$ relative to ATP concentration

Initial velocity and product inhibition Double-reciprocal plots of initial velocity data obtained with variable concentrations of dThd from 5–30 μM with fixed concentrations of 0 05–1 mM ATP, yielded a series of lines that intersected to the left of the vertical axis (Fig 1) Secondary plots of the slope and intercepts were linear and yielded a K_m of 90 μM for ATP Double-reciprocal plots of initial velocity findings, obtained by varying concentrations of ATP against fixed concentrations of dThd, also yielded a series of converging lines (data not given) Secondary plots of the slope and intercepts were linear and yielded a K_m for dThd of 5 μM

Inhibition of human liver thymidine kinase by fixed concentrations of the product of the reaction ADP, with concentrations of the substrate dThd varying from 5 to 30 μM and a final saturating concentration of ATP of 2 mM, produced changes in both slopes and intercepts of double-reciprocal plots, consistent with non-competitive inhibition (data not

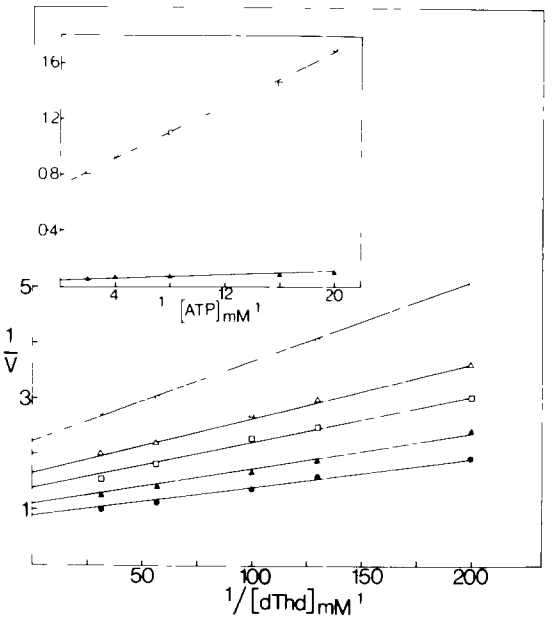


Fig 1 Double-reciprocal plots of initial velocity studies with variable dThd concentrations ranging from 5 to 30 μM and fixed concentrations of ATP ranging from 0 05 to 1 0 mM ○———○, 0 05 mM, △———△, 0 10 mM, □———□, 0 125 mM, ▲———▲, 0 25 mM and ●———●, 1 0 mM The assays contained 50 mM Tris-HCl buffer pH 7 4 and 0 033 units enzyme The inset shows a secondary plot slope (▲———▲) and intercepts (□———□) versus the inverse ATP concentration The K_m for ATP is 90 μM and 5 μM for dThd

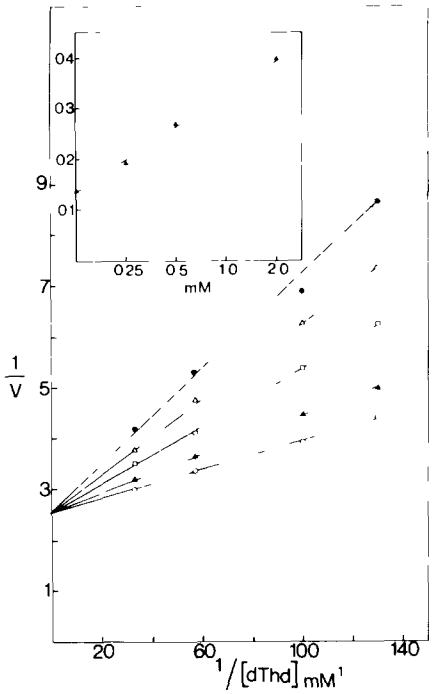


Fig 2 Double-reciprocal plots of product inhibition studies with TMP The variable substrate dThd concentrations ranged from 7 5 to 30 μM and fixed concentrations of TMP from 0 to 2 mM ○———○, 0 mM, ▲———▲, 0 25 mM, □———□, 0 50 mM, △———△, 1 0 mM and ●———●, 2 0 mM Each assay contained 50 mM Tris-HCl buffer, pH 7 4/2 mM ATP/ 4 mM $MgCl_2$/0 033 units enzyme The inset shows a secondary plot of slopes versus the TMP concentrations in mM yielding of K_i of 0 63 mM

given) The K_i for ADP was 6 5 mM With ATP as the variable substrate and dThd at a fixed concentration of 55 μM, inhibition produced by ADP was apparently uncompetitive When inhibition of human liver thymidine kinase by the end product of the reaction TMP was studied with varying concentrations of the substrate dThd and a fixed concentration of ATP, double-reciprocal plots revealed changes in slopes, but not intercepts consistent with competitive inhibition (Fig 2) The K_i for TMP was 0 63 mM With varying concentrations of the substrate ATP and fixed concentrations of thymidine, TMP was found to be non-inhibitory

Specificity of phosphate donors for thymidine kinase Various deoxynucleoside triphosphates were examined for their efficiency as phosphate donors for human liver thymidine kinase Maximal activity was

TABLE I

SUMMARY OF K_m AND V VALUES FOR DIFFERENT PHOSPHATE DONORS OF HUMAN LIVER THYMIDINE KINASE

V values are expressed as percent activities with respect to ATP

Donor	K_m (mM)	Relative V
ATP	0 10	100
CTP	0 15	62
UTP	0 26	48
GTP	0 30	45

observed with ATP while CTP, UTP and XTP were also active donors * The kinetics of ATP, CTP, UTP and GTP as phosphate donors for human liver thymidine kinase were studied in further detail All these nucleoside triphosphates conformed to Lineweaver-Burk kinetics and the appropriate K_m and relative V are shown in Table I

Nucleoside specificity of nucleosides for thymidine kinase The nucleoside substrate specificity for human liver thymidine kinase was examined by determining the effect of various nucleosides on enzyme activity with thymidine as the substrate and these data are presented in Table II Among the compounds tested only deoxycytidine, deoxyuridine, 5-fluorodeoxyuridine and 5-iodocytidine produced significant degrees of inhibition of enzyme activity The K_m for deoxycytidine was determined to be 6 μM

Effect of purine and pyrimidine nucleotides on thymidine kinase activity A number of purine and pyrimidine nucleotides were examined for their effect on human liver thymidine kinase Of the pyrimidine compounds dCTP and TTP were found to be effective inhibitors, while among the purine compounds tested, dGTP exhibited a significant inhibitory effect * The mechanism of inhibition of human liver thymidine kinase by TTP and dCTP was further examined TTP was found to be a competitive inhibitor with respect to either of the substrates, ATP and thymidine, with K_i values of 8 5 and 13 5 μM, respectively With thymidine or ATP as the variable substrate, dCTP exhibited a complex pattern of inhibition. With concentrations of dCTP ranging from

* Results available on request

TABLE II

THE EFFECT OF VARIOUS NUCLEOSIDES ON THE ACTIVITY OF HUMAN LIVER THYMIDINE KINASE

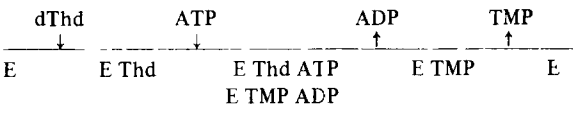
Assays were performed in duplicate with 55 μM [^{3}H]thymidine, 2 mM ATP, 2 mM $MgCl_2$ and 55 μM of the indicated compound Results are expressed as percent TMP formed in the absence of additional pyrimidine or purine nucleoside Each assay contained 0 033 units purified enzyme

Additional nucleosides	Percent activity
None	100
Adenosine	95
Guanosine	90
Cytidine	90
Uridine	80
Deoxyadenosine	89
Deoxyguanosine	83
Deoxycytidine	61
Deoxyuridine	70
Chloroadenosine	76
5-Fluoro-deoxyuridine	71
5-Iodocytidine	53
6-Mercaptopurine riboside	72
6-Azauridine	74

0 to 25 μM, dCTP exhibited progressive degrees of inhibition which was reversed at higher concentrations of 0 05 to 1 mM dCTP This anomalous inhibition occurred independent of the concentration of substrates dThd or ATP

Discussion

The kinetic mechanism of mitochondrial thymidine kinase purified to apparent homogeneity from human liver has been studied Reciprocal plots of initial velocity data are intersecting and suggest a sequential reaction mechanism [17] Product inhibition studies have shown that ADP is non-competitive with respect to dThd, but with the substrate ATP produced a series of lines that were nearly parallel rather than intersecting This property reflects the irreversibility of the reaction in which nearly parallel intersecting lines would be expected TMP was found to inhibit in a manner which is competitive with respect to thymidine but not ATP These observations are most compatible with an ordered reaction mechanism in which thymidine binds first and TMP is released last [17] The reaction mechanism suggested by our data is



Thymidine kinase from calf thymus [9] and the cytosol isoenzyme of human leukemic blast cells [13] exhibit similar reaction mechanisms but, paradoxially, that for the mitochondrial isoenzyme of human leukemia blast cells appears to be a 'ping-pong' reaction mechanism [13] The basis for this difference between the human mitochondrial enzymes is not apparent

With respect to phosphate donors, the enzyme from human liver is not highly specific in that CTP, XTP and UTP could efficiently substitute for ATP, with all nucleoside triphosphate tested conforming to Lineweaver-Burk kinetics (Table I) A similar nucleoside triphosphate donor specificity has been observed for the mitochondrial enzyme of human leukemic blast cells [13], however, thymidine kinases from other mammalian sources including calf thymus [9], proliferating human skin fibroblasts [6], the cytosolic enzyme of human leukemic blast cells [13] and human fetal liver [18] are more restrictive in phosphate donor specificity, with ATP the optimal donor The Michaelis constants for thymidine of the enzymes from various mammalian sources range from 2–57 μM The value of this constant for the human liver enzyme (5 μM) agrees favourably with that found with either the cytosol (2 6 μM) or mitochondrial (5 2 μM) isoenzymes of human leukemic blast cells but is considerably lower than that found with the calf thymus enzyme (57 μM) Leung and his colleagues [19] have suggested that the mitochondrial thymidine kinase can also act as a deoxycytidine kinase The nucleoside substrate specificity of the homogeneous human liver enzyme reported here agrees with this suggestion, as deoxycytidine is an effective phosphate acceptor with a Michaelis constant (6 μM), which corresponds favourably to that of mitochondrial deoxycytidine kinase of human leukemic cells [20]

dTTP and dCTP have been found to act as feedback effectors of some enzymes involved in DNA synthesis [21], and of the various purine and pyrimidine compounds examined, these two deoxynucleoside tri-

phosphates exert a significant inhibitory effect on human liver thymidine kinase The mechanism of inhibition by dTTP was found to be competitive with respect to both substrates thymidine and ATP and similar mechanisms occur with the calf thymus enzyme [9] In keeping with properties of mitochondrial enzyme of human leukemic blast cells, the inhibition produced by dCTP is more complex which in part may reflect the fact that dCTP can act as a phosphate donor

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