Diverging Substrate Specificity of Pure Human Thymidine Kinases 1 and 2 Against Antiviral Dideoxynucleosides*

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The two thymidine (dThd) kinases in human cells, the cytosolic, S-phase-specific TK1 and the mitochondrial, constitutively expressed TK2 were purified to homogeneity as judged from sodium dodecyl sulfate-gel electrophoresis. The substrate specificity of TK1 and TK2 toward natural substrates and important nucleoside analogues was compared. With TK1, the $K_m$ values for 5-fluorodeoxyuridine (FdUrd), 3'-azido-2',3'-dideoxythymidine (AZT), and 3'-fluoro-2',3'-dideoxythymidine (FLT) were 2.2, 0.6, and 2.1 $\mu$M as compared to 0.5 $\mu$M for dThd and 9 $\mu$M for deoxyuridine (dUrd). With TK2, dUrd, deoxythymidine (dCd), and 5'-fluorodeoxyuridine (FdUrd) were efficiently phosphorylated, but with distinctly different kinetics: Michaelis-Menten kinetics with dCyd, dUrd, and FdUrd; negative cooperativity with dThd. Negative cooperativity was also observed with AZT, although this drug was a very poor substrate for TK2 with a $V_{max}$ of 5-6% of that with dThd. FLT, 2',3'-dideoxyadenosine (ddCd), and arabinofuranosylcytosine (araC) were not substrates for TK2, and 2',3'-didehydrodideoxythymidine (D4T) was not a substrate for TK1 or TK2. On the other hand, AZT, FLT, and D4T were competitive inhibitors with $K_i$ values of 0.6, 6, and 2073 $\mu$M for TK1, and 2, 10, and 78 $\mu$M for TK2, respectively. The much lower concentration of 2',3'-didehydro-2',3'-dideoxythymidine (D4T) as compared to TK1 is important for the design of new antiviral nucleoside analogues intended for use in cells with different expression of TK1 and TK2.

Thymidine (dThd)\(^1\) kinases (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.21) catalyze the phosphorylation of dThd to dTMP, but the exact role of the enzyme activity in cell division and DNA synthesis is far from clear. In eucaryotic cells, two different dThd kinases have been found, TK1 and TK2 (1). High levels of TK1 activity are found in proliferating and malignant cells, fluctuating with DNA synthesis during the cell cycle (2-9). In contrast, the considerably lower and fairly constant level of dThd kinase activity in resting cells is due to the presence of TK2 which has been shown to be predominantly localized in mitochondria (3, 9-14). However, both cellular fractionation experiments (9, 13) and the well known fact that $[\text{3H}]$dThd is incorporated into nonproliferating cells with radiation or chemical damaged DNA (15) have shown that TK2 is present in the cytosol and mitochondria in resting and proliferating cells. In human cells, the gene for TK1 is located on chromosome 17 (16) and the gene for TK2 on chromosome 16 (17).

Information about the properties of various forms of the dThd kinases is extensive, and diverging observations have been reported as to the molecular weight and electrophoretic behavior (3, 6, 8, 9) and kinetic properties (6, 11, 12, 18) of these two enzymes at different stages of purification. However, human TK1 has recently been purified to homogeneity from HeLa cells, and the enzyme was a tetramer of 24-kDa polypeptides (19), the gene has been cloned, and its cell cycle regulation was studied in detail (20-22).

TK2 has been purified 1634-fold from acute myelocytic leukemia blood cells (9) and 5625-fold from human liver (10), but, due to high instability and low cellular amounts of TK2, information about its enzymatic and physical properties is very limited.

Here, we report preparation of quantitative amounts of pure and stable TK2 from human leukemic spleen. This enzyme showed a 10-fold higher specific activity than any previously isolated TK2 preparation, and the enzyme was a monomer of a 29-kDa polypeptide. In parallel, human TK1 was also completely purified, and this enzyme showed properties similar to that isolated from HeLa cells (19).

Phosphorylation of the dThd analogues, 3'-azido-2',3'-dideoxythymidine (AZT), 3'-fluoro-2',3'-dideoxythymidine (FLT), and 3'-didehydro-2',3'-dideoxythymidine (D4T), is essential for their effects as inhibitors of human immunodeficiency virus (HIV) replication (23-26). In triphosphate form, these analogues exhibit an antiviral effect by acting partly as DNA chain terminators (27) and partly by competing with the normal deoxyribonucleoside triphosphates for the HIV reverse transcriptase (24). TK1 from human cells has been reported to phosphorylate AZT and FLT (24, 25) almost as efficiently as dThd. D4T is anabolized to the monophosphate in human lymphoblastoid cell lines, but to a 300-600-fold lower extent (28, 29) than AZT.

In the present paper, we have been able to compare the properties of pure preparations of TK1 and TK2 to the...
phosphorylation of important pyrimidine analogues such as AZT, FLT, and D4T. We present evidence that TK1 and TK2 do not phosphorylate D4T and that TK2 has very poor capacity for AZT phosphorylation and does not phosphorylate FLT.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Purification of TK1 and TK2**—In the DEAE-chromatography, two peaks of dThd kinase activity, TK1 and TK2, were completely separated (Fig. 1). Also, there were two peaks of dCyd kinase activity, the major peak eluting with higher salt (fractions 42–58) represented the previously purified 30-kDa dCyd kinase (32). The fractions containing both dThd kinase and dCyd kinase activity (fractions 31–42) were purified during the subsequent steps. The purification procedure is summarized in Tables I and II. Compared to the specific activity of dThd kinase in the desalted ammonium sulfate fraction, the activity was purified at least 27,000-fold for TK1 and 21,000-fold for TK2. In 12% SDS-acrylamide gel electrophoresis, a band at 22–24 kDa correlated with TK1 activity (Fig. 2C), and a band at 29 kDa correlated with TK2 activity (Fig. 2B).

The presence of CHAPS during the last purification steps and storage at −70 °C stabilized both enzymes, whereas more than 80% of the activity was lost in a week at −70 °C in the absence of the detergent. dThd could not replace CHAPS as stabilizing agent.

There was no measurable dTMP kinase activity in the pure preparations of TK1 and TK2, as judged by analysis of the reaction products by PEI-cellulose chromatography. When TK1 or TK2 was incubated with radiolabeled dThd or dTMP, no radioactivity was detectable in the dTDP and dTTP spots.

**Kinetic Properties of TK2**—Since our TK2 preparation has a higher specific activity and different native and subunit molecular weights than previously isolated forms of TK2 (9, 10), we have made a more extensive kinetic characterization of this enzyme.

**Comparison of the Substrate Specificity of TK1 and TK2**—The phosphorylation capacity of TK1 and TK2 with natural substrates and various nucleoside analogues was compared by measuring the initial velocity of the reaction at varied substrate concentrations. The kinetic data are shown in Table III.

**Substrate Specificity of Thymidine Kinases 1 and 2**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Hill</th>
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<th>Kmaxa</th>
<th>Vmax/Kmaxa</th>
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<td>32</td>
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a Portions of this paper (including ”Experimental Procedures,” Figs. 1–5, and Tables I and II) are presented in miniprint at the end of this paper. Mini print is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

³ In the case of negative cooperativity, the Vmax/Km ratio was calculated using the Kcat value.
dThd. By increasing the concentrations of enzyme and the specific radioactivity, it was possible to determine the $V_{max}$ for AZT to approximately 6% of the $V_{max}$ with dThd. The substrate kinetics with AZT gave biphasic Hofstee plots and were similar to the kinetics with dThd obtained with TK2 (Fig. 4). The two $K_v$ values for AZT at low and high substrate concentration were 0.2 and 90 μM. This indicated negative cooperativity which was confirmed by Hill coefficients of 0.4 for AZT. The different phosphorylation kinetics of TK1 and TK2 regarding dThd and AZT are demonstrated by the Hill plots shown in Fig. 5.

No phosphorylation of D4T in a concentration range of 5–200 μM was measurable with either TK1 or TK2, indicating that the $V_{max}$ values were at least below 0.04% for TK1 and 0.1% for TK2 in comparison with the corresponding $V_{max}$ values for dThd. Furthermore, although TK2 was an efficient dCyd kinase, phosphorylation of ddCyd and araC was not measurable.

**Substrate Competition**—The competition between the different natural substrates and the nucleoside analogues was studied by inhibition analysis. The $K_v$ values are given in Table IV, and, for all the combinations shown, the kinetics were noncooperative and competitive. According to the $K_v$ values for the competition between the natural substrates for TK2, dThd appeared to be the preferred substrate. It is noticeable that D4T and FLT were efficient inhibitors of TK2, although they could not be phosphorylated. Also, AZT inhibited TK2 almost as efficiently as TK1 in spite of the fact that TK2 had a very low phosphorylation capacity for this drug.

**DISCUSSION**

The specific activity of our purified TK1 was 50-, 1.8-, and 100-fold higher than earlier purified cytosolic human dThd kinases, *i.e.* acute myelocytic cells (9), HeLa cells (19), and placenta (35, 36), respectively. The subunit molecular mass of TK1 from human lymphocytes (22–24 kDa) (Fig. 3C) is in agreement with those previously found for TK1 from HeLa cells (19) and placenta (36). In contrast to what was reported for the purification of TK1 from placenta (36), we found no dTMP kinase associated with TK1 or TK2 during the dThd Sepharose chromatography.

The subunit molecular mass of our pure TK2 preparation was 29 kDa and thus higher than that of TK1, but lower than the 49 kDa reported for the TK2 isolated from human liver (10). No further comparison can be made, however, since the SDS-gel electrophoresis of the liver dThd kinase was not shown (10). The specific activity of the liver TK2 preparation was approximately 18-fold lower than the present spleen TK2 preparation. As judged by the gel filtration chromatography (Fig. 3), the active enzyme appeared to be a monomer, in the absence as well as in the presence of ATP or dThd. This was an unexpected finding, since previous molecular weight examinations of both TK1 and TK2 have shown dimer or tetramer structures (9, 12, 19, 37).

The purified TK2 phosphorylates dCyd with a $V_{max}$ almost 2-fold the $V_{max}$ of dThd. Since cytosolic dCyd kinase is also present in spleen extracts and partly overlapped the TK2 activity during the DEAE-chromatography (Fig. 1), it is possible that our purified TK2 could contain significant contamination of the cytosolic dCyd kinase. However, the following facts demonstrate that this is not the case. (a) Cytosolic dCyd kinase activity is not retained on the 3'-TMP-Sepharose matrix. (b) In the SDS-gel electrophoresis system that clearly separates the 29-kDa TK2 band and the 30-kDa cytosolic dCyd kinase band, no detectable band at 30 kDa was found in the TK2 preparation. (c) The native molecular weight of cytosolic dCyd kinase is 60 kDa, and no significant activity was observed at this position during gel filtration of TK2 which eluted in the position of a 30-kDa protein. (d) No activity with araC, ddA, or ddCyd was observed with TK2, while these analogues are good substrates for the cytoplasmic dCyd kinase. (e) The ratio between dCyd and dThd kinase activity and the negative cooperative dThd kinetics were retained during Superose 12 chromatography of pure TK2. (f) With dCyd as substrate, the cytoplasmic 30-kDa dCyd kinase exhibits negative cooperativity at the employed assay conditions and substrate range (38), whereas the present TK2 shows Michaelis-Menten kinetics. Thus, we conclude that the broad substrate specificity and complex negative cooperativity with dThd is a genuine property of pure TK2.

In the present investigation, we were able to compare the substrate kinetic properties of TK1 and TK2 with regard to the dThd analogues FuUrd, AZT, FLT, and D4T. In accordance with findings from other laboratories with AZT (24) and FLT (25), we found that these drugs were efficiently phosphorylated by TK1, with $V_{max}$ values approximately 50% and 30% of those observed for dThd, respectively. If the ratio $V_{max}/K_v$ (Table III) was taken as an indicator of the phosphorylation capacity, AZT was phosphorylated by TK1 with almost half the efficiency of dThd and higher than FuUrd and dUrd. FLT was phosphorylated by TK1 with the same efficiency as dUrd. The $K_v$ values for AZT and FLT with respect to dThd were as expected from their respective $K_v$ values and 5- and 2-fold lower than the $K_v$ values for partially purified TK1 from Molt/4F cells, respectively (39). Regarding D4T, no phosphorylation with TK1 was detectable, and dThd phosphorylation was inhibited with a $K_v$ value about 3500-fold higher than the $K_v$ value for AZT inhibition.

TK2 which is a pyrimidine deoxynucleoside kinase, showed a very different specificity towards AZT and FLT as compared to TK1. AZT was a poor substrate with an 18-fold lower $V_{max}$ than that for dThd, and the phosphorylation kinetics were negative cooperative similar to the kinetics with dThd. From biphasic Hofstee plots of the kinetic data, 2 $K_v$ values could be obtained, 1 at submicromolar concentrations and 1 at about 450-fold higher concentrations. The negative cooperative kinetics of TK2 was only obtained with thymidine-containing substrates, while ordinary Michaelis-Menten kinetics were

<table>
<thead>
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<th>Substrate</th>
<th>Inhibitor</th>
<th>$K_v$ [μM]</th>
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</thead>
<tbody>
<tr>
<td>dThd</td>
<td>AZT</td>
<td>0.6</td>
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<tr>
<td></td>
<td>FLT</td>
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</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>dUrd</td>
<td>690</td>
</tr>
</tbody>
</table>

*The $K_v$ values were determined from Dixon plots.*
obtained with dCyd, dUrd, andFdUrd.

FLT, D4T, and ddCyd were not phosphorylated by TK2, and, although this enzyme shows a broad specificity with regard to pyrimidine bases, it appears to be much more restrictive with regard to the deoxyribonucleoside moiety. These results are also in line with the fact that TK2 shows no activity with araC which was demonstrated earlier with TK2 from human acute myelocytic cells (40).

Although FLT and D4T could not serve as substrates for TK2, they were relatively efficient inhibitors with Ki values of 10 and 78 μM, respectively. We also found that dCyd, which is a relatively good substrate for TK2 (Ki = 36 μM), was a poor inhibitor of dThd phosphorylation (Ki = 630 μM). We suspect that this behavior is related to the negative cooperative kinetics of TK2.

Recent studies in this laboratory with pure cytosolic dCyd kinase showed that this enzyme in contrast to TK2 efficiently phosphorylated ddCyd (Ki = 60 μM, Vmax = 80% of that with dCyd).3 However, a partially pure mitochondrial preparation of dCyd kinase which most likely corresponds to TK2, had a much lower capacity (approximately 10% of that with dCyd).3 In both cases the Ki values for ddCyd were around 300 μM.3 With the present TK2 preparation, we would easily have detected that level of ddCyd phosphorylation. The reason for these diverging results is not known.

Phosphorylation of D4T has been studied with whole cells and crude dThd kinase fractions, and it was concluded that this analogue was very inefficient phosphorylated (28, 29). On the other hand, dThd kinase-deficient cells showed only a slight reduction in d4T phosphorylation, while in the same cells, AZT phosphorylation was diminished at least 100-fold. This suggests an alternative pathway of phosphorylation, conceivably a phosphotransferase type of activity. Such an enzyme activity capable of transferring phosphates from phenylphosphates or nucleoside monophosphate to pyrimidine ribo- or deoxyribonucleosides was detected in extracts from human tissues (42). The purine analogue, 2',3'-dideoxyinosine which is now tested in several clinical trials as an anti-HIV agent, has been shown to be phosphorylated by a phosphotransferase (43).

Resting cells lack TK1 but contain TK2 activity, and this limited capacity to salvage dideoxynucleosides may explain why human monocytes/macrophages showed a markedly decreased phosphorylation of AZT as compared to T-cells (44). However, due to a low de novo dTPP production in these cells, a favorable ratio between AZT triphosphate and dTPP was still achieved and thereby an efficient anti-HIV effect. Nevertheless, in order to develop new and improved therapies against HIV, it is essential to know the capacity of TK1 and TK2 to activate candidate nucleoside analogues.

The specificity of TK2 with regard to dideoxynucleoside analogues may be of significance for the toxic effect on mitochondria as has been observed in cells treated with ddCyd (45). However, further biochemical characterization of the tissue and subcellular distribution of TK2 is needed to clarify its relative contribution to the toxic side effects observed with dideoxynucleoside analogues.

Acknowledgments—We are indebted to Anita Herrström and Birgitta Wahlström for their expert technical assistance.

REFERENCES


35. Eng Gan, T., Brunlley, J. L., and Van Der Weyden, M. B. (1983)
Substrate Specificity of Thymidine Kinases 1 and 2

**Experimental Procedures**

**Isolation of Blasts**

Hydroxyethaneperoxide-stimulated lymphocytes were isolated from 50 ml portions of peripheral blood from healthy volunteers by Immuno-Ficol gradient centrifugation. The blast cultures were cultured for 3 days in Falcon tissue culture medium with hydroxyethylperoxide at 100 units/ml. The blast cultures were chilled to 4°C, frozen within two hours and stored at -70°C.

**Assay of dThd Kinase Activity**

The extraction medium for the assay of dThd kinase activity was the same as the extraction medium for the assay of dCyd kinase activity, except that 0.5 ng TK1 or 10 ng TK2 were added to the 3'TMP assay mixture. The fraction volume was 28 nl. TK2 was concentrated and dThd removed by chromatography on a Sephadex G-25 column (10 x 15 mm), pre-equilibrated with buffer C-10. After application of the samples to the column, the column was washed with 30 ml of buffer C-10 (fractions 11-14), and then with 10 ml of buffer C-50 (fractions 15-20) which is the same as buffer C-10, except for the potassium phosphate concentration being 0.01 M. dThd kinase activity was eluted with 20 cts C-10 containing approximately 10 cts dThd and both kinases eluted as single peaks at about 30 cts dThd. The dThd concentration in the fractions was estimated from the abundance at 187 nm. Fig. A4 and A5 show the elution pattern for TK2 and TK1, respectively. The 20 cts of elution of fractions from 3'TMP-Sepharose chromatography of TK2 was concentrated and dThd removed by chromatography on a Sephadex G-25 column (10 x 15 mm), pre-equilibrated with buffer C-10. After application of the pooled fractions from step F, the column was washed with 50 volumes of buffer C-10. The wash solutions were pooled and directly applied to the DEAE Sepharose column. The Sic peak of dThd kinase activity (fractions 10-15) was eluted at 48 cts C-10 (fractions 1-10), with buffer C-100 which is the same as buffer C-10, except for the potassium phosphate concentration being 0.1 M. dThd was eluted from the DEAE Sepharose column with 100 cts C-10 containing approximately 10 cts dThd and both kinases eluted as single peaks at about 40 cts dThd.

**RESULTS**

**Kinetic Studies**

The maximal velocity of the reaction was defined as the amount of enzyme that can phosphorylate 1 nmole of nucleoside/nucleotide per min at 37°C under standard assay conditions.

**Kinetics**

The substrate kinase activity was measured in duplicate micropipettes (1/2 versus 1/2), Rosetree plates (2 versus 2/1) and Wilson plates (1/2 versus 1/2) under standard assay conditions.

**Specific Activity**

The specific activity of the enzyme was defined as the amount of enzyme that can phosphorylate 1 nmole of nucleoside/nucleotide per min at 37°C under standard assay conditions.

**Substrate Kinetics**

The enzyme activity was measured by Hill plots. The substrate kinetics were measured by Hill plots (1/2 versus 1/2) containing 10 cts C-10 (fractions 1-10), with buffer C-100 which is the same as buffer C-10, except for the potassium phosphate concentration being 0.1 M. dThd was eluted from the DEAE Sepharose column with 100 cts C-10 containing approximately 10 cts dThd and TK1 and TK2 eluted as single peaks at about 40 cts dThd.

**TABLE I**

<table>
<thead>
<tr>
<th>Total protein</th>
<th>Spec. Act.</th>
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</thead>
<tbody>
<tr>
<td>Total activity</td>
<td>Activity</td>
<td>TK</td>
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</table>

**DISCUSSION**

The apparent molecular weight of native TK2 and TK1 was determined using a Superose 12 column (16 x 300 mm) connected to an FPLC system (FPLC Protein A chromatography). The column was standardized by the A-580 absorbance of a 0.01 M solution of dThd at 267 nm. The flow rate was 0.5 ml/min and 20 cts C-10 containing approximately 10 cts dThd was applied to the column. TK1 and TK2 were separated by DEAE Sepharose ion-exchange chromatography using 0.01 M potassium phosphate, 0.15 M NaCl, and 0.1 M NaHCO3. TK1 was eluted at 48 cts C-10 (fractions 1-10), with buffer C-100 which is the same as buffer C-10, except for the potassium phosphate concentration being 0.1 M. dThd was eluted from the DEAE Sepharose column with 100 cts C-10 containing approximately 10 cts dThd and both kinases eluted as single peaks at about 40 cts dThd.

**Figures**

**Fig. 1** shows the elution pattern for TK2 and TK1, respectively. The 20 cts of elution of fractions from 3'TMP-Sepharose chromatography of TK2 was concentrated and dThd removed by chromatography on a Sephadex G-25 column (10 x 15 mm), pre-equilibrated with buffer C-10. After application of the pooled fractions from step F, the column was washed with 50 volumes of buffer C-10. The wash solutions were pooled and directly applied to the DEAE Sepharose column. The Sic peak of dThd kinase activity (fractions 10-15) was eluted at 48 cts C-10 (fractions 1-10), with buffer C-100 which is the same as buffer C-10, except for the potassium phosphate concentration being 0.1 M. dThd was eluted from the DEAE Sepharose column with 100 cts C-10 containing approximately 10 cts dThd and both kinases eluted as single peaks at about 40 cts dThd.
Substrate Specificity of Thymidine Kinases 1 and 2

TABLE II

<table>
<thead>
<tr>
<th>Purification of TK1 from human phytohemagglutinin stimulated lymphocytes</th>
<th>Total volume (ml)</th>
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<th>Total Activity (units)</th>
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The activity was measured at standard assay conditions and represents the sum of both TK1 and TK2 activities.

TK1 and TK2 were separated by DEAE ion-exchange chromatography (see "Experimental procedures").

Figure 1

DEAE-Sepharose ion-exchange chromatography of the ammonium sulfate fraction. The proteins were eluted with a 0.0-0.3 M KCl gradient, and the conductivity (---), the difference in absorbance at 280 and 310 nm (---) and the activity of dTdh kinase (—) and dThd kinase (—) were determined in the fractions.

Figure 2A

Affinity chromatography of the pooled fractions from the hydroxylapatite chromatography of TK2. The dTdh (—) and protein concentration (—) and the dThd kinase activity (—) were measured in the fractions.

RESULTS

Molecular weight determination on a Superose 12 column connected to an FPLC system. Pure TK2 was injected together with alcohol dehydrogenase and bovine serum albumin. The arrows indicate from left to right the elution volumes of the marker proteins: alcohol dehydrogenase, 150 kD; bovine albumin, 66 kD; ovalbumin 45 kD; and carbonic anhydrase 29 kD. The relative UV absorption ([]) was recorded, and dTdh kinase (—), and dThd kinase (—) activity were determined (20 μM substrate in the assay) in each fraction.

Figure 2B

SDS gel electrophoresis of the indicated fractions from the affinity chromatography of TK2. The activities (units/mg) in fractions 1, 5 and 7 were 0.1, 14.4 and 0.11, respectively.

Figure 2C

SDS gel electrophoresis of the indicated fractions from affinity chromatography of TK1 from human lymphocytes. The activity (units/mg) in fraction 1, 5 and 7 were 0.1, 14.4 and 0.11, respectively.
The activity of the pure dThd kinase from leukemic spleen at varying dThd and dCyd concentrations. The other assay conditions were standard. The data are plotted according to Hafstee. $v$ is the enzyme activity in units/mg and $s$ is the substrate concentration in $\mu$M. Regression analysis were used for calculation of the kinetic constants.

Figure 4

Figure 5

Lin plots of the kinetic data with TK1 (○) and TK2 at (●) various concentrations of dThd (A) and AZT (B).