Purification and Characterization of Herpes Simplex Virus (Type 1) Thymidine Kinase Produced in *Escherichia coli* by a High Efficiency Expression Plasmid Utilizing a λ P<sub>L</sub> Promoter and a cI857 Temperature-sensitive Repressor

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The structural gene for herpes simplex virus (type 1) thymidine kinase was cloned downstream from the λ phage high efficiency leftward promoter in a plasmid (pHETK2) also containing the gene for the λ cI857 temperature-sensitive repressor. Thymidine kinase is synthesized as a run-on product containing the NH<sub>2</sub> terminus of the λ N protein. Heat inactivation of the λ repressor by growth at 42 °C results in the accumulation of thymidine kinase as approximately 4% of the total soluble cellular protein. Thymidine kinase has been purified to greater than 95% homogeneity by high speed centrifugation, ammonium sulfate fractionation, and Sephadex G-100 and hydroxylapatite column chromatography. Thymidine kinase has a subunit Mr = 42,000 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and behaves as a dimer during Sephadex G-100 chromatography and glycerol gradient centrifugation. Thymidine kinase is enzymatically active from pH 6 to 10 with maximum activity at pH 8.5. The enzyme is protected from heat inactivation by thymidine and has a half-life at 40 °C of 30 min in the presence of thymidine and 3 min in its absence. Thymidine kinase displays Michaelis-Menten kinetics with apparent Michaelis constants of 0.6 and 118 μM for thymidine and ATP, respectively. Iododeoxyxanthine is a competitive inhibitor of thymidine with an apparent K<sub>i</sub> of 14 μM. The anti-herpes drug acyclovir (9-(2-hydroxyethoxy)methyl)guanine) also appears to be a competitive inhibitor of thymidine (K<sub>i</sub> of approximately 300 μM) but requires 3,000-fold higher concentrations than thymidine to give 50% inhibition. Other nucleoside triphosphates can substitute for ATP in the kinase reaction with the exception of dTTP which appears to inhibit thymidine kinase activity by about 50% when present in concentrations equal to that of thymidine.

The structural expression of a virus has different properties from the endogenous cellular thymidine kinase. These differences have been exploited both in the design and development of anti-herpes drugs (1) and in the detection of viral thymidine kinase in cells (2). HSV-1 enzyme has been isolated from several mammalian cell lines and partially purified and characterized (3-10). The expression of HSV-1 thymidine kinase in *Escherichia coli* has also been described (11, 12).

Thymidine kinase is a valuable selective gene in somatic cell genetics, in DNA transfection and selection experiments in eukaryotic cells, and in studies of eukaryotic promoters (13, 14). Cells can be selected which lack or contain thymidine kinase by use of the drugs bromodeoxyuridine or aminopterin (methotrexate), respectively. In this report we describe the high level bacterial synthesis of herpes thymidine kinase and the purification and biochemical characterization of the enzyme.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

Plasmids used in the construction of the high expression thymidine kinase plasmids, pHETK1 and pHETK2, are derivatives of pBR322. References to nucleotide positions in the λ genome refer to the λ nucleotide sequence published by Sanger et al. (15). The organization of plasmids pHETK1 and pHETK2 is illustrated in Fig. 1 and confirmed by restriction digests with appropriate enzymes. The orientation of the repressor gene in pHETK1 appears to be important because cells containing the plasmid with the repressor in the opposite orientation grew slowly and yielded barely detectable levels of plasmid DNA. The details of plasmid construction are described in Miniprint. The insertion of bacterial guanine-xanthine phosphoribosyltransferase in an analogous plasmid (pHEGPT) and its high expression is described elsewhere (24).

1 The abbreviations used are: HSV, herpes simplex virus; SDS, sodium dodecyl sulfate; kb, kilobase; DTT, dithiothreitol; BSA, bovine serum albumin; TK, thymidine kinase; PAGE, polyacrylamide gel electrophoresis; 5′-I'G, 5′-iododeoxyxanthine.

2 Portions of this paper (including "Experimental Procedures" and Table III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-778, cite the authors and include a check or money order for $2.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
The presence of protease inhibitors during cell lysis does not appear to affect the thymidine kinase activity (Table I). These data suggest that the fused protein is proteolytically cleaved by enzymes released during bacterial lysis, and that the two most sensitive cleavage sites appear to be directly adjacent to the normal start of the thymidine kinase protein and between the N gene and the upstream sequences of the thymidine kinase protein.

HSV-1 thymidine kinase has been purified 25-fold to near homogeneity from extracts of cells containing pHETK2 (Table II). The enzyme represents 4% of the soluble protein in induced cells. We can obtain 7 mg of purified thymidine kinase/liter of cells with a 15% cumulative recovery of enzyme activity. The details of purification are described in Miniprint. Ammonium sulfate fractionation provides major purification after which the enzyme represents greater than 50% of the total protein. Additional contaminants are removed by Sephadex G-100 and hydroxylapatite column chromatography. The protein compositions at different stages of purification are illustrated in the SDS-polyacrylamide gel in Fig. 3. A single protein band for the hydroxylapatite fraction represents a 25-fold purification and a 15% cumulative yield of the thymidine kinase activity. Purification of thymidine kinase activity is correlated with the purification of the M, = 42,000 band on SDS-polyacrylamide gels. Although chromatography on hydroxylapatite does not appear to increase the specific activity 11.

<table>
<thead>
<tr>
<th>Thymidine kinase activity from cultures grown at</th>
<th>30 °C</th>
<th>42 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Extract of HB101 containing:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plasmid</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>pHETK2</td>
<td>0.9</td>
<td>12.0</td>
</tr>
<tr>
<td>pHETK2 (protease-inhibited)</td>
<td>ND</td>
<td>9.0</td>
</tr>
<tr>
<td>pHETK1</td>
<td>ND</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**TABLE I**

Induction of thymidine kinase

Cultures (100 ml) were grown at 30 °C to mid-log phase. Cultures were then either shifted to 42 °C or left at 30 °C and incubated an additional 16 h. High speed supernatant fractions were prepared and assayed for thymidine kinase activity and total protein. ND, not determined.

Fig. 1. **Construction of pHETK1 and pHETK2**. Plasmids were constructed as described under "Experimental Procedures." DNA sequences are as follows: pBR322 (•); λ BglII fragment nucleotide sequences 35711 to 38103 from pRLM5 (□); λ HpaI to BglII fragment nucleotide sequences 35261 to 35711 from pAY2131 (■); HSV-1 thymidine kinase gene (BglII to PvuII fragment from pGH6 (■)).

Plasmids pHETK2 and pHEGPT produce large quantities of gene products cloned downstream from the λ leftward and rightward promoters. The expression of genes in these plasmids is 4% or more of the cellular proteins. The high speed supernatant fraction of bacteria containing pHETK2 grown at 42 °C shows a 30-fold greater thymidine kinase activity than that of HB101 without pHETK2 (Table I). Maximum increase in specific activity and yield of enzyme occur after overnight incubation at 42 °C. At 42 °C, bacteria containing pHETK1 produce thymidine kinase at about one third the level found in induced cultures containing pHETK2 (Table I).

Continuous high expression leads to slower growth and may be lethal to the cell. Therefore, it is useful to repress expression of the λ promoters during cell propagation with λ repressor. The inclusion of the λ temperature-sensitive repressor c1857 gene on the plasmid allows growth in E. coli strains not lysogenized with λ phage. Thus, the induction of product by temperature shift from 30 to 42 °C does not result in the induction of λ proteins, as would be the case if the plasmid were placed in a λ lysogen.

Efficient translation of mRNA in bacteria requires an active bacterial ribosome-binding site which may not be present in eukaryotic genes. In pHETK2, the thymidine kinase structural gene is linked to the NH₂ terminus of the λ N protein and makes use of the N protein’s ribosome-binding site. The thymidine kinase mRNA codons are in phase with those of the N protein, and the expected protein is a fused product consisting of the first 59 amino acids of N protein, the 20 amino acids coded for by the upstream sequence of the thymidine kinase gene (between the BglII site and the normal AUG start codon), and the 376 amino acids of the normal thymidine kinase protein. A protein of this size would be expected to have M, = 42,000 (Fig. 2). However, when induced cells are lysed in the presence of protease inhibitors (10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 100 Kallikrein-inactivating units/ml of aprotinin) or boiled directly in SDS, the amount of M, = 42,000 product is diminished, while two new products appear having apparent M, = 51,500 and 44,300 which are those predicted for the full length fused protein and the thymidine kinase protein including upstream sequences but missing the 59 amino acids from the N protein.

Fig. 2. **SDS-polyacrylamide gel electrophoresis of high speed supernatant fractions of induced and noninduced cultures.** High speed supernatants of: lane a, bacteria grown at 42 °C; lane b, bacteria containing pHETK2 grown at 30 °C; lane c, bacteria containing pHETK2 grown at 42 °C (M, = 42,000 band indicated by arrow); lane d, bacteria containing pHETK2 grown at 42 °C and lysed in the presence of protease inhibitors (M, = 51,500 and 44,300 bands indicated by arrows). Bovine serum albumin (M, = 68,000, 68k), ovalbumin (45k), and carbonic anhydrase (31k) are the molecular weight markers.
ammonium sulfate fraction, 15 μg; lone b, of HSV-1 thymidine kinase to be 40,000 to 44,000 (3, 4, 25) 

PM 80,000. 

Polyacrylamide gel electrophoresis. No contaminating poly-

peptides are observed in SDS-polyacrylamide gels when 4.5 μg of hydroxylapatite fraction thymidine kinase are applied. Using 0.25 μg of protein as the lower limit of sensitivity for proteins stained by Coomassie blue, we estimate that greater than 95% of the protein in the hydroxylapatite fraction is thymidine kinase.

Sephadex G-100 column chromatography and glycerol gra-
dient centrifugation indicate that HSV-1 thymidine kinase has a native molecular weight of approximately 70,000 to 80,000. A subunit Mr = 42,000 determined by SDS-polyacryl-

amide gels implies that HSV-1 thymidine kinase is a dimer. Other investigators have found the subunit molecular weight of HSV-1 thymidine kinase to be 40,000 to 44,000 (3, 4, 25) and reported that the HSV-1 enzyme is a dimer (4-6, 25). Thus, the structure of HSV-1 thymidine kinase synthesized in bacteria is apparently the same as that of HSV-1 thymidine kinase in mammalian cells.

Both substrates ATP and thymidine increase the stability of HSV-1 thymidine kinase at elevated temperatures. At 40 °C thymidine kinase has a half-life of 3 min in the absence of substrate and half-lives of 30 or 6 min in the presence of 120 μM thymidine or 2 mM ATP, respectively. β-Mercaptoethanol is an important component in the enzyme buffer because in its absence, thymidine kinase is rapidly inactivated at room temperature. The Sephadex G-100 fraction of thymidine kinase has been stored in thymidine kinase enzyme buffer at 4 °C for 1 month with less than a 10% loss in enzyme activity. The freezing (at −70 °C) and thawing of the Sephadex G-100 fraction does not appear to inactivate the enzyme.

HSV-1 thymidine kinase displays Michaelis-Menten kinetics when one of the substrates is in limiting amounts and the other is in excess. The apparent Kₘ values are 118 μM for ATP and 0.6 μM for thymidine. Thymidine analogs distinguish between the herpes, mammalian, and bacterial thymidine kinase enzymes. 5'-Iododeoxycytidine is not a substrate for bacterial thymidine kinase (26) or cellular thymidine kinase. However, iododeoxycytidine is a substrate of herpes thymidine kinase and has been used to specifically assay for HSV-1 thymidine kinase in the presence of mammalian cellular thymidine kinases (2). Our results indicate that for the bacterially synthesized herpes thymidine kinase, iododeoxycytidine is a competitive inhibitor of thymidine with a Kᵢ of 14 μM.

Acyclovir (9-[(2-hydroxyethoxy)methyl]guanine), an anti-

herpes drug manufactured by Burroughs-Wellcome, derives its antiviral capability by first serving as a substrate for herpes thymidine kinase. The phosphorylated product then acts as an inhibitor of herpes DNA polymerase or as a chain termi-

nator during DNA synthesis (1). Our results suggest that acyclovir is a substrate of herpes thymidine kinase but re-

quires concentrations nearly 3000-fold in excess of thymidine to inhibit enzyme activity by 50%. Acyclovir inhibition appears to be competitive with thymidine because it is overcome by increasing the concentration of thymidine. From a Dixon plot, we estimate that acyclovir has a Kᵢ of approximately 300 μM. Similar results have been reported for HSV-1 thymidine kinase isolated from mammalian cells (1, 10).

The biochemical properties of HSV-1 thymidine kinase synthesized in bacteria are similar to those reported for the enzyme isolated from HSV-1-infected cells. The specific activity of hydroxylapatite fraction thymidine kinase is 130 units/mg, which compares with 45 and 67 units/mg reported for the HSV-1 thymidine kinase by Kit et al. (7) and Cheng and Ostrander (8), respectively. The apparent Kₘ for thymidine observed for HSV-1 thymidine kinase is identi-

cal with that reported by Elion (1) and nearly identical with the value of 0.4 μM reported by Jamieson and Subak-Sharpe (9) for the HSV-1 enzyme. By contrast, the Kₘ for thymidine for the E. coli thymidine kinase is reported to be 17 μM (26). HSV-1 thymidine kinase appears to have maximal enzymatic activity at pH 8.5, which is in contrast to the pH optimum of 6.0 found by Jamieson and Subak-Sharpe (9). However, these investigators employed crude cell lysates whose other com-

ponents could affect the apparent pH optimum of the enzyme.

Several nucleoside triphosphates are potential substrates (phosphate donors) for herpes thymidine kinase. At a concentra-

tion of 1 mM, the nucleoside triphosphate dATP, GTP, dGTP, CTP, and dCTP phosphorylate thymidine at 75 to 100% the rate given by ATP. However, dTTP is not a sub-

strate and appears to inhibit thymidine kinase activity by 50% when present in concentrations equal to that of thymi-

dine. Thin layer chromatography of the dTTP on polyeth-


TABLE II
Purification of thymidine kinase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Total activity</th>
<th>Cumulative yield</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>25</td>
<td>23.5</td>
<td>5.2</td>
<td>3055</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>High speed supernatant</td>
<td>20</td>
<td>18.0</td>
<td>9.7</td>
<td>3492</td>
<td>114</td>
<td>1.9</td>
</tr>
<tr>
<td>35 to 45% ammonium sulfate</td>
<td>1.2</td>
<td>15.0</td>
<td>90</td>
<td>1620</td>
<td>53</td>
<td>17</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>12</td>
<td>1.0</td>
<td>130</td>
<td>1560</td>
<td>51</td>
<td>25</td>
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<tr>
<td>Hydroxylapatite</td>
<td>36</td>
<td>0.1</td>
<td>130</td>
<td>468</td>
<td>15</td>
<td>25</td>
</tr>
</tbody>
</table>

HSV-1 thymidine kinase displays Michaelis-Menten kinetic values for the hydroxylapatite fraction are those that would be obtained had the entire Sephadex G-100 fraction been applied to hydroxylapatite.
yleneimine-cellulose (23) indicates that the inhibition by dTTP is not the result of isotope dilution by contaminating thymidine.

REFERENCES

HSV Thymidine Kinase

HSV Thymidine Kinase

Experiential Material: HSV thymidine kinase (EC 2.7.1.67), a 5'-nucleotidase, was obtained from a commercial source. Thymidine kinase (EC 2.7.1.67) was assayed by a modification of the method of Pataki and Pataki (1972). The assay was performed in a 1.0 ml reaction mixture containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM KCl, and 200 µM [3H]-thymidine. The reaction was started by adding 20 µM ATP. The reaction was stopped after 10 min by the addition of 1 ml of 10% trichloroacetic acid. The precipitate was collected by filtration and the radioactivity was measured in a liquid scintillation counter.

Results: The specific activity of thymidine kinase was 44.6 units/mg protein. The assay was linear for at least 30 min and the reaction was complete within 10 min.

Discussion: The high specific activity of thymidine kinase in HSV indicates that this enzyme is a significant target for antiviral therapy. The assay can be used to monitor the activity of thymidine kinase inhibitors in vitro.

Table 1: Thymidine Kinase Activity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV TK 1</td>
<td>44.6</td>
</tr>
</tbody>
</table>

**References**


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Purification and characterization of herpes simplex virus (type 1) thymidine kinase produced in Escherichia coli by a high efficiency expression plasmid utilizing a lambda PL promoter and cI857 temperature-sensitive repressor.

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