Deoxythymidine Kinase Induced in HeLa TK- Cells by Herpes Simplex Virus Type I and Type II

II. PURIFICATION AND CHARACTERIZATION*

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Deoxythymidine kinase activities were induced in HeLa TK- (deoxythymidine kinase-deficient) cells infected with either herpes simplex virus type I or herpes simplex virus type II. The herpes simplex virus type I-induced enzyme was found in the cytoplasmic and nuclear fractions of the infected cells, whereas the herpes simplex type II-induced deoxythymidine kinase could only be found in the cytoplasm.

Herpes simplex virus type I and II specific deoxythymidine kinases were purified by affinity column chromatography. Both purified deoxythymidine kinases retained the deoxycytidine kinase activity present in the crude preparation. The purified herpes simplex virus type I deoxythymidine kinase had a different mobility on electrophoresis, but the same sedimentation rate on a glycerol gradient as the corresponding unpurified enzyme, whereas the purified herpes simplex virus type II deoxythymidine kinase had the same mobility and sedimentation rate as the corresponding unpurified enzyme.

In the presence of Mg2+ATP and dithiothreitol, herpes simplex virus type II deoxythymidine kinase was more stable than herpes simplex virus type I deoxythymidine kinase at both 45° and 4°. The deoxycytidine kinase activity present in the purified preparations was inactivated at the same rate as the deoxythymidine kinase activity. In the presence of the other substrate, deoxycytidine, herpes simplex virus type I deoxythymidine kinase was more stable than herpes simplex virus type II kinase. The purified herpes simplex virus type I and II deoxythymidine kinases had different activation energies when Mg2+ATP and deoxythymidine were used as substrates, but showed the same sensitivity toward ammonium sulfate inhibition.

Deoxythymidine kinase has been found to increase in various mammalian cells after infection with herpes simplex virus type I or type II. The induced dThd kinases differ from the host cell dThd kinases with regard to immunogenicity (1-3), molecular weight (4, 5), substrate specificity (3, 4), electrophoretic mobility (4), and thermostability (5). Host cell mitochondrial and HS-I virus-induced dThd kinases are able to use ATP, GTP, UTP, and CTP as phosphate donors, whereas the host cytosol dThd kinase can use only ATP as the phosphate donor (4). The host mitochondrial enzyme is relatively more sensitive to dCTP inhibition than either the HS-I virus enzyme or the host cytosol enzyme. The sedimentation coefficient of HS-II virus dThd kinase is slightly less than that of HS-I virus dThd kinase (3). The HS-II virus enzyme is somewhat less stable at 40° than the HS-I virus enzyme (5). Recently, it was suggested that the herpes-induced dThd kinase also possesses dCyd kinase activity (2, 6-8); these data were obtained with either crude or partially purified enzyme.

Because herpes type viruses can cause many human diseases and also have the potential to induce cell transformation (9-11), there is need for chemotherapeutic agents which inhibit the growth of these viruses. This laboratory has been interested in developing anti-herpes drugs by exploiting the unique properties of the herpes-induced dThd kinase. The unexpected presence of HS-I virus-induced enzyme in the nucleus, time course of induction, and the cellular distribution of the induced enzymes as well as the isolation, purification, and some properties of these enzymes are reported here.

MATERIALS AND METHODS

Cells and Virus—The stock viruses HS-I virus (KOS strain) and HS-II virus (333 strain) were given to us by Dr. W. Munyon of this Institute. They were passed at low multiplicity and assayed in a CV-1 monolayer culture.

HeLa TK- (BU 25), a dThd kinase-deficient human cell line, was originally developed in Dr. S. Kit's laboratory (12) and given to us by...
Dr. W. Munyon. The cells were grown in Falcon flasks (75 cm²) in Eagle's minimum essential medium containing 25 µg/ml of BrdUrd and 10% calf serum. BrdUrd was omitted from the medium for two or three passages immediately preceding an experiment. Cells were passaged when monolayers were confluent and were obtained by trypsinization. Cells were harvested for the experiments by scraping with a rubber policeman.

Infection of HeLa TK- Cells by Viruses—Confluent monolayer cultures of HeLa TK- cells were infected with either HS-I virus or HS-II virus at an input of 10 to 20 plaque-forming units/cell. After an absorption period of 1 hour, growth medium was added and the culture incubated for the desired time period at 37°C. Cells were washed twice and then harvested in cold phosphate-buffered saline.

Extraction and Fractionation of dThd Kinase from Cells—The harvested cells in suspension were centrifuged and then resuspended in 4 volumes of hypotonic buffer containing 0.01 M Tris-HCl, pH 7.5, 0.01 M KCl, 1 mM MgCl₂, 1 mM mercaptoethanol, and 50 µM dThd (Buffer A). The cell suspension was allowed to stand for 20 min at 4°C and then disrupted with a Dounce homogenizer (about 30 strokes); a sample of the homogenate was examined under the light microscope to verify that cells were broken. The homogenate was centrifuged at 15,000 rpm for 25 min in a Beckman model J-21B centrifuge with a JA-20 rotor. Under these conditions, all nuclei and mitochondria are pelleted at the bottom of the centrifuge tube. To the supernatant were added glycerol to 10% and KCl to 0.15 M. This will be referred to as the "cytosol fraction." The pellets were resuspended in 4 volumes of original cell pellet of Buffer A containing 10% glycerol and 0.15 M KCl, and sonicated; the sonicated samples were centrifuged at 15,000 rpm for 25 min in a Beckman model J-21B centrifuge with a JA-20 rotor, and the supernatant of this preparation will be referred to as the "nucleotyocysol fraction." The procedures followed to obtain the organelle fractions from the infected HeLa TK- cells were the same as described by Kit et al. (13). All operations were performed at 4°C, and the samples were kept frozen at -70°C until assayed.

Radiolabeling of Cellular Protein—[^H]-Amino-acid hydrolysate (50 µCi) was added to HS-I virus- or HS-II virus-infected HeLa TK- cells 2 hours post infection. The rest of the procedures were the same as described above.

Enzyme Assays—The enzyme assays were performed at 37°C for 30 min. The dThd kinase assay mixture contained 94 µM [^3H]dThd, 9 mM MgCl₂ ATP, 3 mM creatine phosphate, 1 unit of creatine phosphokinase, 0.5 mg/ml of albumin, and 25 mM NaF. The assays were linear with time for at least 2 hours of incubation even when crude enzyme preparations were used. The other procedures were as described previously (14). The dCyd kinase assay mixture contained 0.4 mM [^3H]dCyd, 5 mM MgCl₂ ATP, 0.3 mM tetrahydrodridine, and 25 mM NaF. The unit used was nanomoles of dTMP or dCMP formed/min at 37°C. Protein concentrations were determined by the method of Lowry et al. (15).

Electrophoresis and Glycerol Gradient Centrifugation—Electrophoresis was performed as described in the preceding paper (16). Glycerol gradient (10 to 30%, v/v) centrifugation conditions were the same as those described by Kit and co-workers (4) except that the running time was 50 hours.

Materials—The affinity column matrix was prepared according to Kowal and Markus (17) with the modifications described in the preceding paper (16). [^3H]dCyd (52 Ci/mol) and[^H]-Amino-acid hydrolysate were purchased from New England Nuclear Chemical Co. All the compounds used were reagent grade.

RESULTS

Induction and Subcellular Distribution of dThd Kinase Activity—The time course of induction of dThd kinase activity in the cytosol and noncytosol fractions of HS-I virus- or HS-II virus- or mock infected HeLa TK- cells is shown in Fig. 1. The specific activity of dThd kinase was increased in both cytosol and noncytosol fractions of HS-I virus-infected cells and reached a peak around 18 hours post infection. The cells were fractionated after 18 hours of HS-I virus infection and the distribution of the dThd kinase activity among the organelles was determined as follows: 73% in the 100,000 x g supernatant, 24% in the nuclei, 3% in the mitochondria, and no activity detectable in the microsomal fraction. There seems to be a longer induction lag period in the noncytosol fraction than in the cytosol fraction. In contrast, in HS-II virus-infected cells, induction of dThd kinase activity can be observed only in the cytoplasmic fraction. The activity in the cytosol fraction of HS-II virus-infected cells reached a peak around 12 hours post infection.

For the purification reported below, the following starting preparations were used: (a) the cytosol fraction of HS-I virus-infected HeLa TK- cells, 18 hours post infection and (b) the cytosol fraction of HS-II virus-infected HeLa TK- cells, 12 hours post infection.

Purification of HS-I virus- and HS-II virus-specific dThd Kinase—Cytosol fractions from HS-I virus- and HS-II virus-infected cells were centrifuged at 39,000 rpm (100,000 x g) for 1 hour in a Beckman preparative ultracentrifuge using a 50 Ti rotor. To the supernatant ("100,000 x g fraction"), streptomycin sulfate solution (20%) was added dropwise to a final concentration of 1%, and the precipitate was removed by centrifugation. Solid ammonium sulfate was added slowly to give 20% saturation, the precipitate was removed by centrifugation, and additional ammonium sulfate was added to give 90% saturation. The precipitate formed was removed by centrifugation, dissolved in 0.02 M Tris-HCl buffer, pH 7.5, containing 10% glycerol and 3 mM dithiothreitol and applied to the affinity column. The elution buffers used are shown in Fig. 2. HS-I virus-specific dThd kinase was eluted from the column with 0.3 M Tris-HCl buffer containing 0.3 M Tris-HCl buffer containing 0.2 µM dThd (Fig. 2A). HS-II virus-specific dThd kinase could only be eluted with 0.8 M Tris-HCl buffer containing 0.2 µM dThd. The recovery from the column was greater than 70%. The purification of both types of dThd kinase is shown in Table I. The final purification of the HS-I virus enzyme was 67-fold and that of HS-II virus enzyme was 123-fold. It is unclear at this time whether the
Purification and Characterization of HS-I Virus and HS-II Virus dThd Kinase

purified preparations for both types of dThd kinase were homogeneous or not, due to the fact that the protein concentrations in the final preparations were too low to give visible bands when Amido black was used as the staining agent for protein on gel electrophoresis. Attempts to concentrate the purified enzymes by either Diaflo ultrafiltration or ammonium sulfate precipitation resulted in a loss of activity. The purified enzymes were stable at −70° in the presence of 200 μM dThd, 3 mM dithiothreitol, and 10% glycerol.

The results of an experiment in which 3H-amino-acid-labeled cell extracts from HS-I virus- or HS-II virus-infected HeLa TK− cells were used as starting materials for the purification are shown in Fig. 3. The final purification was by affinity column chromatography. A 3H-labeled peak with dThd activity was eluted from the column in both cases when 200 μM dThd was added to the elution buffers. These fractions also exhibited dCyd kinase activity.

Electrophoresis—The results of electrophoresis of the 100,000 × g supernatant preparations derived from the cytosol fraction of HS-I virus- and HS-II virus-infected cells and of the purified HS-I virus and HS-II virus dThd kinase preparations are presented in Fig. 4. The electromobility of the crude preparation of dThd kinase from HS-II virus-infected cells was the same as that of the purified HS-II virus dThd kinase, whereas the electromobility of the crude preparation of dThd kinase from HS-I virus-infected cells was 0.95 fold higher than that of the purified enzyme.

TABLE I
Purification of dThd kinases

| Procedure                                     | Volume (ml) | Units/ml | nmol/min/mg | Yield (%) | Purification fold |
|-----------------------------------------------|-------------|----------|-------------|-----------|------------------|
| HS-I virus                                    |             |          |             |           |                  |
| 100,000 × g supernatant                       | 13          | 0.91     | 1.00        | 100       | —                |
| 1% Streptomycin sulfate supernatant           | 14          | 0.82     | 0.95       | 98        | 0.95             |
| 20 to 50% (NH₄)₂SO₄ fractionation             | 2           | 3.50     | 1.41       | 59        | 1.40             |
| Affinity column chromatography                | 3           | 1.60     | 67.2       | 41        | 67               |
| HS-II virus                                   |             |          |             |           |                  |
| 100,000 × g supernatant                       | 13          | 1.59     | 1.56       | 100       | —                |
| 1% Streptomycin sulfate supernatant           | 14          | 1.42     | 1.34       | 96        | 0.86             |
| 20 to 50% (NH₄)₂SO₄ fractionation             | 2           | 9.75     | 3.02       | 94        | 1.90             |
| Affinity column chromatography                | 3           | 3.50     | 192         | 55        | 1.25             |
kinase from HS-I virus-infected cells was different from that of the purified HS-I virus dThd kinase. Relative mobility with respect to bromphenol blue for either the crude or the purified HS-II virus dThd kinase was 0.42 and for the crude or the purified HS-I virus dThd kinase, relative mobilities were 0.45 and 0.70, respectively. When the purified HS-I virus-specific dThd kinase was incubated with the 50% ammonium sulfate precipitate from mock infected cells, the electrophoretic mobility of dThd kinase was shifted back to that of the unpurified or partially purified enzyme (ammonium sulfate fractionated); the activity of the enzyme was not affected.

**Glycerol Gradient Centrifugation**—The results of an experiment in which H3-amino-acid-labeled purified HS-I virus or HS-II virus dThd kinase preparations were layered on top of a 10 to 30% glycerol gradient and then centrifuged for 30 hours are depicted in Fig. 5. The radioactive H3 peak coincided with the enzymatic activity peak in both cases. The recovery of the activity of purified HS-I virus or HS-II virus dThd kinase after centrifugation was 25% and 98%, respectively. The sedimentation rate for both purified preparations was the same as that of the corresponding unpurified preparation, since 50 μM dThd used to stabilize dThd kinase during centrifugation is sufficient to inhibit the dCyd kinase activity in our assays, the activity of dCyd kinase was not measured after centrifugation.

**Heat Inactivation**—Purified HS-I virus and HS-II virus dThd kinase were incubated at 45°C in 0.2 M Tris-HCl buffer at pH 7.5 in the presence of 2 mM Mg2+ ATP, 3 mM dithiothreitol, 0.5 mg/ml of albumin, and 10% glycerol. The dThd kinase and dCyd kinase activities were measured at designated time intervals as indicated in Fig. 6A and B. The HS-I virus dThd kinase was more unstable than the HS-II virus dThd kinase under these conditions with, a half-life of 28 min and 120 min respectively (Fig. 6A). The rate of inactivation of dCyd kinase

![FIG. 4. Electrophoresis of dThd kinase. Method and assay were the same as that described by Lee and Cheng (16). The samples (25 μl with protein concentration and the specific activity as indicated in Table I) used were as follows: A, 100,000 × g supernatant of HS-I virus enzyme. B, affinity column-purified HS-I virus enzyme. C, 100,000 × g supernatant of HS-II virus enzyme. D, affinity column-purified HS-II virus enzyme.](http://www.jbc.org/)

![FIG. 5. Fractionation pattern of dThd kinase by glycerol gradient centrifugation. The glycerol density gradient centrifugation conditions were the same as those described by Dr. S. Kit's laboratory (4) except that the running time was 30 hours. Hemoglobin (Hb) and pyruvate kinase (PK) were used as markers. The dThd kinase samples (0.5 ml) layered on the top of each gradient were as follows: A, purified H3-labeled HS-I virus dThd kinase, 0.15 unit/ml. B, crude HS-I virus dThd kinase, 0.3 unit/ml. C, purified H3-labeled HS-II virus dThd kinase, 2.5 unit/ml. D, crude HS-II virus dThd kinase, 1 unit/ml.](http://www.jbc.org/)

![FIG. 6. Heat inactivation of dThd or dCyd kinase activity of the purified HS-I virus and HS-II virus dThd kinase. The purified HS-I virus enzyme (0.1 unit/ml) (O-O) or HS-II virus enzyme (0.2 unit/ml) (C-C) were incubated either with 0.2 M Tris-HCl buffer at pH 7.5 containing 10% glycerol, 2 mM Mg2+ ATP and 3 mM dithiothreitol at 45°C (A and B) or with 0.2 M Tris-HCl buffer at pH 7.5 containing 10% glycerol, 2 mM Mg2+ ATP, 3 mM dithiothreitol, and 100 μM dThd at 43°C (C). Protein concentrations were adjusted to 0.5 mg/ml by adding albumin. Samples were removed at the designated time intervals and assayed for dThd activity (A and C) or for dCyd kinase activity (B).](http://www.jbc.org/)
was the same as that of the corresponding dThd kinase (Fig. 6B). Inactivation of dThd or dCyd kinase by ammonium sulfate fractionation gave half-lives similar to those of the corresponding purified enzymes when heated at 45°C. When the purified preparations were stored under the same conditions at 4°C for 48 hours, there was no inactivation of HS-II virus dThd or dCyd kinase activity, but there was a greater than 50% loss of HS I virus dThd or dCyd kinase activities.

In contrast, when the stability of the purified enzymes was studied at 43°C in 0.2 M Tris-HCl buffer at pH 7.5 containing 10% glycerol, 0.5 mg/ml albumin, 3 mM dithiothreitol and 200 μM dThd, the HS-I virus dThd kinase activity was more stable than that of the HS-II virus enzyme as shown in Fig. 6C. Both HS-I virus and HS-II virus dThd kinase were stabilized at 43°C for at least 30 min when Mg2+ATP (2 mM) and dThd (100 μM) were present.

**Temperature Dependence of dThd Kinase Reaction**—Because both the HS-I virus and HS-II virus enzymes were stable under the assay conditions used in the temperature range of 27-43°C, these studies were performed within that temperature range. Linear Arrhenius plots are presented in Fig. 7. The activation energy was 17.2 kcal/mol for HS-I virus dThd kinase and 25.4 kcal/mol for the HS-II virus enzyme.

**Inhibition by Ammonium Sulfate**—Both purified HS-I virus and HS-II virus dThd kinase were inhibited by ammonium sulfate to the same extent (Fig. 8). The effect of sodium sulfate was the same as that of ammonium sulfate, whereas NaCl of the same ionic strength exerted much less of an inhibitory effect on both enzymes.

**DISCUSSION**

The basis for the appearance of virus-specific dThd kinase in the nuclei of HS-I virus-infected HeLa TK- cells and its absence in that of cells infected with HS-II virus is not known. Conceivably this may be due to different effects produced in the nuclear membrane by these viruses, or to differences in the structures of the two types of viral dThd kinase such that only one of these enzymes could penetrate the nuclear membrane. The physiological significance, if any, of the differences in the cellular distribution of this enzyme is not apparent. It should be noted that the dThd kinase from the nuclei of the HS-I virus-infected cells had the same mobility on electrophoresis as that from the cytoplasm of the HS-I virus-infected cells.3

Affinity column chromatography has been used in the purification of dThd from many sources such as mouse intestinal mucosa (17), cytoplasm and mitochondria of blast cells of acute myelogenous leukemia (16) and the cytoplasm of HS-I virus- and HS-II virus-infected cells. The buffer strength required for the elution of these enzymes from the affinity column is different. This could be attributed to factors such as affinity of the enzyme for the bound ligand.

HS-I virus-specific dThd kinase was purified 67-fold with 41% recovery and the HS-II virus-specific enzyme was purified 128-fold with 55% recovery. Due to the low protein content and the instability of the purified enzymes under various concentrating procedures it was uncertain whether they were homogeneous with respect to protein. Using radiolabeled protein from the cytosol fraction of virus-infected cells, a distinct radioactive peak was eluted from the affinity column which coincided with the dThd kinase activity peak. Such coincidence was also present in the glycerol gradient profile of both enzymes. This seems to suggest that the preparations obtained were reasonably pure.

The purified dThd kinase from both sources possessed dCyd kinase activity. Also, dThd and dCyd kinase activity could be inactivated at 45°C at the same rate. This supports the assumption that the virus-specific dThd kinase can catalyze dCyd phosphorylation, although the K_m of dThd was at least 20-fold lower than that of dCyd for both HS I virus and HS II virus-specific dThd kinase.

In the absence of substrates, HS-I virus and HS-II virus dThd kinase undergo inactivation very rapidly; however, in the presence of either dThd or Mg2+ATP each of these enzymes is stabilized (data not shown), indicating that either of these substrates can bind to the enzymes in the absence of the other. The effects of saturating amounts of either substrate on the stability of these kinases are shown in Fig. 6. It is suggested that Mg2+ATP is a better agent for stabilizing HS-II virus dThd kinase, whereas dThd is more effective for stabilizing HS-I virus dThd kinase.

The mobility of purified HS-I virus-specific dThd kinase on
polyacrylamide gel electrophoresis was different from that of
the unpurified preparation, whereas they had similar sedimenta-
tion rates in the glycerol gradient. When purified HS-I
virus-specific dThd kinase was incubated with the 50% ammo-
nium sulfate-precipitated fraction of mock infected cells, the
activity of the dThd kinase did not change but the electropho-
retic mobility of the dThd kinase was changed to that of the
unpurified or ammonium sulfate fractionated HS-I virus-
specific enzyme. Mg²⁺ATP, dThd or both did not change the
electrophoretic mobility of the purified preparation (data not
shown). This suggested that a host cell factor, precipitable by
50% ammonium sulfate, could influence the electrophoretic
mobility, but not the apparent molecular weight of the
enzyme; the mechanism for this effect is unknown. These
effects on the HS-I virus-specific dThd kinase were reversible.
The properties and physiological role of this host cell factor are
currently under investigation. Unlike the HS-I virus enzyme,
the unpurified and purified HS-II virus-specific dThd kinase
had similar electrophoretic mobilities and sedimentation rates
in the glycerol gradient.

In summary, each of HS-I virus and HS-II virus could induce
specific dThd kinase in cells. The induced enzymes differed
from each other in general properties as well as intracellular
distributions. The use of the newly developed affinity column
chromatography technique allowed us to purify the enzymes to
the extent that we were able to study the properties of the host
dThd kinase and those of the virus-induced enzymes. It is
hopeful that a comparative study of this nature could lead us to
developing specific antiviral agents.

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