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Altered Thymidine-Thymidylate Kinases from Strains of Herpes Simplex Virus with Modified Drug Sensitivities to Acyclovir and (*E*)-5-(2-Bromovinyl)-2′-deoxyuridine

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SUMMARY

Virus-coded thymidine (dThd) kinases were purified by affinity chromatography from a parental strain (SC16) and two strains (SC16 B3 and SC16 S1) of herpes simplex virus, Type 1, with altered drug sensitivities. These latter two strains were less sensitive, respectively, to *E*-5-(2-bromovinyl)-2′-deoxyuridine (BrVdUrd) and to both BrVdUrd and 9-(2-hydroxyethoxymethyl)guanine (acyclovir). The enzymes were characterized with respect to physical and catalytic properties. The enzyme from SC16 B3 was very similar to the parental enzyme except in its substrate specificity and kinetic constants. It catalyzed the phosphorylation of BrVdUrd at a relative rate that was 110% of the rate with dThd versus a relative rate of 140% with the parental enzyme. The apparent K_m value for BrVdUrd was 6 μM versus 0.1 μM for the parental enzyme. The reaction kinetics with acyclovir were similar for the two enzymes. The SC16 B3 enzyme catalyzed the phosphorylation of dTMP, but at only 2% the efficiency of the parental enzyme; phosphorylation of the monophosphate of BrVdUrd (BrVdUMP) was not detected with the SC16 B3 enzyme. The enzyme from the SC16 S1 variant had a much narrower phosphate acceptor specificity than the enzyme from the parental virus. BrVdUrd was a substrate but with a relative rate of 30% and an apparent K_m value of 4 μM; acyclovir was neither detectably phosphorylated nor a good inhibitor. BrVdUMP was not detectably phosphorylated. The relative efficiencies of the two variant enzymes for acyclovir phosphorylation correlated well with the sensitivities of the viruses to this compound. In contrast, the relative efficiencies of the second phosphorylation step (BrVdUMP to BrVdUDP) were most consistent with the sensitivities of the viruses to BrVdUrd.

INTRODUCTION

Thymidine kinase (EC 2.7.1.75) induced by HSV[1] (1) catalyzes the initial phosphorylation of the two antiviral compounds, acyclovir and BrVdUrd (2–4). These compounds are not efficiently phosphorylated in uninfected cells. Subsequent phosphorylation to the triphosphate can lead to greater inhibition of the viral than of the host DNA polymerase and to interference of DNA replication (5, 6).

Another possible enzymatic basis for selectivity involves the thymidylate phosphorylating activity that is associated with virus-coded dThd kinase. These two activities have been shown to belong to this bifunctional enzyme by the criteria of biospecific coelution from an affinity column, cosedimentation in glycerol density gradient centrifugation, and comigration during isoelectric focusing (7) (with coseparation into several components). Host cell dTMP kinase was well separated from viral dThd-dTMP kinase with all of these techniques. Inhibition studies indicated that dThd and dTMP were phosphorylated at the same or overlapping active sites (8). Furthermore, transformation of dThd kinase-deficient mouse cells (LMTK$^-$) to a TK$^+$ phenotype with UV-irradiated HSV-1 (or by a small DNA fragment containing the TK gene) produced a bifunctional dThd-dTMP kinase. Two HSV-1 mutants with a normal-size dThd kinase polypeptide but reduced activity and one with a changed size without activity produced corresponding changes in dTMP phosphorylating activities (9). The virus-coded dThd kinases purified from HSV-2 and varicella zoster virus also phosphorylated dTMP (10).

Previous data (10) indicated that the dThd-dTMP kinase specified by HSV-1 also catalyzed the phosphorylation of BrVdUMP to BrVdUDP. This product was not detected with the enzyme specified by HSV-2. This selectivity at the second step of BrVdUrd phosphorylation correlated well with the effectiveness of the analogue

[1] The abbreviations used are: HSV-1, herpes simplex virus, Type 1; acyclovir, 9-(2-hydroxyethoxymethyl)guanine; BrVdUrd, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; BrVdUMP and BrVdUDP, the 5′-monophosphate and 5′-diphosphate of BrVdUrd; acyclic, any substitution for the carbohydrate moiety of a nucleoside analogue which is not a cyclic group; HPLC, high-pressure liquid chromatography; PEI, polyethyleneimine.

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to inhibit HSV replication in cell culture (11). This locus of discrimination is not relevant with acyclovir, since the monophosphate of this antiviral compound is phosphorylated by a host cell kinase (2, 12).

Recently Darby *et al.* (13) described the selection *in vitro* of an HSV-1 strain which appeared to have a dThd kinase with an altered substrate specificity. The dThd kinase from this strain (SC16 S1) catalyzed the phosphorylation of dThd but not of acyclovir. Virus replication was resistant to acyclovir as well as to BrVdUrd (14). Another dThd kinase-positive strain (SC16 B3) was sensitive to acyclovir but not to BrVdUrd (15). The sensitivities of these viruses in cell culture to BrVdUrd were recently published [ED_{50} values = 0.03, 0.54, and 30 μM for the parental and mutants, respectively (16)].

In this study, the dThd kinases from these parental and resistant strains were purified and characterized. The substrate specificities were studied to determine whether any of a variety of acyclic nucleoside analogues would serve as a phosphate acceptor in spite of the loss of phosphorylation activity for acyclovir. In particular, we wanted to establish whether the dTMP-phosphorylating activity of the viral dThd-dTMP kinase plays a role in the altered sensitivities of the SC16 S1 and SC16 B3 strains to BrVdUrd.

EXPERIMENTAL PROCEDURES

Materials. Most materials were from the sources used previously (2, 10, 17). In addition, arabinosyluracil was obtained from Pfanstiehl (Waukegan, Ill.); Bio-Gel was from Bio-Rad Laboratories (Richmond, Calif.); Polybuffer exchanger (PBE 94) and Polybuffer 74 for chromatofocusing (see below) were from Pharmacia Fine Chemicals (Piscataway, N. J.); *Staphylococcus aureus* cells (Pansorbin) from Calbiochem (San Diego, Calif.); [2-^{14}C]thymine (54 Ci/mole) from New England Nuclear Corporation (Boston, Mass.); [*methyl*-^{3}H]dThd (80 Ci/mmole) from ICN (Irvine, Calif.); and [2-^{14}C]dTMP (60 Ci/mole) from Moravek Biochemicals (Brea, Calif.) or from ICN. Diaflo membranes were obtained from Amicon (Danverse, Mass.). Chemically synthesized BrVdUMP and BrVdUDP (for HPLC markers) were kindly provided by Dr. Erik De Clercq, Rega Institute (Leuven, Belgium). Creatine kinase (EC 2.7.3.2) and creatine phosphate were from Boehringer-Mannheim (Indianapolis, Ind.). Purified thymidine phosphorylase (EC 2.4.2.4) and uridine phosphorylase (EC 2.4.2.3) were generously provided by Dr. T. A. Krenitsky (18). The HSV-1 strains of virus, SC16, SC16 B3 (15), and SC16 S1 (13) were kindly provided by Dr. H. J. Field, Department of Pathology, Cambridge University (Cambridge, England). The mutant strains (SC16 B3 and SC16 S1) had been selected from serum-starved cells with drug pressure from BrVdUrd (SC16 B3) or acyclovir (SC16 S1) and subsequently plaque-purified. The viruses were grown in Vero cells (5).

dTMP and ATP were purified before use. The dTMP (P-L Biochemicals, Milwaukee, Wisc.) contained 0.7% dThd, which was substantially removed (<0.01%) with a reverse-phase column (Waters, μBondapack C18, 3.9 × 300 mm) equilibrated with water. For situations when an ATP-regenerating system (10) could not be used during the reaction, contaminating ADP (1–9%) was phosphorylated to ATP prior to use. Creatine kinase was removed by filtration thru a Diaflo, YM-10 filter after which the concentration of ADP was <0.02% of the ATP concentration (>99.9%) as determined by HPLC. In assays with a regenerating system for ATP, the concentration of ADP after a standard reaction was <0.01%.

To synthesize [2-^{14}C]arabinosylthymine, 1.4 mM [^{14}C]thymine, 4 mM KPi (pH 7.0),[2] 30 mM arabinosyluracil, and 60 IU of uridine phosphorylase (18) in a total of 1.3 ml were incubated for 40 hr at about 24°. Protein was removed and the solution was chromatographed on reverse-phase HPLC with 20 mM KPi (pH 7.0)/2% methanol. The purified material had the same retention time and UV characteristics as the commercial arabinosylthymine and was >98% homogeneous as judged by HPLC. The solution was lyophilized before use to remove the methanol.

The enzymatic synthesis of BrVdUMP was accomplished as described before (10), with some exceptions. The product was purified with a reverse-phase HPLC column equilibrated with 20 mM (NH_4)Pi (pH 5.8). Water was used to elute the BrVdUMP. This product was rechromatographed in water to remove salts and traces of BrVdUrd. One major symmetrical peak (>99%) and a small peak of BrVdUrd (0.01%) were observed with anion exchange HPLC. The product was identified as BrVdUMP as previously described (10). To test whether 0.01% BrVdUrd contamination would interfere with the assessment of BrVdUMP inhibition of dTMP phosphorylation (see Results), reactions with the SC16 dThd-dTMP kinase and 10 μM dTMP ± 0.02 μM BrVdUrd (corresponding to the contaminant from 200 μM BrVdUMP) were compared. No difference in reaction rates was observed. Presumably, the small amount of BrVdUrd that bound to the enzyme was rapidly phosphorylated to the much less inhibitory BrVdUMP.

Assays. Direct assays for nucleoside phosphorylation were performed at pH 7.5 (Tris·HCl) with radioactive phosphate acceptors. Thymidine (0.1 mM) and dCyd (1 mM) phosphorylations were measured with the standard DEAE-paper method as described previously (10). To determine K_m values of dThd, [*methyl*-^{3}H]dThd was substituted for [^{14}C]dThd. An identical procedure was used to measure arabinosylthymine phosphorylation, substituting 40 μM [2-^{14}C]arabinosylthymine (20 cpm/pmole) for dThd. Phosphorylation of dTMP was measured by a thin-layer chromatographic method with PEI cellulose (10) and that of acyclovir (to assay column fractions) by the DEAE-paper method (2). For K_m determinations, phosphorylation of acyclovir was measured by using styrene cation exchange (Bio-Rad AG50W-X8, 100–200 mesh) columns to separate product from substrate. This method resulted in less background interference and greater reproducibility than the DEAE-paper or PEI chromatography methods (2). Reaction mixtures were as for the standard assay, but [^{14}C]acyclovir (60 cpm/pmole) was substituted for dThd. Reactions were terminated by mixing a 15-μl aliquot with 0.5 ml of 0.1 M HCl. This was percolated through a cation exchange column (0.5 × 3 cm) equilibrated with 0.1 M HCl. Columns were rinsed with 5 ml of the acid and then with 1 ml of 30% methanol; finally, the acyclovir monophosphate was eluted with an additional 4 ml of 30% methanol and counted in 14 ml of Aquasol 2 (New England Nuclear Corporation). The acyclovir substrate remained adsorbed to the columns. Results with this method were consistent with PEI thin-layer chromatography (2) to within 10% for both rates of reactions and quantitation of substrates and products. There was a linear relationship between product formation and counts observed over at least a 900-fold range, and observed product formation was not affected by variation of ATP·Mg concentrations from 0.05 to 5 mM.

To verify the nonhyperbolic substrate saturation kinetics that were observed with dThd and acyclovir (SC16 and SC16 B3 enzymes only), the reaction velocities were determined with and without the ATP-regenerating system, with the DEAE-paper and the PEI chromatography (and cation exchanger for acyclovir) assays, at pH 7.5 and 6.0, with freshly desalted versus desalted and stored (−80°) enzyme (for dThd), with rechromatographed (HPLC, reverse-phase, water eluant) and commercially obtained [^{3}H]dThd, and by several different individuals.

Reaction velocities with nonradioactive phosphate acceptor substrates for the dThd kinases were determined by measuring the conversion of [α-^{32}P]ATP to [α-^{32}P]ADP as previously described (10). Control reactions (phosphate acceptor omitted) typically had an ATP hydrolysis rate of about 5% of the dThd phosphorylation rate. After the reactions, at least 99% of the radioactivity was associated with ADP and/or ATP (PEI chromatography).

In all velocity determinations except those depicted in Figs. 1 and 2, three- to five-point time courses were used. Product formation (less

[2] pH measurements were made at 24°.

than 20% substrate depletion) was a linear function of both time and enzyme concentration. The data from double-reciprocal plots of the reaction velocities versus the substrate concentrations, with or without inhibitor, were analyzed by least-squares fits of the data to hyperbolae with the aid of a computer (19). Secondary replots of the slopes versus inhibitor concentrations were analyzed by linear regression to determine K_i values. The analyses did not assume an inhibition model.

One unit of dThd-dTMP kinase catalyzes the formation of 1 pmole of dTMP per minute at 37° under the standard conditions described above. Protein concentration was estimated by the Coomassie brilliant blue dye method (20).

Immunoprecipitation procedures. Purified dThd kinases were incubated with several dilutions of serum from a rabbit immunized with purified HSV-1 (H29) dThd kinase (21). These mixtures (160 μl) were incubated at 24° for 40 min in buffer [15 mM Tris (pH 7.5), 4 mM ATP·Mg, 0.03 mM dThd, 1 mM dithiothreitol, and albumin (1 mg/ml)]. Control mixtures contained serum from preimmune rabbits. Antibodies and antigen-antibody complexes were precipitated by the addition of *S. aureus* cells. The supernatants were assayed for uncomplexed kinase activity as described above. With the dTMP-phosphorylating activities, dThd was omitted from the incubation buffer.

Enzyme separations. Cytosol extracts of infected cells (18 hr post-infection, 90–100% cytopathic effect) were prepared for affinity chromatography as described previously (2). The dThd-agarose column was rinsed with Buffer A, then sequentially eluted with 1 mM dTMP in Buffer A, Buffer A, Buffer B [same as Buffer A except with 800 mM Tris·HCl (pH 6.8)], and finally Buffer B containing 1 mM ATP, albumin (1 mg/ml), and a dThd gradient from 0 to 0.2 mM (for the SC16 and SC16 B3 enzymes) or from 0 to 0.6 mM (for the SC16 S1 enzyme). Recovery of dThd-phosphorylating activity was about 90% with the extracts from SC16- and SC16 B3-infected cells and about 70% with that from SC16 S1-infected cells. The dThd kinases were stable when stored at −80° in the eluant from the column. Before use, dThd and ATP were removed (<0.01 μM dThd and 1–2 μM ATP) from the enzyme preparations by gel filtration.

As a diagnostic procedure, enzyme activities were separated by chromatofocusing (22) at 4° with a column (0.5 × 26 cm) of exchanger (PBE 94) equilibrated with a buffered solution (pH 7.3) containing 25 mM imidazole·HCl, 2 mM dithiothreitol, 0.01 mM dThd, and 10% glycerol. After equilibration, 1 ml of polybuffer 74 (1:8 diluted) (pH 4.0) containing 2 mM dithiothreitol, 10 μM dThd, and 10% glycerol was loaded onto the column, followed by 1.5–2 ml of the above enzyme preparation (2,000–15,000 units of dThd-phosphorylating activity), which had been treated by gel filtration to exchange the dialysis buffer for equilibration buffer. The enzymes were eluted (5 ml/hr) with 80 ml of the above Polybuffer solution. Recovery of dThd-phosphorylating activity was 60–70% in each case.

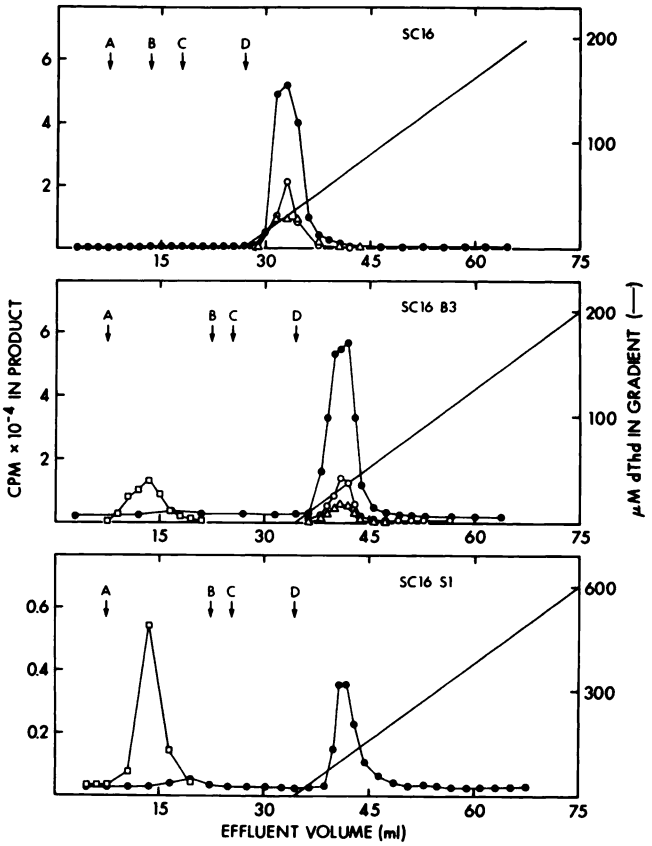

FIG. 1. *Profiles of kinase activities eluted from a dThd-agarose affinity chromatography column*

The samples added onto the column contained 8 × 10⁴, 8 × 10⁴, and 1.2 × 10⁴ units of dThd phosphorylating activity from cytosol fractions of cells infected with SC16, SC16 B3, and SC16 S1 viruses, respectively. Column fractions were assayed for phosphorylation of dThd (●), acyclovir (○), arabinosylthymine (△), and dTMP (□) by the direct radiochemical assays. The fractions had a final dilution of 1:2 in the reaction mixtures, and incubation times were 10 min except with the SC16 S1 fractions (45 min). Before being assayed for activities with acyclovir or arabinosylthymine, fractions were treated with thymidine phosphorylase (2) to remove the dThd. The column was eluted with low salt and dTMP at *arrow A*; low salt at *arrow B*; high salt at *arrow C*; and high salt with ATP, albumin, and a dThd gradient at *arrow D* (see Experimental Procedures).

RESULTS

Purification of the Virus-Coded dThd Kinases

Cytosol extracts of cells infected with the strains SC16, SC16 B3, and SC16 S1 contained about 3700, 3700, and 800 units of dThd phosphorylating activity per milligram of extracted protein, respectively. The virus-coded dThd kinase from each strain was purified by previously described methods of affinity chromatography (2, 7, 23) with a dThd-agarose matrix.

The virus-coded dThd kinases from both the parental strain (SC16) and the SC16 B3 strain of virus were eluted biospecifically in 30 μM dThd (Fig. 1). Activities for acyclovir and arabinosylthymine phosphorylation coeluted with that for dThd. Two activities for dTMP phosphorylation were observed. In agreement with earlier studies, host cell dTMP kinase was eluted with dTMP and was well separated from the virus-coded dThd-dTMP kinase (7, 10). This separation was also achieved with the enzymes from cells infected with the SC16 B3 strain of virus (Fig. 1, *middle panel*). Fractions containing this viral dThd-dTMP kinase catalyzed the phosphorylation of dTMP and dThd with a constant ratio of rates (0.17 ± 0.02 for the fractions from 38–44 ml). Furthermore, this activity for dTMP phosphorylation could be fully inhibited by dThd under conditions that did not inhibit the host cell dTMP kinase. No clear indication of host cell dThd kinase was detected during the purification of dThd-dTMP kinase from either strain of virus. This was not completely unexpected, since the infected cells showed almost complete cytopathic effects when harvested, and previous studies have shown that the amount of extractable cellular dThd kinase decreased with increased times of infection (2).

The enzyme from cells infected with strain SC16 S1 (Fig. 1, *bottom panel*) eluted in a concentration of dThd (100 μM) close to that expected for elution of the host

cell dThd kinase (2). Even though no detectable host cell dThd kinase was observed with the extracts from SC16 and SC16 B3 infections, with SC16 S1 it was necessary to verify that the purified enzyme was not contaminated with that of the host cell. Samples of extracts from uninfected cells and from cells infected with SC16 S1 were combined and chromatographed on an ion exchange column with a self-generating pH gradient. This technique (chromatofocusing; see Experimental Procedures) separates proteins according to their isoelectric points. Good separation of the host cell dThd kinase and the enzyme induced by SC16 S1 was achieved (Fig. 2A and B). When a portion of the extract used for purification of the virus-coded dThd kinase was analyzed by chromatofocusing (Fig. 2C), the extract was free of detectable host cell dThd kinase. Thus, the purified SC16 S1 enzyme from the dThd-agarose column would also be free of host cell activity. Further evidence of this assumption was that dTMP completely inhibited the SC16 S1 enzyme at concentrations that did not inhibit the cellular dThd kinase.

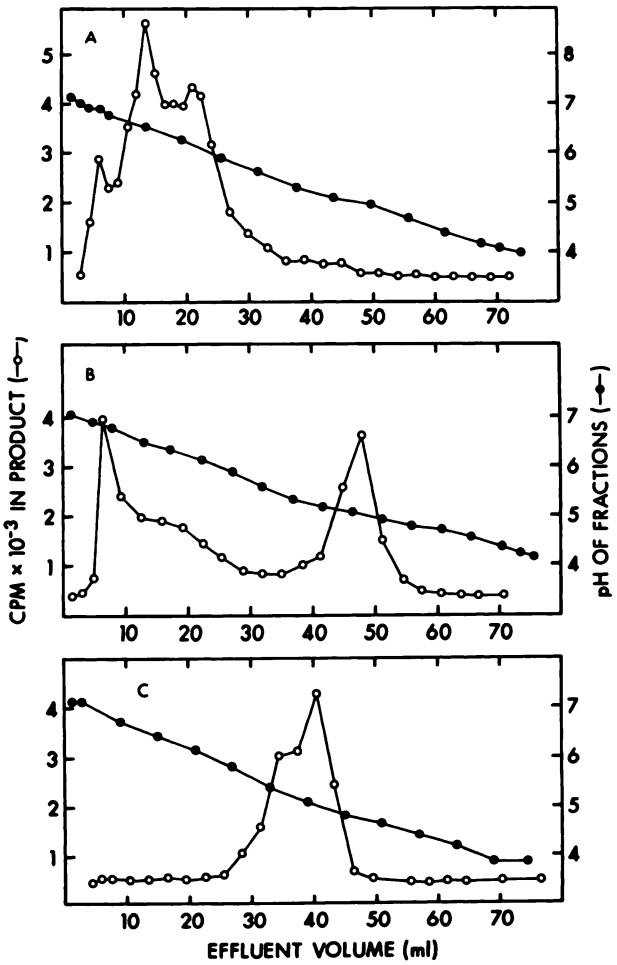

FIG. 2. *Activity profiles of dThd phosphorylation from fractions after chromatofocusing*

The samples chromatographed were from (A) a cytosol extract of uninfected Vero cells (6.5×10^3 units of dThd-phosphorylating activity), (B) combined extracts from uninfected cells (3.2×10^3 units) and cells infected with SC16 S1 virus (1.3×10^3 units), and (C) a cytosol extract of cells infected with SC16 S1 virus (2.7×10^3 units). Fractions were incubated for 60 min to assay for dThd phosphorylation as otherwise described in Fig. 1.

Characteristics of the Virus-Coded dThd Kinases

Isoelectric point similarities. Each enzyme was eluted from the chromatofocusing column (Fig. 2C and unreported data) within the range of isoelectric points reported previously for the HSV-1 kinase [pH 4.5–6.6 (7, 9)]. Each of the viral dThd kinases showed evidence of multiple forms. Chen and Prusoff (7) and Chen *et al.* (9) similarly observed multiple forms with isoelectric points from pH 4.5–6.6 after isoelectric focusing.

Enzyme stability. The SC16 S1 dThd kinase in cell extracts has previously been shown to be more heat-labile than the SC16 enzyme (24). The purified enzyme from SC16 B3 was also more heat-labile. The SC16 enzyme (8 units/μl) was incubated in Buffer A [20 mM Tris (pH 7.5) 10% glycerol, 3 mM dithiothreitol] at 40.5° and was inactivated with a half-life of about 50 min; that with the SC16 B3 enzyme (5 units/μl) was about 15 min.

Effects of salts on enzyme activity. The effect of anions on the activity of the dThd kinase from the SC16 B3 strain was not markedly different than that from the SC16 strain. However, large differences were observed with the enzyme from the SC16 S1 strain. For example, 250 mM potassium phosphate (pH 7.5) inhibited this enzyme by 99% but the SC16 enzyme by only 20%. Acetate, chloride, and nitrate were also more inhibitory.

Stimulation of dThd phosphorylation. Previous data by Chen *et al.* (8) showed that the phosphorylation of dThd with a HSV-1 dThd kinase was stimulated by the addition of dTMP when a saturating concentration of dThd (43 μM) was used. At a much lower concentration of dThd (1.5 μM), dTMP inhibited the phosphorylation. Similar results were seen with the SC16 and SC16 B3 enzymes, but quantitative differences were observed. With these enzymes, hyperbolic activation was observed throughout the dTMP concentration range. An apparent activation constant (concentration to give half-maximal activation) for dTMP was calculated to be 370 ± 30 (SE) μM for the SC16 enzyme and 400 ± 170 μM for the SC16 B3 enzyme. These values were substantially higher than that reported earlier (45 μM) for another HSV-1 enzyme (8). Since commercially available dTMP was contaminated with dThd (0.7%), in the present study the dThd was removed before use. Traces of dThd in dTMP could have caused the nonhyperbolic activation observed previously (8).

In contrast to the activation seen with the SC16 and SC16 B3 enzymes, the SC16 S1 enzyme was only inhibited by dTMP (e.g., 98% inhibition at 500 μM dTMP with 40 μM dThd).

Interaction of virus-coded dThd kinases with specific antiserum. The three purified enzymes and that from the H29 strain of HSV-1 were treated with antiserum raised against purified (H29) dThd kinase (21). No major differences in antigenic determinants were detected among the four enzymes. The dTMP-phosphorylating activities from the virus-induced dThd-dTMP kinases and from the host cell dTMP kinase were also measured after treatment with the antiserum. Although only 20% of the purified host cell (Vero cells) dTMP-phosphoryl-

ating activity was removed by the antiserum treatment, 94–>99% of the dTMP-phosphorylating activity associated with the virus-coded dThd kinase was removed.

Phosphate acceptor specificity of the dThd kinases. The purified enzymes were compared for their abilities to catalyze the phosphorylation of a variety of nonradioactive pyrimidine and purine derivatives by utilizing [α-^{32}P]ATP (see Experimental Procedures). In general, the enzyme from the SC16 B3 strain had a broad but different specificity as compared with that from the parental strain; the enzyme from the SC16 S1 strain had a much narrower specificity. The rates determined with common deoxynucleosides (1 mM) and expressed as the relative rates as compared with the rate of dThd phosphorylation (set at 100) were (SC16, SC16 B3, and SC16 S1, respectively): dUrd (44, 94, 10), dCyd (390, 175, <5), dGuo (7, 14, <5), and dAdo (30, 8, <5). All three enzymes catalyzed the phosphorylation of the thymidine analogue antiviral compounds, arabinosylthymine (32, 58, 60 in the same order as above), 5-trifluoromethyldeoxyuridine (140, 50, 50), and BrVdUrd (140, 130, 10). The SC16 B3 enzyme catalyzed the phosphorylation of some acyclic nucleoside analogues with faster relative rates than the parental enzyme, whereas none was detectably phosphorylated with the SC16 S1 enzyme (Table 1). Only the SC16 and SC16 B3 enzymes readily utilized dTMP (200, 58, <5). The SC16 B3 enzyme catalyzed this phosphorylation at a relative rate of less than one-third that of the parental strain. Some activity was detected with the SC16 S1 enzyme by using a direct radiochemical assay, but only at a level of 0.6% of the rate with dThd. This activity did appear to be associated with the viral dThd kinase, since it could be completely inhibited by dThd or partially inhibited to an extent equal to that calculated from its kinetic constants. In addition to dTMP, the rates with dCyd and arabinosylthymine were verified with [^{14}C] phosphate acceptors. The analogue of dTMP, BrVdUMP, was detectably phosphorylated only with the enzyme from the parental strain (40, <5, <5).

Direct detection of BrVdUMP phosphorylation. Because of the pharmacological importance of the reaction (10), verification of BrVdUMP phosphorylation by direct detection of BrVdUDP was attempted with the present enzymes. BrVdUMP was incubated with ATP·Mg and purified dThd kinases from the SC16, SC16 B3, and SC16 S1 strains of HSV-1. When the SC16 enzyme was used,

TABLE 1

Phosphorylation of acyclic nucleoside analogues with purified HSV-1 deoxythymidine kinases

Radioactive product ([α-^{32}P]ADP) was measured from reactions with 1 mM ATP·Mg, 1 mM phosphate acceptor, buffer, and enzyme. Details of the assay procedure are described under Experimental Procedures.

COMPOUND NUMBER	PHOSPHATE ACCEPTOR (1 mM)	RELATIVE PHOSPHORYLATION RATES[a] SC16	SC16 B3	SC16 S1
1	Guo–O–OH[b]	52	67	<5
2	Guo–O–(OH)	62	120	<5
3	"–O–OH	36	90	"
4	"–O–O–OH	8	9	"
5	"–O–(OH, CH_3)	28	54	"
6	"–O–(OH, OH)	20	58	"
7	"–O–(OH, CH_3)	6	<5	"
8	"–S–OH	24	50	"
9	"–OH	5	18	"
10	Thy–O–OH	20	<5	"
11	2-(SCH_3)-Hyp–O–OH	40	64	"

[a] The rate with dThd was arbitrarily set at 100. The ranges of replicate determinations were routinely within ±5 of the values shown.

[b] Relative rates with this compound were verified with a direct [^{14}C] acyclovir assay.

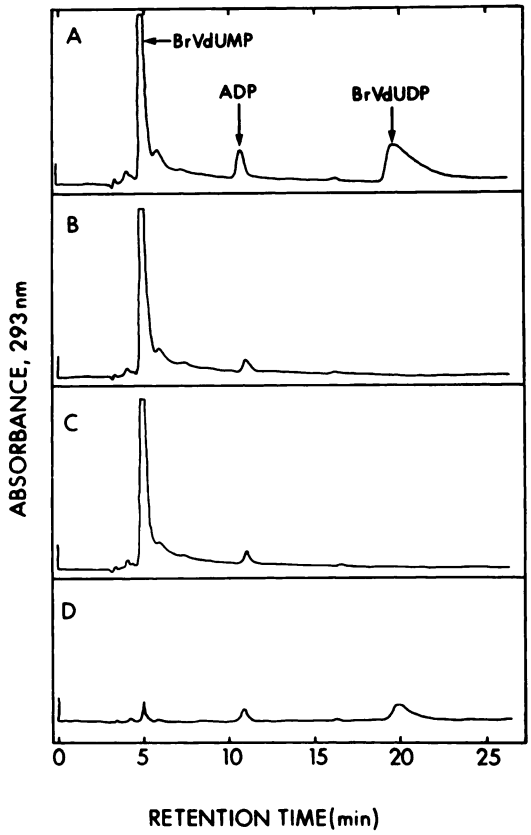

FIG. 3. *Chromatograms from HPLC analysis of reaction mixtures with BrVdUMP and purified viral dThd kinases*

A, The results with SC16 enzyme (1100 units/ml); B, with SC16 B3 enzyme (800 units/ml); C, with SC16 S1 enzyme (130 units/ml); and D, with chemically synthesized BrVdUDP substituted for BrVdUMP and enzyme. The reaction mixtures contained 0.4 mM enzymatically synthesized BrVdUMP,10 mM ATP·Mg, 75 mM Tris·HCl (pH 7.5), and albumin (0.2 mg/ml) and were incubated for 4 hr at 30°. Protein was removed by filtration (Diaflo, YM-10 membranes), and 0.1-ml samples were analyzed with anion exchange HPLC using 0.2 M KPi (pH 3.5) as the eluant. Product formation was quantitated by direct comparison of peak areas with that of chemically synthesized BrVdUDP. Each panel has the same relative ordinate dimensions.

about 50% of the monophosphate was converted to material that was eluted from HPLC with the same retention time as chemically synthesized BrVdUDP (Fig. 3A and D). The product was identified by spectral, chromatographic, kinetic, and enzymatic degradation means as described previously (10). No BrVdUDP formation was detected from chromatograms of incubations with the SC16 B3 or SC16 S1 enzymes (Fig. 3B and C). The detectable limits of BrVdUDP with these two enzymes were 0.4% and 3% of the amount formed with the SC16 enzyme (percentages were normalized to equal units of dThd phosphorylating activity). In control reactions without BrVdUMP, enzyme activity was retained over the 4-hr incubation period.

Kinetic constants. Kinetic constants for several of the substrates and related compounds were determined with the three enzymes (Table 2; Fig. 4). When radiolabeled phosphate acceptor substrates were not available or when the reaction velocities were very slow, apparent K_i values were determined instead of apparent K_m values.

With either the SC16 or SC16 B3 enzyme, the double-reciprocal plots ($1/v$ versus 1/[dThd]) were not linear at high concentrations of dThd (Fig. 4A and B). On the other hand, with the SC16 S1 enzyme, classical Michaelis-Menten kinetics were observed (Fig. 4C). The nonlinear kinetics were observed with several different assay procedures under many sets of conditions (see Experimental Procedures) with consistent results. The kinetics do not fit a substrate inhibition model (19). Since the nonlinear portion of the double-reciprocal plots were at concentratioins above the apparent K_m values for dThd, the values reported here (Table 2; Fig. 4G and H) are those extrapolated from the linear portion of the plots.

Substrate saturation kinetics with acyclovir were non-

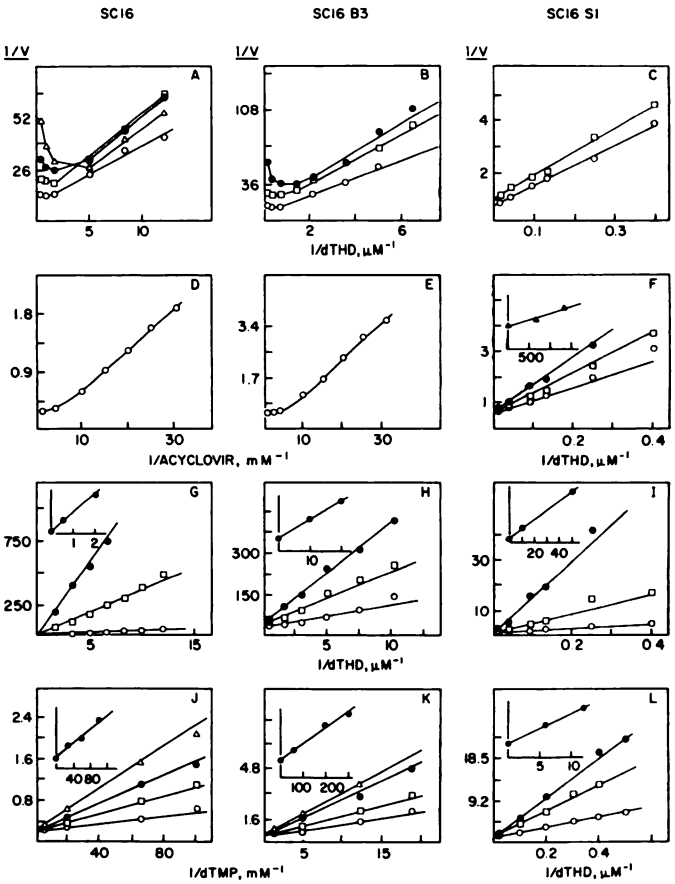

FIG. 4. *Double-reciprocal plots of velocities versus substrate concentrations*

Radiolabeled substrates were as indicated (○) with inhibitors in A, (□) 20 μM, (●) 50 μM, and (△) 100 μM ADP; B, (□) 30 μM and (●) 60 μM ADP; C, (□) 1500 μM ADP; F, (□) 660 μM and (●) 1330 μM acyclovir; G, (□) 0.5 μM and (●) 2 μM BrVdUrd; H, (□) 10 μM and (●) 20 μM BrVdUrd; I, (□) 10 μM and (●) 50 μM BrVdUrd; J, (□) 30 μM, (●) 60 μM, and (△) 100 μM BrVdUMP; K, (□) 60 μM, (●) 200 μM, and (△) 300 μM BrVdUMP; L, (□) 6 μM and (●) 12 μM BrVdUMP. Velocities are expressed as (picomoles per minute) × 10^{-2} per milliliter of reaction solution. Other details are described in Table 2. *Insets*, Replots of slopes versus concentrations of inhibitors.

TABLE 2

Kinetic constants of purified virus-coded dThd-dTMP kinases

The apparent K_m and K_i values were determined from the data shown in Fig. 4 except for the values for dTMP (with the SC16 S1 enzyme), ATP, and ADP. These latter two were determined with ATP·Mg as the varied substrate, with dThd as the nonvaried substrate (5 μM, 5 μM, and 60 μM with the SC16, SC16 B3, and SC16 S1 enzyme, respectively), and with ADP·Mg as the inhibitor. With the remainder of the determinations, 2 mM ATP·Mg was the nonvaried substrate. The K_i value for dTMP with the SC16 S1 enzyme was determined with dThd as the varied substrate (see Results). In all cases where K_i values are reported, the inhibition was competitive. The K_i values were from linear secondary plots of slope versus inhibitor concentration. Other details are described under Experimental Procedures.

Substrate or inhibitor	Apparent K_m or K_i values		
	SC16	SC16 B3	SC16 S1
	μM	μM	μM
dThd	0.4	0.6	10
BrVdUrd	0.1[a]	6[a]	4[a]
acyclovir	(100)[b]	(100)[b]	2000[a]
dTMP	20	300	5[a]
BrVdUMP	25[a]	140[a]	4[a]
ADP	1.5[a]	3[a]	800[a]
ATP	40	50	3000

[a] Determined as K_i values. In cases where the compound is a substrate (see Results), the value should equal the apparent K_m value (26).

[b] Not K_m values. These were the approximate concentrations of acyclovir at one-half the experimental maximal velocity.

hyperbolic functions with the enzymes from SC16 and SC16 B3 (Fig. 4D and E). Similar kinetics with HSV-1 dThd kinase have been reported previously (2, 17). Apparent substrate activation also seemed to occur, so concentrations of acyclovir for half-maximal velocities are reported rather than K_m values. These values were similar to those obtained in another laboratory by an apparent K_i determination method (25). The K_i value for acyclovir with the SC16 S1 enzyme obtained in the conventional manner (Fig. 4F) was about 10-fold the operational "K_m" values obtained with the other two enzymes.

Chen *et al.* (8) reported that the inhibition of dThd kinase by ADP·Mg versus dThd was nonlinear; the inhibition was a function of the saturation of the dThd kinase with dThd. This phenomenon was also observed here with the SC16 and SC16 B3 enzymes (Fig. 4A and B). This was in addition to the slightly nonlinear plots noted above without added ADP·Mg. The two characteristics do not seem to be directly related, since analyses

of reactions without added ADP (see Experimental Procedures) detected less than 0.2 μM ADP. In contrast to these observations with the SC16 S1 enzyme linear inhibition plots were obtained (Fig. 4C).

Apparent K_i values for ADP were determined using a single saturating concentration of dThd (Table 2). The substrate saturation kinetics with ATP were classical hyperbolic relationships, and the ADP inhibition was competitive with ATP. The K_i values were in the low micromolar range with the SC16 and SC16 B3 enzymes, but the K_i was 800 μM for the SC16 S1 enzyme. In each case the replots of slope versus inhibitor concentration were linear. No inhibition by 35 μM AMP (2 mM ATP·Mg) was observed (data not shown).

Notable differences between the kinetic constants for the SC16 and SC16 B3 enzymes were obtained with BrVdUrd, dTMP, and BrVdUMP as substrates or inhibitors (Fig. 4; Table 2). With the parental enzyme (SC16), the K_m values for BrVdUrd and dTMP were similar to those (0.24 and 13–25 μM) reported by other investigators (3, 7–9). The SC16 B3 enzyme had higher apparent K_m (or K_i) values than the SC16 enzyme. In contrast, using the SC16 S1 enzyme with dTMP or with BrVdUMP, both very poor substrates, the K_i values were considerably lower than the corresponding apparent K_m values with the SC16 enzyme. Since dTMP was a poor substrate for the SC16 S1 enzyme, the apparent K_m value was determined for dTMP as a competitive inhibitor of dThd phosphorylation [K_i value = apparent K_m value (26)]. Linear competitive inhibition was obtained (data not shown). The K_i value for BrVdUMP with the SC16 S1 enzyme was obtained (Fig. 4L) in a manner analogous to that for dTMP.

DISCUSSION

The virus-coded dThd kinases from two variants of the SC16 strain of HSV-1, one strain that was resistant to BrVdUrd (SC16 B3) and one that was less sensitive to both acyclovir and BrVdUrd (SC16 S1), were purified and characterized. Of the two, the SC16 S1 enzyme had the most striking differences from the parental strain enzyme. It was much more sensitive to anion inhibition, was eluted at a higher concentration of dThd from a dThd-agarose affinity column, was not stimulated by dTMP, had a much narrower substrate specificity, and had different kinetic constants. It is tempting to speculate that the lower apparent K_m value for dTMP and higher value for dThd may be related to its behavior on the dThd-agarose affinity column, since the ligand moiety is actually a dTMP moiety (albeit a 3′-phosphate). The substrate specificity of the SC16 S1 enzyme excluded all of the acyclic compounds and purine nucleosides as effective phosphate acceptors. It only weakly bound acyclovir and, even when partly saturated, did not detectably catalyze its phosphorylation. Phosphorylation of dCyd was not detected either. This coordinate relationship of activities with acyclovir and dCyd has been observed with vaccinia virus (1, 2) and several herpes viruses of nonhuman origin (27). Whether a structural correlation exists between these enzymes and the SC16 S1 enzyme is not known.

In contrast to the enzyme from the SC16 S1 enzyme, the SC16 B3 enzyme had an equally broad but quantitatively different specificity as compared with that of the parental enzyme. Acyclovir and several other analogues had higher relative phosphorylation rates with the SC16 B3 enzyme. Interestingly, and in contrast to the parental enzyme, the SC16 B3 enzyme would not accept an acyclic thymine derivative as a substrate (Table 1, Compound 10).[3]

The substrate saturation kinetics with the SC16 and SC16 B3 enzymes deviated slightly from a classical hyperbolic function with dThd and obviously so with acyclovir. These effects have been seen consistently with all of the HSV-1 enzymes from parental strains (2, 17) and with many different sets of assay condition (see Experimental Procedures), with different assay methods, and by several individuals. On the other hand, we observed classical hyperbolic kinetics with the SC16 S1 enzyme and with the virus-coded dThd kinase from varicella zoster virus[4] (10). We have no good explanation as to why the nonhyperbolic kinetics have not been noted by other workers. Recently, Cheng *et al.* (28) have reported nonhyperbolic kinetics with the virus-coded dThd kinases from several strains of HSV-1 with an acyclic guanosine analogue as substrate.

The basis for the resistance of the SC16 S1 strain of virus to acyclovir appears to be straightforward. Acyclovir was not detectably phosphorylated by the SC16 S1 dThd kinase, and this strain of virus was not sensitive to acyclovir. In contrast, acyclovir was phosphorylated by the enzyme from SC16 B3 (as well as the parental strain), and this strain of virus was fully sensitive to the drug[5] (15).

Two possible causes for the lower sensitivities of the SC16 B3 and SC16 S1 strains to BrVdUrd may be related to two enzyme activities needed to activate the drug. An examination of the relative efficiencies of the enzymes from each of the three virus strains for phosphorylation of the drug (relative velocity/apparent K_m) at each phosphorylation step suggests the following. The efficiencies calculated for BrVdUrd phosphorylation with the SC16, SC16 B3, and SC16 S1 enzymes (Results; and Table 2) were 1400, 18, and 8. Cells infected with the SC16 S1

[3] Coincident with the findings reported here were some ascertained by Larder *et al.* (25). Good agreement was found with regard to most of those physical and catalytic properties of the enzymes that were duplicated. The only major differences were with the value for the relative phosphorylation rate with dTMP (66% versus 0.6% reported here) for the SC16 S1 enzyme and the apparent K_m value for dTMP with the SC16 B3 enzyme (10 μM versus 300 μM reported here). The use of different extraction procedures, purification procedures, and assay pH values by the two research groups might account for some differences. In the present study, an extraction procedure to obtain the cytosol fraction, essentially a one-step purification procedure with stabilizers present, and assays near physiological pH were used. Care was taken to remove host cell dTMP kinase from the dThd-agarose column before eluting the virus-coded dThd-dTMP kinases. Selective precipitation of the virus-coded dTMP phosphorylating activities with antibody raised against the purified dThd kinase corroborated that these activities were associated with the virus-coded dThd kinase. Additionally, the virus-coded activity for dTMP was fully inhibited by dThd at concentrations consistent with the kinetic constants.

[4] J. A. Fyfe, unpublished results.

[5] P. A. Furman, personal communication.

virus induced less dThd-phosphorylating activity than did the parental strain (ref. 13; Results). To account for this difference, the efficiency values were normalized to 1400, 18, and 2. The sensitivities of the virus strains to BrVdUrd [ED_{50} values of 0.03, 30, and 0.54 μM (16)] do not correlate with these enzyme efficiencies.

For BrVdUMP phosphorylation, only the SC16 enzyme had a measurable relative rate (Results). From the direct detection of BrVdUMP phosphorylation (Results), it was calculated that the SC16 B3 and SC16 S1 enzymes had relative rates of <0.4% and <3% of the rate with the SC16 enzyme (i.e., relative rates of 40, <0.16, and <1.2 for the three enzymes). Using these rates and the values in Table 2, and normalizing as above, the calculated efficiencies were 1.6, <0.001, and <0.06. These are much less than those with BrVdUrd for all three enzymes, implying that BrVdUMP phosphorylation is more likely to be a rate-limiting step in triphosphate formation. In addition, these results are in qualitative agreement with the sensitivities to the drug. Since the phosphorylation of BrVdUMP was not detected with the SC16 B3 or SC16 S1 enzyme under the conditions that could be employed, complete quantitative correlations could not be made.

If the relative maximal rates of phosphorylation are the more pertinent values for comparison (e.g., when concentrations of substrates are greater than K_m values), the phosphorylation of BrVdUMP again correlated better with the drug sensitivity data. This situation is reminiscent of that with the enzymes from HSV-1 and HSV-2 and the susceptibilities of these viruses to BrVdUrd (10). Anabolism studies with cell cultures (4) supported the suggestion that the rate-limiting step was BrVdUMP phosphorylation. Similar studies with radiolabeled BrVdUrd and the virus strains studied here should help to establish whether one of the phosphorylation steps is rate-limiting and crucial for sensitivity to the drug.

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