Characterization of Abnormal Thymidine Kinases Induced by Drug-resistant Strains of Herpes Simplex Virus Type 1

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SUMMARY

Two TK+ acyclovir-resistant variants of herpes simplex virus (HSV) (S1 and Tr7) and one TK+ BVdU-resistant variant (B3) induce abnormal thymidine kinases with impaired ability to phosphorylate the drugs used in their isolation. These enzymes have been purified and their properties compared with those of the wild-type (wt) parent, SC16. The enzyme induced by S1 differed markedly from the other three in both its responses to salt and to pH. B3 TK recognized the enzyme's natural substrates, thymidine, deoxycytidine, dTMP and ATP as well as the wt enzyme. In contrast, Tr7 and S1 TKs failed to bind deoxycytidine and bind thymidine less well than wt. Tr7 and S1 TKs had affinities for dTMP similar to those of B3 and the wt enzymes. ATP binding to wt, Tr7 and B3 enzymes was similar but this substrate bound only weakly to S1 TK. Each mutant displayed a characteristically distinct pattern of affinities for a range of nucleoside analogue substrates, suggesting that they will show some cross-resistance to drugs which have a similar mechanism of action to acyclovir and BVdU.

INTRODUCTION

Several nucleoside analogues have recently been recognized as potent inhibitors of herpes simplex virus (HSV) replication (for review, see De Clercq, 1981), and two which are attracting considerable interest, acyclovir [ACV or 9-(2-hydroxyethoxymethyl)guanine] (Elion et al., 1977; Schaeffer et al., 1978) and BVdU [E-5(2-bromovinyl)-2'-deoxyuridine] (De Clercq et al., 1979) are now being used to treat herpes infections in humans (e.g. Brigden et al., 1981; De Clercq et al., 1980a). Each compound has to be activated to its triphosphate form in the infected cell and it is this form which disrupts virus DNA synthesis (Elion et al., 1977; Furman et al., 1979; Derse et al., 1981; Allaudeen et al., 1981). The enzyme, thymidine kinase (TK), which is encoded by the virus, has an important role in their activation since it is this enzyme which selectively converts each drug to its monophosphate (Fyfe et al., 1978; Cheng et al., 1981a). Virus variants which fail to induce TK (TK−) are resistant to these compounds (Coen & Schaffer, 1980; Schnipper & Crumpacker, 1980; Field et al., 1980; De Clercq et al., 1980b). However, although such variants are viable in tissue culture systems, many recent studies have shown them to be avirulent in animal models and they appear to establish latent infections only with difficulty (Field & Wildy, 1978; Field & Darby, 1980; Tenser & Dunstan, 1979; Tenser et al., 1979).

There appear to be two strategies by which HSV may acquire resistance to these compounds and at the same time retain pathogenicity, both strategies leading to retention of the TK+ phenotype. The first mechanism to be recognized is mutation of the DNA polymerase gene so that DNA replication becomes insensitive to inhibition by the analogue triphosphates (Coen & Schaffer, 1980; Schnipper & Crumpacker, 1980; Field et al., 1980; Furman et al., 1981). The second mechanism, recognized more recently, is mutation in the TK gene resulting in the induction of an enzyme with altered substrate specificity so that its ability to phosphorylate the...
drug is severely impaired whilst at the same time its ability to phosphorylate thymidine is retained (Darby et al., 1981; Larder & Darby, 1982).

Three drug-resistant mutants of HSV-1 recently isolated in this laboratory (Darby et al., 1981; Field & Neden, 1982; B. A. Larder, unpublished results) all have TK activity and retain pathogenicity. Studies with purified enzymes encoded by these variants indicate that they induce apparently normal DNA polymerases but that there are lesions in their TK genes which probably account for their drug resistance (Larder et al., 1983).

In this paper we describe the characterization of the TKs, purified by affinity column chromatography from mutant virus-infected TK− cells. We compare both physical and kinetic properties of these enzymes with purified TK induced by wt virus. We provide clear evidence that the mutant enzymes differ, not only from wt, but also from each other. The changes in TK are discussed in relation to the normal functions of the enzyme, and their likely implications for cross-resistance to other anti-herpesvirus drugs are considered.

**METHODS**

**Cells and tissue culture.** The cells used in this study were baby hamster kidney cells (BHK-21) and 5-bromo-2'-deoxyuridine (BUDR)-resistant BHK cells, which express no cellular TK (BU-BHK). Cells were maintained in Glasgow modified Eagle’s medium containing 10% newborn calf serum and 10% tryptose-phosphate broth.

**Viruses.** The parental HSV-1 strain, SC16 (Hill et al., 1975) and drug-resistant mutants made from it were used. The isolation of SC16 S1 (S1), an ACV-resistant strain, has been described by Darby et al. (1981). The BVdU-resistant mutant, SC16 B3 (B3) which was kindly provided by Dr H. J. Field, Department of Pathology, Cambridge, U.K., was isolated by passage of the parent virus (SC16) in BHK cells in the presence of 30 μM-BVdU (Field & Neden, 1982). SC16 Tr7 (Tr7), an ACV-resistant strain, was isolated from BHK TK− cells (BU-BHK cells biochemically transformed to the TK+ phenotype with cloned HSV-1 TK gene) infected with SC16 in the presence of 4 μM-ACV.

Viruses stocks were all cloned twice by plaque purification and grown in BHK cells using low multiplicity infections.

**Virus infection of BU-BHK cells.** Confluent monolayers of BU-BHK cells (about 2 × 10^6) were infected with either the parental (SC16) or drug-resistant strains of virus at 10 p.f.u./cell. After 18 h infection, cells were harvested into the medium, pelleted and frozen at −70 °C until required.

**Extraction and purification of virus thymidine kinase.** Infected cell pellets were thawed and resuspended in 4 vol. of extraction buffer containing 250 mm-KCl, 2 mm-dithiothreitol (DTT), 1 mm-EDTA, 1 mm-phenylmethylsulphonyl fluoride (PMSF), 50 μM-thymidine and 20% glycerol. Cells were disrupted by ultrasonic vibration and the sonicate was centrifuged at 15000 rev/min in a Sorvall RC 2B rotor. The supernatant was passed through two DEAE-cellulose columns, equilibrated with 250 mm- and 50 mm-phosphate buffer (pH 7-5) respectively. Fractions containing TK activity recovered in the flow-through from the second DEAE-cellulose column were pooled and solid ammonium sulphate (to 60%) was added to this material. The precipitate formed was dissolved in affinity column buffer A (0.05 M-Tris-HCl pH 7.5, 2 mM-DTT and 10% glycerol) and TK was purified by affinity column chromatography as described previously using a thymidine-Sepharose affinity matrix (Kowal & Markus, 1975; Cheng & Ostrander, 1976).

Enzymes were stored at −20 °C in 30% glycerol, 0.5 mg/ml bovine serum albumin (BSA) and 100 to 200 μM-thymidine. When stored in this way, TK activity remained stable for at least 5 months.

The TK preparations were desalted by Sephadex G-25 filtration prior to use.

**Enzyme assays.** TK was assayed essentially as described by Klemperer et al. (1967). However, the reaction mixture for the purified enzymes also contained 2 mM-DTT and 0.5 mg/ml BSA in addition to 0.02 M-sodium phosphate buffer pH 6, 5 mM-ATP, 5 mM-MgCl2 and 50 μM[^14]C]thymidine (1-6 μCi/ml). When thymidine was replaced by alternative substrates the following concentrations were used:[^14]C]deoxycytidine, 2 mM (3 μCi/ml) and[^14]C]thymidine-5'-monophosphate (dTMP), 100 μM (3 μCi/ml).

Reaction products from thymidylate kinase assays were separated from substrate by thin-layer chromatography on Polygram C el 300 PEI/UV_254 sheets (Macherey-Nagel & Co., F. R. G.), pre-spotted with unlabelled dTMP and thymidine-5'-diphosphate (dTDP) as markers. The chromatograms were developed in 0.5 M-LiCl, 2 M-acetic acid. The dTDP band was located, cut out and radioactivity determined by liquid scintillation counting.

A unit of TK activity is defined as the amount of enzyme catalysing the conversion of 1 pmol substrate/min at 37 °C under the standard reaction conditions described.

Radiolabelled substrates were obtained from Amersham International.

**Antiviral compounds and chemicals.** The following drugs were gifts: ACV from Dr G. B. Elion, Burroughs Wellcome Co., Research Triangle Park, N.C., U.S.A.; E-5(2-bromovinyl)-, E-5(2-chlorovinyl)- and E-5(2-
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iodovinyl)-2'-deoxyuridines and Z-5(2-bromovinyl)-2'-deoxyuridine from Dr E. De Clercq, Rega Institute for Medical Research, Katholieke Universiteit, Leuven, Belgium; 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) and 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) from Dr J. Fox, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, N.Y., U.S.A.; 1-β-D-arabinofuranosyl thymine (Ara-T) from Dr G. A. Gentry, Department of Microbiology, School of Medicine, University of Mississippi, Jackson, Mississippi, U.S.A.; E-5-propenyl-2'-deoxyuridine and 5-propyl-2'-deoxyuridine were prepared by Dr D. E. Bergstrom, Department of Chemistry, University of North Dakota, Grand Forks, N.D., U.S.A.

Other nucleosides and nucleotides. These were purchased from Sigma.

Isoelectric focusing. Isoelectric focusing was performed at 2 °C using an LKB 8101 column (110 ml) with 2.24% ampholine (pH range 3.5 to 10) in a 0 to 60% glycerol gradient. The gradient also contained 2 mM-DTT and 50 μM-thymidine. The column was run at 5 W constant power, with voltage not exceeding 1500 V, until the current had decreased to and stabilized at a minimum (about 20 h). Fractions (2 ml) were collected from the gradient, the pH was measured and a sample was removed from each to assay for TK activity using the standard assay described.

Protein determinations. Protein concentration was determined by the method of Bradford (1976).

RESULTS

ACV-resistant mutants, Tr7 and S1, and the BVdU-resistant mutant, B3, were all derived from the same wt parental strain, SC16. The TK induced by each mutant was purified, the final stage in the purification being thymidine affinity column chromatography. Fuller details of the purification procedure will be published elsewhere; however, it is relevant to point out that the final yields and specific activities of the purified wt and mutant enzymes (all between 1 × 10⁵ and 3.3 × 10⁶ units/mg protein) were very similar. The specific activities were also similar to those obtained previously with purified wt HSV-1 TK (Cheng & Ostrander, 1976). Representative purification data for SC16 are summarized in Table 1.

Our initial experiments were designed to investigate whether there were any major differences in the enzyme activities resulting in changes in their response to ionic strength or pH.

Effect of ionic strength on enzyme activity

The activities of the three mutant enzymes and wt were tested at various salt concentrations. The results are shown in Fig. 1. B3, Tr7 and wt TKs all showed maximum activity with 0.25 M-NaCl in the reaction mixture and their responses to increased salt concentration were very similar. The enzyme induced by S1 was quite different, showing maximum activity at very low salt concentrations and decreasing activity with increased salt.

Effect of pH on enzyme activity

The effect of varying the pH of the assay system on the amount of activity measured was assessed in the range pH 5.8 to 8.0. Once again, the enzyme induced by S1 was different from the other three. Increasing pH correlated with increasing activity in the case of S1 TK, whereas other enzymes were less active at higher pH (Fig. 2). The difference in pH dependence of the S1 TK activity suggested a possible alteration in a charged amino acid species at or affecting the active site of the enzyme. In fact, we had suggested earlier on the basis of studies with crude enzymes that S1 TK might differ in charge from the wt (Larder & Darby, 1982). This possibility was further investigated by use of isoelectric focusing.

Table 1. Purification of SC16 TK

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>Units/ml</th>
<th>Units/mg protein</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
<th>Total protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell sonicate</td>
<td>3-5</td>
<td>4 × 10⁴</td>
<td>2.4 × 10³</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>1st DEAE-cellulose</td>
<td>7-5</td>
<td>1.6 × 10⁴</td>
<td>4.2 × 10³</td>
<td>86</td>
<td>1.75</td>
<td>49</td>
</tr>
<tr>
<td>2nd DEAE-cellulose</td>
<td>40</td>
<td>3 × 10³</td>
<td>6 × 10⁴</td>
<td>86</td>
<td>25</td>
<td>3.4</td>
</tr>
<tr>
<td>Affinity column</td>
<td>6</td>
<td>3.5 × 10³</td>
<td>3.3 × 10⁵</td>
<td>15</td>
<td>137</td>
<td>0.1</td>
</tr>
</tbody>
</table>
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1. Effect of NaCl on the rate of thymidine phosphorylation. Activity of TK purified from SC16 and mutant virus-infected cells was measured at different concentrations of NaCl in the standard TK assay reaction mixture (described in Methods). Reaction velocities were measured and are expressed as a percentage of the velocity of the control to which no NaCl was added. ■, SC16; ○, S1; ○, B3; ▲, Tr7.

2. Effect of pH on the rate of thymidine phosphorylation. Activity of the purified TKs was measured over the pH range of 5-8 to 8.0. Thymidine phosphorylation rates were measured at different pH values of the reaction mixture, the pH being adjusted using a phosphate buffer system. TK activities are expressed as a percentage of the activity measured at pH 6. ■, SC16; ○, S1; ○, B3; ▲, Tr7.

Isoelectric focusing of S1 and wt TKs

It was hoped that we might demonstrate a difference in charge between S1 and wt TKs by isoelectric focusing, since the loss or gain of a charge on the mutant enzyme would have resulted in a change in the isoelectric point. In fact, the isoelectric points for the enzymes were not significantly different being 7.0 and 6.8 for SC16 and S1 TK respectively.

Phosphorylation of natural substrates

HSV-specific TK is a multi-functional enzyme which recognizes several different natural substrates. The enzyme is able to phosphorylate both pyrimidine deoxynucleosides (thymidine and deoxycytidine) and also the nucleoside monophosphate, dTMP (Kit & Dubbs, 1965; Klemperer et al., 1967; Jamieson et al., 1974; Jamieson & Subak-Sharpe, 1974; Chen & Prusoff, 1978; Chen et al., 1979). We firstly looked at the phosphorylation of radiolabelled substrates. High substrate concentrations were used and under these conditions it is possible to compare the phosphorylation rates of different substrates for a particular enzyme. The data from these experiments are shown in Table 2. In each case the rate of phosphorylation is compared to the phosphorylation rate for thymidine. The wt enzyme phosphorylated deoxycytidine at a similar rate to thymidine, although this was only true at high deoxycytidine concentration and may not reflect the situation in the infected cell. dTMP was phosphorylated at a rate double that at which thymidine was phosphorylated but once again higher concentrations were required to achieve this. Neither of the ACV-resistant mutants (S1 or Tr7) induced a TK with detectable deoxycytidine kinase activity, although B3 TK, like wt, phosphorylated deoxycytidine as well as thymidine. Data with dTMP were variable, both S1 and B3 TKs phosphorylating the nucleotide less well than thymidine, and Tr7 TK phosphorylating it more efficiently.

We next measured Michaelis-Menten constants (K_m values) using radiolabelled substrates, to provide an indication of binding affinity of the enzymes for these substrates. These data are shown in Fig. 3 and are also summarized in Table 2. In addition to the phosphate acceptors, we measured the K_m for ATP, the phosphate donor. Several interesting points emerged from these experiments. The TKs of the ACV-resistant mutants, S1 and Tr7, both had increased K_m values for thymidine (10 and 2.5 μM respectively), whereas that induced by B3 had a similar K_m (0.3 μM).
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Fig. 3. Reaction kinetics for natural substrates of thymidine kinase induced by SC16 (■), S1 (●), B3 (○) and Tr7 (▲). Initial reaction rates were measured at different concentrations of substrate, with saturating levels of ATP–Mg\(^{2+}\) (5 mM) when measuring thymidine, deoxycytidine and dTMP phosphorylation, and saturating levels of thymidine (150 μM) when ATP–Mg\(^{2+}\) \(K_m\) values were determined. Lineweaver–Burk plots (reciprocal of velocity versus reciprocal of substrate concentration) are shown for (a) thymidine, (b) deoxycytidine, (c) dTMP and (d) ATP–Mg\(^{2+}\) \(K_m\) determinations. The plots shown were constructed to illustrate differences in reaction kinetics between the different enzymes; however, \(K_m\) values (shown in Table 2) were calculated from this data redrawn on more appropriate scales. \(V = \) pmol substrate converted per min per ml enzyme.

Table 2. Relative phosphorylation rates and kinetic constants for natural substrates of TK

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SC16</th>
<th>S1</th>
<th>B3</th>
<th>Tr7</th>
<th>SC16</th>
<th>S1</th>
<th>B3</th>
<th>Tr7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0.2</td>
<td>10</td>
<td>0.3</td>
<td>2.5</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>103</td>
<td>NP</td>
<td>96</td>
<td>NP</td>
<td>500</td>
<td>NP</td>
<td>500</td>
<td>NP</td>
</tr>
<tr>
<td>dTMP</td>
<td>196</td>
<td>66</td>
<td>37</td>
<td>330</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>ATP–Mg(^{2+})</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20</td>
<td>1000</td>
<td>20</td>
<td>12</td>
</tr>
</tbody>
</table>

* Phosphorylation rates by purified TK were measured using the standard reaction mixture described in Methods, containing either thymidine (50 μM), deoxycytidine (2 mM) or dTMP (100 μM) as substrate. Reaction velocities are expressed as a percentage of the thymidine phosphorylating rate for each enzyme.

† \(K_m\) values were evaluated for thymidine, deoxycytidine, dTMP and ATP–Mg\(^{2+}\) from the Lineweaver–Burk plots, as described in the legend to Fig. 3.

‡ NP, No phosphorylation (phosphorylation rate < 1%); therefore, \(K_m\) values could not be evaluated.

Table 2. Relative phosphorylation rates and kinetic constants for natural substrates of TK

- Phosphorylation rates by purified TK were measured using the standard reaction mixture described in Methods, containing either thymidine (50 μM), deoxycytidine (2 mM) or dTMP (100 μM) as substrate. Reaction velocities are expressed as a percentage of the thymidine phosphorylating rate for each enzyme.
- \(K_m\) values were evaluated for thymidine, deoxycytidine, dTMP and ATP–Mg\(^{2+}\) from the Lineweaver–Burk plots, as described in the legend to Fig. 3.
- NP, No phosphorylation (phosphorylation rate < 1%); therefore, \(K_m\) values could not be evaluated.

The TK of B3 also had a similar \(K_m\) for deoxycytidine (500 μM) to that of the \(wt\) enzyme (500 μM). As no phosphorylation of deoxycytidine by S1 or Tr7 TKs could be detected, \(K_m\) values could not be determined. One other major difference between the enzymes was seen when the \(K_m\) values for ATP were measured. In this case B3 and Tr7 TKs were very similar (\(K_m\) values of 20 μM and 12 μM respectively) but S1 showed a 50-fold increase (\(K_m\) 1 mM) compared to \(wt\) (\(K_m\) 20 μM). Surprisingly, all enzymes had the same \(K_m\) for dTMP (10 μM), further confirmation that this substrate probably binds to a different site on the enzyme than
Table 3. \( K_i \) values for nucleoside analogues

<table>
<thead>
<tr>
<th>Nucleoside analogue</th>
<th>SC16</th>
<th>S1</th>
<th>B3</th>
<th>Tr7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine†</td>
<td>0-2</td>
<td>10(50)</td>
<td>0-3(1.5)</td>
<td>2-5(12)</td>
</tr>
<tr>
<td>ACV</td>
<td>200</td>
<td>2200(11)</td>
<td>225(1-1)</td>
<td>2500(12)</td>
</tr>
<tr>
<td>BVdU</td>
<td>0-10</td>
<td>5(50)</td>
<td>4-5(45)</td>
<td>1-5(15)</td>
</tr>
<tr>
<td>Ara-T</td>
<td>3-6</td>
<td>420(116)</td>
<td>6-6(1-8)</td>
<td>60(17)</td>
</tr>
<tr>
<td>IUdR</td>
<td>0-14</td>
<td>6(43)</td>
<td>1-1(7-8)</td>
<td>0-72(5-1)</td>
</tr>
<tr>
<td>FLAC</td>
<td>0-50</td>
<td>138(276)</td>
<td>2-9(5-8)</td>
<td>29(58)</td>
</tr>
<tr>
<td>FMAU</td>
<td>0-24</td>
<td>50(208)</td>
<td>1-3(5-4)</td>
<td>1-6(6-7)</td>
</tr>
</tbody>
</table>

* Purified TKs were used from wt virus- and mutant virus-infected cells. Initial reaction velocities of thymidine phosphorylation were measured in the presence and absence of fixed concentrations of inhibitor at a saturating concentration of ATP-Mg\(^{2+}\) (5 mM). \( K_i \) values were either evaluated from Lineweaver-Burk plots or by the procedure of Cheng & Prusoff (1973). Numbers in parentheses indicate the fold increase compared with the value for SC16.

† Thymidine \( K_m \) was included for comparison.

Table 4. \( K_i \) values for 5-substituted 2'-deoxyuridine analogues*

<table>
<thead>
<tr>
<th>Nucleoside analogue</th>
<th>SC16</th>
<th>B3</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-propyl-2'-deoxyuridine</td>
<td>0-2</td>
<td>1-1</td>
<td>5-5</td>
</tr>
<tr>
<td>E-5-propenyl-2'-deoxyuridine</td>
<td>0-064</td>
<td>1-5</td>
<td>23</td>
</tr>
<tr>
<td>E-5-chlorovinyl-2'-deoxyuridine</td>
<td>0-11</td>
<td>3-4</td>
<td>31</td>
</tr>
<tr>
<td>E-5-bromovinyl-2'-deoxyuridine</td>
<td>0-10</td>
<td>4-5</td>
<td>45</td>
</tr>
<tr>
<td>E-5-iodovinyl-2'-deoxyuridine</td>
<td>0-10</td>
<td>6-2</td>
<td>62</td>
</tr>
<tr>
<td>Z-5-bromovinyl-2'-deoxyuridine</td>
<td>0-86</td>
<td>10-2</td>
<td>12</td>
</tr>
</tbody>
</table>

* Purified TK from the wt virus (SC16) and the BVdU-resistant mutant, B3, was used. Inhibition of thymidine phosphorylation was determined by measuring initial reaction velocities in the presence and absence of fixed concentrations of inhibitor at a saturating level of ATP-Mg\(^{2+}\) (5 mM). \( K_i \) values were evaluated by the procedure of Cheng & Prusoff (1973).

that binding thymidine. Conflicting results have been obtained elsewhere with the TK induced by S1, since Dr J. A. Fyfe at Burroughs Wellcome (personal communication) has been unable to detect dTMP phosphorylating activity in purified preparations of this enzyme.

Inhibition studies using nucleoside analogues

We investigated the implications of these alterations in TK with respect to the recognition of other nucleoside analogue inhibitors of HSV. It was felt that such studies might throw light on possible patterns of cross-resistance. All analogues tested are known substrates of HSV TK (Cheng \textit{et al.}, 1981\textit{a, b}; Larder & Darby, 1982). The interactions of these analogues with TK were assessed by measuring the extent to which they inhibited the phosphorylation of thymidine. The \( K_i \) values for these compounds are shown in Table 3. The TKs of the ACV-resistant variants, S1 and Tr7, showed similar binding patterns, with increased \( K_i \) values (decreased binding affinities) for all analogues relative to the wt enzyme. The \( K_i \) values for ACV were very similar, but with all other analogues the increases were greater for S1 TK than for Tr7. In fact, with S1 TK the smallest increase in \( K_i \) was seen with ACV (11-fold), all other \( K_i \) values increasing by at least 40-fold.

The situation with TK induced by the BVdU-resistant mutant, B3, was different. There was a large decrease in affinity for BVdU (45-fold compared to wt) but the enzymes' affinities for ACV and Ara-T showed only minor changes. In addition to BVdU, significant increases in \( K_i \) were seen with IUdR (8-fold), FLAC (6-fold) and FMAU (5-fold).

Since the alteration in binding affinity of nucleoside analogues to B3 TK seemed to be somewhat more specific than the changes in S1 and Tr7 enzymes we thought it worthwhile to look at the affinity of this enzyme for a family of compounds related to BVdU. The affinities were compared with those for the wt enzyme (Table 4). The first notable feature is that these
compounds bound to \textit{wt} TK with similar affinities ($K_i$ values 0.06 to 0.2 $\mu M$). The only exception was the Z-5-bromo compound which bound more weakly ($K_i$ 0.9 $\mu M$). These results are consistent with earlier observations using TK purified from HSV-1-infected cells (Cheng, 1976; Cheng \textit{et al.,} 1980, 1981a). In each case, the binding of these compounds to the mutant enzyme was weaker and a clear pattern emerged. Both the absolute $K_i$ values and the differences in $K_i$ values between the mutant and \textit{wt} enzymes increased in magnitude as the size of the trans-substituted halogen on the 2-position increased. Smaller changes were seen with a methyl substituent at that position (E-5-propenyl-2'-deoxyuridine). The smallest change was seen with 5-propyl-2'-deoxyuridine. In this case, the double bond is removed and a methyl substituent is present on the 2-position.

\textbf{DISCUSSION}

Three TKs induced by drug-resistant mutants of HSV have been purified and their properties compared with those of the \textit{wt} enzyme. All the viruses are at least 100-fold less sensitive than \textit{wt} virus to the drug used in their selection (Darby \textit{et al.,} 1981; Field & Neden, 1982) as judged by their ED$_{50}$ values determined by plaque-reduction assay in BHK cells, and data will be presented elsewhere showing that these viruses induce apparently normal DNA polymerases. A consequence of their TK$^+$ phenotype is that they remain pathogenic in animal model systems and are also capable of establishing latent infections (Darby \textit{et al.,} 1981; Field & Neden, 1982).

Experiments designed to assess the response of these enzymes to changes in salt concentration and pH revealed the TKs induced by Tr7 and B3 to be very similar to that of \textit{wt}. S1 TK, in contrast, behaved differently, showing sensitivity to increased salt concentration and an increase in activity with increased pH. Although we felt that the changes in the S1 enzyme might best be explained by an amino acid substitution at the active site thus affecting the charge on the protein, we could detect no significant difference in the isoelectric points of S1 and \textit{wt} TKs. The isoelectric point for \textit{wt} (pI 7.0) is close to values reported previously for HSV TK from crude infected extracts (Leung \textit{et al.,} 1975) but in contrast to other work using purified TK where broad, multicomponent peaks were seen (Chen & Prusoff, 1978) we observed a single peak of enzyme activity.

Since TK is a multifunctional enzyme it was of considerable interest to investigate the ability of each enzyme to perform its 'normal' functions. The TK from the mutant B3 was competent in this respect. The \textit{K}_m values for thymidine, deoxycytidine and dTMP were very similar to those of the \textit{wt} enzyme, indicating similar affinities for these substrates and apart from a significant decrease in the relative rate of phosphorylation of dTMP little difference could be seen between this enzyme and the \textit{wt}. In view of these results it would be expected that the biological properties of B3 would be very similar to those of the \textit{wt}, and preliminary results in this laboratory support this conclusion (Field & Neden, 1982).

A different pattern was seen with the ACV-resistant mutants Tr7 and S1. Most striking was the failure of either enzyme to phosphorylate deoxycytidine. However, the \textit{K}_m of the \textit{wt} TK for deoxycytidine (500 $\mu M$) suggests that this nucleoside is a poorer substrate for the enzyme than thymidine or any of the analogues tested. This may indicate that the ability of \textit{wt} TK to phosphorylate deoxycytidine is purely fortuitous, a result of the broad spectrum of substrate recognition inherent in the structure of the enzyme. Although the relative rates of phosphorylation of thymidine and deoxycytidine were similar, these experiments were carried out in substrate excess, 50 $\mu M$ in the case of thymidine but 2 mM for deoxycytidine. In view of the difference in concentrations of substrates required to achieve these rates it is unlikely that similar rates would be achieved \textit{in vivo} where the concentration of deoxycytidine would almost certainly be considerably lower than the \textit{K}_m. However, these arguments may be spurious. It is interesting that the only major discrepancy in kinetic values obtained using purified \textit{wt} TK rather than crude infected cell extracts was in the \textit{K}_m for deoxycytidine. The value of 500 $\mu M$ is considerably higher than that measured when using crude infected cell extracts (\textit{K}_m 20 $\mu M$) (Larder & Darby, 1982). This anomaly, which is consistent with observations reported by others (Jamieson & Subak-Sharpe, 1974; Chen & Prusoff, 1979), suggests that purification results in a decrease in the affinity of the enzyme for this substrate. In view of these differences in \textit{K}_m values
observed using crude and purified TK preparations, the significance of the deoxycytidine kinase activity in vivo must remain in some doubt.

The failure of Tr7 and S1 TKs to phosphorylate deoxycytidine was probably a result of the general decrease in affinities of these enzymes for nucleoside substrates. [It is worth noting in this context that we have been unable to demonstrate phosphorylation of deoxycytidine using crude enzymes where stronger binding might be expected (Larder & Darby, 1982).] When a wide range of analogues were tested with these enzymes each showed decreased affinity compared to wt, the changes apparently reflecting the changes in affinity for thymidine. The correlation was not absolute but a clear trend emerged with S1, showing lower affinity than Tr7 in all cases. The only exception to this rule was with ACV binding where the affinities were similar. It is attractive to speculate on the basis of these kinetic data that S1 and Tr7 will show a degree of cross-resistance to all nucleoside analogues whose selective antiviral activity is dependent upon phosphorylation by HSV TK. This possibility is currently being investigated.

We should not conclude on the basis of the data obtained with S1 and Tr7 TKs that all TK mutants exhibiting a TK+ phenotype will exhibit the pattern of resistance suggested by these variants. In fact, data obtained with B3 argue against this idea. Although B3 TK has a considerably reduced affinity for BVdU its affinity for ACV is unchanged and its affinity for Ara-T is only 2-fold lower than that of the wt virus. In fact, B3 has already been shown to be sensitive to ACV both in vitro and in vivo in the mouse (Field & Neden, 1982).

The more subtle changes in TK observed with B3 prompted us to look at the affinity of this enzyme for a number of compounds related to BVdU. In a series of experiments using 2-substituted vinyl compounds where the bromine atom of BVdU was replaced by other substituents, a clear pattern emerged. All compounds had similar affinity for the wt enzyme. However, the decrease in affinity of the mutant TK relative to that of wt enzyme for each substrate depended on the nature of the substituent. Where the halogen was replaced by a methyl group the decrease was 23-fold. Greater decreases in affinity were observed with Cl, Br or I substituents, the decrease increasing in magnitude as the size of the halogen atom was increased. It therefore seems possible that the change in this enzyme has a specific effect on the topology of the active site affecting entry of these substrates, the magnitude of the effect depending on both the size and electronegativity of the substituent.

In conclusion, we have described three TK mutants of HSV-1 which all exhibit a TK+ phenotype. They were selected for resistance to either ACV or BVdU and the resistance appeared in each case to be due to impaired ability to phosphorylate the appropriate drug. Each variant exhibits a characteristically distinct pattern of recognition of natural substrates and nucleoside analogues. Since they combine drug resistance with pathogenicity and ability to establish latent infections they may be the first representatives of a large and important group of HSV mutants.

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