Kinetic studies of the predicted substrate-binding site of varicella-zoster virus thymidine kinase

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To investigate the mechanism of kinetic action and substrate recognition of varicella-zoster virus (VZV) thymidine kinase (TK), we designed and isolated a site-directed mutant VZV TK which has double amino acid substitutions, 136threonine to leucine and 137isoleucine to leucine (SDM TK). This mutant was designed to alter the substrate-binding site of the VZV TK to duplicate that of the herpes simplex virus type 2 enzyme. Kinetic studies of the activity of wild-type TK indicated that the binding order of ATP and thymidine is random and that wild-type VZV TK possessed high thymidylate kinase (TM-K) activity. The sensitivity of VZV TK to bisubstrate analogues, dinucleotides of adenosine and thymidine, showed that the optimum distance between the ATP- and substrate-binding sites is two phosphoryl groups greater than with the natural substrate for TK activity. SDM TK lost deoxycytidine kinase activity and had reduced TK and TM-K activities. Inhibition studies on both WT and SDM TK by 5-halogenovinyluridine analogues and their 5' monophosphate derivatives revealed that amino acids at positions 136 and 137 are involved in substrate binding, probably through a role in the formation of the binding pocket for bulky substrates.

Introduction

Thymidine kinase (TK) is one of the enzymes induced by herpesviruses during lytic infection. Several studies have been carried out on the enzyme because of its unique characteristics, differing significantly from TK in mammalian cells and in other DNA viruses. For example, herpesvirus TKs have multi-enzyme activities in one polypeptide (Hay et al., 1971; Chen & Prusoff, 1978; Kit, 1985; Veerisetty et al., 1990). The broad range of phosphorylation substrates recognized by herpesvirus TKs provides a basis for the specific antiviral action of nucleoside analogues such as acyclovir (ACV) (Fyfe et al., 1978).

Previous studies of the sequences of herpesvirus TKs showed various degrees of similarity between them, demonstrated their evolution and divergence, and aroused interest in the relationship between their structure and function (Gentry et al., 1983; Honess et al., 1989; Harrison et al., 1991).

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) TKs are very similar polypeptides with 79.0% of the amino acids of HSV-1 TK identical to those of HSV-2 TK. Varicella-zoster virus (VZV) TK has only limited similarity to HSV-1 and HSV-2 TKs (identity of 28.6 and 29.3%, respectively), but highly conserved primary structures are observed in some regions, especially in the predicted ATP- and substrate-binding sites (Honess et al., 1989; Balasubramaniam et al., 1990). However, significant differences in the enzymes' properties have been reported in these TKs, particularly in their affinities for 5-halogenovinyluridine analogues and their thymidylate kinase (TM-K) activities (Cheng et al., 1981; Fyfe, 1982; Ayisi et al., 1987; Suzutani et al., 1988b; Yokota et al., 1989). Therefore, continued investigation of the relationship between the structure and function of these enzymes and those of other herpesviruses is important in order to understand the enzymology and the evolutionary relationship of cell and virus TKs, and to develop anti-herpesvirus chemotherapy.

In this study, to investigate the mechanism of kinase action and the origin of substrate specificity of VZV TK, we isolated a site-directed mutant (SDM) VZV TK which has an amino acid sequence identical to part of the predicted substrate-binding site of HSV-2 TK. Wild-type (wt) and SDM VZV TKs were expressed in the TK-deficient Escherichia coli C600 strain and were partially purified. Kinetic studies were then performed on the TKs to investigate the effect of the mutation on catalytic activity and substrate specificity.

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Methods

Materials and chemicals. Arabinofuranosylthymine (araT), 1-β-D-arabinofuranosyl-E-5-(2-chlorovinyl)uracil (CV-araU), 1-β-D-arabinofuranosyl-E-5-(2-bromovinyl)uracil (BV-araU), 1-β-D-arabinofuranosyl-E-5-(2-iodovinyl)uracil (IV-araU), E-5-(2-bromovinyl)-2″-deoxyuridine (BV-dU) and their 5′-monophosphate derivatives (araTMP, CV-araUMP, BV-araUMP, IV-araUMP and BV-dUMP, furaTMP, E-5-(2-bromovinyl)-2″-deoxyuridine (BV-dU) and their 5′-monophosphate derivatives (araTMP, CV-araUMP, BV-araUMP, IV-araUMP and BV-dUMP, respectively) were generously provided by H. Machida of the Yamasa Shoyu Co. Ltd, Choshi, Japan. ACV was a gift from S. F. Lacey, Wellcome Research Laboratories, Beckenham, U.K. Dinucleotides of adenosine and thymidine, P1-(adenosine-5′)-P1-(thymidine-5′)-triphosphate (Ap4T), P1-(adenosine-5′)-P4-(thymidine-5′)-tetraphosphate (Ap4P), P1-(adenosine-5′)-P5-(thymidine-5′)-pentaphosphate (Ap5P), P1-(adenosine-5′)-P6-(thymidine-5′)-hexaphosphate (Ap6P), P1-(adenosine-5′)-P7-(thymidine-5′)-heptaphosphate (Ap7P), P1-(adenosine-5′)-P8-(thymidine-5′)-octaphosphate (Ap8P) were synthesized as described previously (Davies et al., 1988). [3H]Thymidine (84 Ci/mmol) and [3H]deoxyctydine (23 Ci/mmol) were obtained from Amersham. [Methyl-3H]Thymidine monophosphate (methyl-[3H]TMP) and [5-3H]deoxyctydine monophosphate ([5-3H]dCMP were purified from the phosphorylation products of [methyl-3H]thymidine or [5-3H]deoxyctydine by VZV TK using DEAE-Sephadex A-25 chromatography (Pharmacia) as described previously (Karlstrom & Gronowitz, 1987). [methyl-3H]TMP and [5-3H]dCMP were separated by thin-layer chromatography. Other nucleotides and compounds were purchased from Sigma.

Protein-expressing system (pT7-7, mGPI-2 system) were described previously (Tabor & Richardson, 1985; Lacey et al., 1991).

Site-directed mutagenesis. A mutant VZV TK was derived by mutagenesis of pT7-7VZTK using PCR. PCR was performed using 200 ng pT7-7VZTK as the template and 1 µM-SN1 and -SN2 primers of TK (Cheng et al., 1985) but with several modifications. The crude enzyme solution was loaded onto a Sephadex G-150 (Pharmacia) column equilibrated with TMG buffer and then eluted with the same buffer. The eluate peak fractions of TKs, monitored by TK assay, were applied to a Mono Q column (Pharmacia) and were eluted with TMG buffer with a concentration gradient of 0 to 0.5 M-NaCl. The fractions containing TK activity were applied to a Blue Sepharose (Pharmacia) column, which was then washed extensively with 0.4 M-Tris-HCl pH 7.5, and the enzyme was eluted with 1.5 M-Tris-HCl pH 7.5 containing 300 µM-thymidine (dT). After dialysis against TMG buffer for 3 days to remove dT from the purified enzyme solution, VZV TKs were stored at -80 °C.

Results and Discussion

Isolation and purification of wild-type and site-directed mutant of varicella-zoster virus thymidine kinase

As a result of analysis of the sequences of drug-resistant HSV-1 TKs and their similarity to HSV-2 TK, Darby et al. (1986) proposed that some residues from positions 161 to 193 of HSV-1 TK contribute to the substrate-binding site for dT, corresponding to the region between residues 128 to 160 of VZV TK. They also reported that the presence of amino acids with larger side-chains at position 168 of HSV-1 TK, instead of alanine, lead to a decrease in the affinity for BV-dU. The alignment of this region shows that VZV TK has a similar structure at the substrate-binding site (Fig. 1), but has significantly more affinity for BV-dU and 5-halogenovinylarabinofuranosyluracils, i.e. BV-araU and IV-araU, than does HSV-2 TK (Cheng et al., 1981; Yokota et al., 1989). In order to analyse the functions of this region in binding nucleosides and nucleoside 5′-monophosphates and in the kinetics of VZV TK reactions, we designed and cloned an SDM TK (pT7-7SDM-VZTK) expressing a site-directed single amino acid substitution, la6Thr to Leu, into the pT7-7 expression plasmid using PCR. This mutant TK has an identical sequence to HSV-1 TK except for the single substitution, which was then washed extensively with 0.4 M-Tris-HCl pH 7.5, and the enzyme was eluted with 1.5 M-Tris-HCl pH 7.5 containing 300 µM-thymidine (dT). After dialysis against TMG buffer for 3 days to remove dT from the purified enzyme solution, VZV TKs were stored at -80 °C.

The enzymatic activities of the wt and SDM TKs were determined after purification (Table 1). The SDM TK lost dCK activity and had reduced TK and TM-K activities, which were 41.9 % and 11.8 % those of wt TK, respectively. For the separation of [methyl-3H]TMP indicated in the Tables and Fig. 2. The products of the enzyme reaction were separated by adsorption onto DE81 discs (Whatman) or by PEI-cellulose thin-layer chromatography (Macherey-Nagel) and radioactivity was measured in a Beckman scintillation counter as described previously (Suzutani et al., 1988a).

Purification of VZV TKs. The pellets of bacteria expressing VZV TKs were lysed in 25 mm-Tris-HCl pH 7.5, 50 mm-glucose, 5 mm-2-mercaptoethanol and 0.5 % Nonidet P40 by three cycles of freezing and thawing. The disrupted cells were centrifuged at 100 000 g for 1 h and the supernatants were used as the crude enzyme solutions. The purification of VZV TKs was performed generally as described by Shirai et al. (1985) but with several modifications. The crude enzyme extracts were fractionated using ammonium sulphate precipitation by addition first to 20 % and then to 50 % saturation (Cheng et al., 1979). The precipitates were dissolved in TMG buffer (20 mm-Tris-HCl pH 8.0, 3 mm-2-mercaptoethanol, 10 % glycerol). This solution was loaded onto a Sephadex G-150 (Pharmacia) column eluted with TMG buffer and then eluted with the same buffer. The eluate peak fractions of TKs, monitored by TK assay, were applied to a Mono Q column (Pharmacia) and were eluted with TMG buffer with a concentration gradient of 0 to 0.3 M-NaCl. The fractions containing TK activity were applied to a Blue Sepharose (Pharmacia) column, which was then washed extensively with 0.45 M-Tris-HCl pH 7.5, and the enzyme was eluted with 1.5 M-Tris-HCl pH 7.5 containing 300 µM-thymidine (dT). After dialysis against TMG buffer for 3 days to remove dT from the purified enzyme solution, VZV TKs were stored at -80 °C.
Kinetic studies of VZV TK

Kinetic studies of wild-type varicella-zoster virus thymidine kinase

To understand the mechanism of the enzymatic reactions of VZV TK, kinetic studies were carried out on purified wt TK. Double reciprocal plots of substrate concentration against the initial velocity for TK activity are shown in Fig. 2. Under the experimental conditions both dT and ATP behaved as Michaelis-Menten substrates throughout the concentration range studied. Kinetic constants, $K_m^{ATP}$, $K_m^{dT}$, $K_m^{dCK}$ and $K_m^{dCMP-K}$ were 15.2, 17.9, 0.617 and 0.700 μM, respectively.

$K_m^{ATP} \times K_m^{dT}$ and $K_m^{dCK} \times K_m^{dCMP-K}$ were 10.6 and 11.0, respectively.

Table 1. Enzymatic activity of purified wild-type and mutant VZV TKs

<table>
<thead>
<tr>
<th>Activity</th>
<th>Wt</th>
<th>SDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK</td>
<td>0.43*</td>
<td>0.18</td>
</tr>
<tr>
<td>TM-K</td>
<td>8.88</td>
<td>1.05</td>
</tr>
<tr>
<td>dCK</td>
<td>0.16</td>
<td>ND</td>
</tr>
<tr>
<td>dCMP-K</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* All values are given in pmol of product/μg protein/min.
† ND, Not detected. Upper limit for the detection level of dCK and dCMP-K activities was 0.08 pmol of product/μg protein/min.

using DEAE–Sephadex chromatography a gradient of ammonium carbonate was employed so contamination of the TMP solution for the TM-K assay with ammonium carbonate cannot be ruled out. Consequently, TM-K activity of the VZV enzyme could not be directly compared to the TK activity because we observed that VZV TK activity was enhanced about 1.5-fold in 500 mM-ammonium carbonate (data not shown). However, very high TM-K activity was observed in wt VZV TK. dCMP kinase (dCMP-K) activity was not detected in wt VZV TK.

Site-directed mutagenesis

Consensus sequence for nucleotide binding

Fig. 1. Alignment of the amino acid sequences for the predicted substrate-binding site of HSV-1, HSV-2 and VZV TKs and the sequence of the SDM VZV TK. The portion similar to the consensus sequence for nucleotide binding is indicated (Amzel & Pedersen, 1983; Suzutani et al., 1992). Identical and similar amino acids are boxed by lines and dotted lines, respectively.

Fig. 2. Double reciprocal plots of substrate concentration against the initial velocity for the TK activity of wt VZV TK. (a) The reaction mixture contained 100 (○), 50 (●), 33 (△) or 25 (▲) μM-ATP. (b) The reaction mixture contained 4 (○), 2 (●), 1.25 (△), or 1 (▲) μM-thymidine. The insets are re-plots of the ordinate intercept (apparent $V_{max}$) with respect to the molar concentration of ATP (a) and thymidine (b).
Table 2. Analysis of phosphorylated products of \[^3H\]dT and \[^{14}C\]dTMP by wild-type VZV TK

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Isotope</th>
<th>Concentration ((\mu M))</th>
<th>Isotope</th>
<th>Concentration ((\mu M))</th>
<th>Isotope</th>
<th>Concentration ((\mu M))</th>
<th>Isotope</th>
<th>Concentration ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(^3H)</td>
<td>0</td>
<td>(^{14}C)</td>
<td>0</td>
<td>(^3H)</td>
<td>0</td>
<td>(^{14}C)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>(^3H)</td>
<td>0</td>
<td>(^{14}C)</td>
<td>3.19</td>
<td>(^3H)</td>
<td>0</td>
<td>(^{14}C)</td>
<td>1.53</td>
</tr>
<tr>
<td>6</td>
<td>(^3H)</td>
<td>0</td>
<td>(^{14}C)</td>
<td>2.19</td>
<td>(^3H)</td>
<td>0</td>
<td>(^{14}C)</td>
<td>2.81</td>
</tr>
</tbody>
</table>

respectively, almost the same. This result indicated that the binding order of the two substrates, ATP and dT, is random, as with HSV-1 TK (Dixon & Webb, 1964; Chen et al., 1979), and the ternary complex ATP-Mg\(^{2+}\)-TK-dT is formed in the reaction.

In order to clarify whether VZV TK catalyses the conversion of dT to deoxythymidine diphosphate (dTDP) without releasing deoxyTMP (dTMP) as the first phosphorylation reaction product from the enzyme, the rate of formation of dTDP from dT and dTMP was evaluated by using \([2-^{14}C]\)dT and \([\text{methyl-}^{3}H]\)dTMP as substrates in one enzyme reaction (Table 2). The amount of \(^3H\)-labelled dTDP was always higher than \(^{14}C\)-labelled dTDP and accumulation of \(^{14}C\)-labelled dTDP was observed before phosphorylation of \(^{14}C\)-dTMP to \(^{14}C\)-dTDP. These results indicate that the majority of the first phosphorylated product, dTMP, probably leaves the enzyme while ADP is replaced with ATP for the next reaction.

Analysis of binding affinity of varicella-zoster virus thymidine kinase for nucleosides and nucleoside monophosphates

To determine the characteristics of substrate recognition by VZV TK, the Michaelis and inhibition constants were determined for nucleoside analogues and their 5'-monophosphate derivatives for wt and SDM VZV TKs. The mutations, \(^{128}\)Thr to Leu and \(^{137}\)Ile to Leu, did not influence the binding affinity of VZV TK for dT (Table 3), but reduced TK activity (Table 1) and reduced the affinity of the enzyme for 5-halogenovinyluridine analogues by about 2-7 to 7-5 times those of wt TK. However, although reduced, the affinity of SDM VZV TK was still much higher than those of HSV-2 and mammalian cellular TKs (Cheng et al., 1981; Yokota et al., 1989). A previous report demonstrated that both TK and TM-K activities reside at the same site (Chen et al., 1979). Therefore, the kinetic constants of nucleoside monophosphate analogues for TK activity were evaluated (Table 4). The \(K_i\) values showed that VZV TK had a high affinity for BV-dUMP. The affinity of BV-araUMP for VZV TK was 16-fold lower than that of BV-dUMP but 21-fold higher than that of ara-TMP. The SDM TK demonstrated low affinity for various nucleoside 5'-monophosphates, especially for dTMP.

Analogue of 5-halogenovinyluridine and nucleoside monophosphates have bulky and strongly negatively charged groups at the 5 and 5' positions, respectively.

Table 3. Kinetic constants of various nucleosides as the substrate or inhibitor for TK activity of wild-type and mutant VZV TKs

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Wt (K_m) ((\mu M))</th>
<th>Wt (K_i) ((\mu M))</th>
<th>SDM (K_m) ((\mu M))</th>
<th>SDM (K_i) ((\mu M))</th>
<th>SDM (K_i) (SDM)/(K_i) (wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dT</td>
<td>0.72*</td>
<td>0.74</td>
<td>3.55</td>
<td>1.53</td>
<td>1.53</td>
</tr>
<tr>
<td>araT</td>
<td>2.32</td>
<td>3.01</td>
<td>2.01</td>
<td>4.67</td>
<td>4.67</td>
</tr>
<tr>
<td>CV-araU</td>
<td>0.043</td>
<td>1.05</td>
<td>1.27</td>
<td>7.47</td>
<td>7.47</td>
</tr>
<tr>
<td>BV-araU</td>
<td>0.06</td>
<td>2.32</td>
<td>0.32</td>
<td>2.67</td>
<td>2.67</td>
</tr>
<tr>
<td>IV-araU</td>
<td>0.17</td>
<td>0.32</td>
<td>0.32</td>
<td>2.67</td>
<td>2.67</td>
</tr>
<tr>
<td>BV-dU</td>
<td>37.5</td>
<td>&gt;500</td>
<td>1.7</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>dC</td>
<td>&gt;500</td>
<td>NT†</td>
<td>NT†</td>
<td>NT†</td>
<td>NT†</td>
</tr>
</tbody>
</table>

* \(K_m\) and \(K_i\) values (\(\mu M\)) were calculated from a minimum of three determinations.
† NT, Not tested.

Table 4. Kinetic constants of various nucleoside 5'-monophosphates for TK activity of wild-type and mutant VZV TKs and of dTMP for TM-K activity of wild-type VZV TK

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Wt (K_m) ((\mu M))</th>
<th>Wt (K_i) ((\mu M))</th>
<th>SDM (K_m) (SDM)/(K_i) (wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTMP</td>
<td>9.09*</td>
<td>6.18</td>
<td>129</td>
</tr>
<tr>
<td>araTMP</td>
<td>1493</td>
<td>41.66</td>
<td>30.0</td>
</tr>
<tr>
<td>CV-araUMP</td>
<td>411</td>
<td>51.70</td>
<td>2.79</td>
</tr>
<tr>
<td>BV-araUMP</td>
<td>72.5</td>
<td>39.8</td>
<td>5.50</td>
</tr>
<tr>
<td>BV-dUMP</td>
<td>4.51</td>
<td>50.3</td>
<td>11.2</td>
</tr>
<tr>
<td>dCMP</td>
<td>4071</td>
<td>NT†</td>
<td>NT†</td>
</tr>
</tbody>
</table>

* \(K_m\) and \(K_i\) values (\(\mu M\)) were calculated from a minimum of three determinations.
† NT, Not tested.
For binding these nucleosides and nucleotides, the substrate-binding site of VZV TK must be able to accommodate large changes in both size and charge. It is probable that this site, at residues 136 and 137, has a role in formation of the substrate-binding pocket for bulky substrates.

**Inhibition of varicella-zoster virus thymidine kinase by dinucleotides**

To clarify the optimum distance of phosphate donor (e.g. ATP) and acceptors (e.g. dT and dTMP) of wt and SDM VZV TKs, inhibition of TKs by bisubstrate analogues was studied (Table 5). The wild-type TK demonstrated high sensitivity to Ap₃T and Ap₄T and maximum inhibition was achieved with Ap₂T which contains two phosphate groups more than the natural substrate for TK. This inhibition strongly supports the existence of the ternary complex, ATP·Mg²⁺·TK·dT or ATP·Mg²⁺·TK·dTMP, and indicates that the kinase reaction of VZV TK probably proceeds via phosphate transfer directly from ATP to dT or dTMP. The optimum distance between the ATP- and substrate-binding sites measured by this technique was the same as for mammalian cellular TK, as described by Davies et al. (1988). The sensitivity of SDM VZV TK to Ap₃T was almost equal to that of wt TK, but SDM required a precise distance between both substrates and was four- to fivefold less sensitive to Ap₂T and Ap₄T.

**Conclusions**

In this study we investigated the characteristics of VZV TK, especially the mechanism of kinase action and the origin of substrate specificity, comparing wt VZV TK with SDM VZV TK, which has an amino acid sequence identical to part of the predicted substrate-binding site of HSV-2 TK. The TM-K activity was much greater than the TK activity of VZV TK and this is different from HSV-1 TK (Chen & Prusoff, 1978; Fyfe, 1982). However, the optimum distance between the VZV TK ATP- and substrate-binding sites was the same as for cellular TK and was shorter than that of cellular TM-K by a length equivalent to one phosphate group (Davies et al., 1988). Robertson & Whalley (1988) and Smith et al. (1989) suggested that the origin of herpesvirus TKs may be a host cell TM-K and recently Harrison et al. (1991) suggested that herpesvirus TKs have evolved from a captured cellular dCK gene. Analyses of the distance between the ATP- and substrate-binding sites of cellular kinases may aid the understanding of which kinase is the origin of the enzymes. The greater affinity for dTMP and 5-halogenovinyluridine analogues was reported as a feature of VZV TK (Chen et al., 1984; Yokota et al., 1989). We were able to show that the site, 1³⁵Thr to 3¹⁰Ile, has a role in formation of the substrate-binding pocket and relates to the substrate specificity of this enzyme. Further studies clarifying the functions of the amino acids in this region, between positions 128 and 160, of VZV TK should help elucidate the evolution of herpesvirus TKs and the development of anti-herpesvirus chemotherapy.

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**References**


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