Evidence that the 'Active Centre' of the Herpes Simplex Virus Thymidine Kinase Involves an Interaction between Three Distinct Regions of the Polypeptide

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SUMMARY

The nucleotide sequence of the coding region of the thymidine kinase gene from each of three mutant strains of herpes simplex virus type 1 and from the parental strain, SC16, has been determined. The mutants were known to express thymidine kinase enzymes with distinct substrate binding properties. Consideration of the lesions in the genes responsible for these altered biochemical properties has led us to postulate a preliminary model for the active centre of the enzyme, involving the cooperation of three distinct regions of the polypeptide.

The thymidine kinase (TK) enzyme specified by herpes simplex virus (HSV) is of special interest because it plays an important role in the mode of action of several potent nucleoside analogue inhibitors of virus replication, being responsible for the initial phosphorylation and consequent activation of these compounds in the infected cell (see Larder & Darby, 1984). The design of more effective anti-herpes drugs would be facilitated by a better understanding of the structure of the active site of the enzyme and of how the enzyme interacts with its various substrates. In order to identify regions of the polypeptide which are involved in the formation of the active site we have identified here the lesions in three mutant TK enzymes which display altered substrate interactions. The mapping of these has led us to postulate a model in which three distinct regions of the polypeptide are involved in the active centre of the enzyme.

HSV TK is composed of two identical subunits (Jamieson & Subak-Sharpe, 1974) each containing 376 amino acids (McKnight, 1980; Wagner et al., 1981), and the native protein has binding sites for both the natural nucleoside substrates thymidine and deoxycytidine, and also for the phosphate donor, ATP. None of these sites on the enzyme has been formally identified but there is reason to believe that a region close to the N-terminus of the polypeptide is involved in ATP binding. A recent comparison of the predicted amino acid sequences of the TK polypeptides of HSV-1 and HSV-2 with that of a marmoset herpesvirus (Otsuka & Kit, 1984) has revealed a region of homology in all three polypeptides corresponding to the sequence of amino acid residues between positions 49 and 66 in the primary sequence of HSV-1 TK. Furthermore, amino acid residues 51 to 63, contained in this sequence, show homology with a sequence implicated in ATP recognition present in other, unrelated, ATP-binding enzymes (Otsuka & Kit, 1984; Walker et al., 1982).

In this study, we have attempted to elucidate other features of the active centre of the enzyme by characterizing mutations that alter the affinity of the enzyme for nucleoside substrates, thus identifying residues in the polypeptide that have a role in nucleoside binding.

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Fig. 1. Sequencing of HSV-1 TK coding regions. (a) Strategy for DNA sequence determination. The diagram shows the region of DNA from HSV-1 strain SC16 which contains the coding sequence for TK (shaded). Those restriction enzyme sites employed for cloning into M13 mp8 to provide the templates for sequencing are marked (T, Taq1; Hp, HpaI; A, AluI; Th, Thal; Ha, HaeIII). Arrows indicate the direction and extent of sequence determined from individual clones. The sequence of the entire coding region of SC16 wild-type was determined for both DNA strands using overlapping clones. The sequences of the SC16-derived mutants B3, Tr7 and S1 were mainly determined for one strand only using Taq1 fragment clones and one Thal fragment clone (marked c) for each as templates. (b, c) Portions of comparative sequencing gels showing the sequences of the fragments marked b and c in (a). (b) A stretch of the sequence of the coding strand of DNA and the positions of the single C → T transitions in the sequences of B3 (nucleotide 502) and Tr7 (nucleotide 527), compared with SC16 wild-type, are indicated (arrows). (c) A stretch of sequence of the non-coding DNA strand illustrating the single G → A transition (equivalent to a C → T transition on the coding strand) found in S1 (nucleotide 1007). No other changes were detected in the nucleotide sequences of the mutants compared with the wild-type.
Table 1. Substrate-binding properties of mutant thymidine kinases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km or Kᵢ value relative to wild-type</th>
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<tbody>
<tr>
<td></td>
<td>B3</td>
</tr>
<tr>
<td>Thymidine</td>
<td>1-5</td>
</tr>
<tr>
<td>ATP</td>
<td>1</td>
</tr>
<tr>
<td>BVdU</td>
<td>45</td>
</tr>
</tbody>
</table>

* This value has been calculated using the formula Km (or Kᵢ) mutant/Km (or Kᵢ) wild-type. Values greater than 1 indicate decreased affinity of the mutant enzyme for the particular substrate or inhibitor. These data have been adapted from previously published kinetic studies (Larder et al., 1983a, b).

We have previously described the biochemical properties of three abnormal thymidine kinases induced by drug-resistant variants of HSV-1 strain SC16 (Larder et al., 1983a, b). Some substrate binding properties of these enzymes are summarized in Table 1. The enzymes induced by all three variants (B3, Tr7 and S1) have distinct alterations in apparent affinities for nucleoside substrates and one of them (S1) also appears to have an altered affinity for ATP. We have now defined the lesion in each variant by comparing the nucleotide sequence of the TK coding region with that of the wild-type parental strain, SC16. Initially, the 3.5 kb BamHI fragment, which contains the entire TK coding sequence, was purified from each virus genome and was cloned into the bacterial plasmid pBR322. The nucleotide sequences of the TK genes were then determined using the dideoxynucleotide chain termination method of Sanger et al. (1977), following subcloning of restriction fragments into M13 mp8. The sequencing strategy is outlined in Fig. 1 (a). In each mutant a single C → T transition had occurred in the coding strand of the DNA. Areas of comparative sequencing gels illustrating the changes are included in Fig. 1 (b, c).

The complete nucleotide sequence obtained for SC16 TK and the amino acid sequence deduced from this is shown in Fig. 2. The single mutation in each SC16-derived variant is shown in a box above the sequence; in each case the mutation resulted in an amino acid substitution in the TK polypeptide. Two of the observed substitutions (alanine → threonine at residue 168 in B3 and arginine → glutamine at residue 176 in Tr7) were situated close to one another in the primary sequence of the polypeptide but the third (cysteine → tyrosine at residue 336 in S1) was located some distance away towards the C-terminus. Since each of these amino acid substitutions affects the apparent affinity of TK for nucleoside substrates (Table 1) it is likely that all three are involved in the formation of the nucleoside binding site.

Fig. 2 also includes a comparison of the wild-type SC16 TK amino acid sequence with previously published sequences of two other HSV-1 strains (McKnight, 1980; Wagner et al., 1981) and an HSV-2 strain (Swain & Galloway, 1983). It is notable that the amino acid substitutions in B3 and Tr7 are in a region of the polypeptide which is highly conserved between types 1 and 2. In fact, the amino acid sequence between residues 161 and 193 in HSV-1 shows 97.6% homology with the corresponding region in HSV-2. This is far higher than the average amino acid homology across the whole coding region (approximately 73%) and suggests a functional role for this conserved sequence.

The substitution of threonine for alanine at position 168 in the TK of the E-5-bromovinyl-2'-deoxyuridine (BVdU)-resistant mutant B3 (Field & Neden, 1982) has little effect on the normal functions of the enzyme (Table 1) but it is responsible for a dramatic reduction in the apparent affinity of the enzyme for BVdU (Larder et al., 1983a). In this respect, the phenotype of B3 resembles that of HSV-2 strains which are similarly resistant to BVdU and induce TKs with low affinity for the analogue (Cheng et al., 1981). It is notable that the only difference between the amino acid sequences of the wild-type strains of HSV-1 and HSV-2 in the conserved region between residues 161 and 193 discussed above again involves the replacement of alanine 168 of HSV-1, this time with a serine residue in HSV-2. This substitution is remarkably similar to that in the mutant B3, and it is therefore tempting to speculate that the nature of the side-chain on
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Fig. 2. Nucleotide sequence and derived amino acid sequence of wild-type HSV-1 TK from strain SC16. The change in each of the TK substrate specificity mutants is indicated in a box above the sequence and below are shown comparisons with the published sequences for the other HSV-1 strains (Wagner et al., 1981) and MP (McKnight, 1980) and the HSV-2 strain 333 (Swain & Galloway, 1983). In these comparisons only amino acid differences are indicated. The broken line above the sequence indicates the putative ATP-binding site.

this particular amino acid residue is responsible for the differential sensitivity of HSV-1 and -2 strains to BVdU. Residue 168 presumably does not have a direct role in thymidine binding, which remains normal in mutant B3, but may be positioned in close proximity to the nucleoside binding site so that when a bulkier side-chain is substituted the binding of substrate analogues carrying large substituents is hindered.

Finally, the amino acid substitution at residue 336 in the TK of mutant S1 has a marked effect on ATP binding as well as on nucleoside binding (Table 1). While it is possible that this is due to an overall disruption of the three-dimensional structure of the protein caused by a lesion at a
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ATP-binding site

N-terminus

C-terminus

Nucleoside-binding site

Fig. 3. Preliminary model for the active centre of HSV TK. The main feature of this model is that three regions of the polypeptide, which appear to be important in nucleoside and ATP binding, cooperate in the generation of overlapping ATP- and nucleoside-binding sites which would ensure that the two substrates bind in close proximity on the surface of the enzyme.

distant site, a simpler interpretation is that the wild-type cysteine 336 residue is situated close to, or constitutes part of, both the ATP-binding site (previously assumed to involve residues 51 to 63) and the nucleoside-binding site.

On the basis of these results we have proposed a tentative model for the active centre of HSV TK (Fig. 3). We do not attempt to define the precise three-dimensional structure at the site but rather we identify three distinct and widely separated regions of the primary structure of the polypeptide which, we propose, cooperate in the native protein to generate overlapping ATP- and nucleoside-binding sites. Such an arrangement would provide a region, presumably on the surface of the protein, which could interact with both nucleoside substrate and phosphate donor molecules, bringing about nucleoside phosphorylation. We are currently testing the roles of further amino acid residues in the regions of the polypeptide forming the putative nucleoside-binding site by means of site-directed mutagenesis experiments.

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REFERENCES


Short communication


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