Analysis of the Role of the Cysteine 171 Residue in the Activity of Herpes Simplex Virus Type 1 Thymidine Kinase by Oligonucleotide-directed Mutagenesis

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SUMMARY

The thymidine kinase (TK) gene from herpes simplex virus type 1 strain SC16 was cloned into bacteriophage M13 mp8 so that functional HSV-1 TK was expressed in bacteria infected with the recombinant bacteriophage, M13/TK. Oligonucleotide site-directed mutagenesis was then employed to introduce single nucleotide changes into the TK gene in M13/TK in order to alter the codon for cysteine 171 in the wild-type enzyme to a codon specifying either serine or glycine. Analysis of the mutant enzymes in bacterial extracts showed that these substitutions had little effect on the activity of the enzyme, indicating that the side chain of this residue is not involved in nucleoside binding and is not essential for the catalytic activity of the enzyme.

INTRODUCTION

The thymidine kinase (TK) enzyme of herpes simplex virus (HSV) (Kit & Dubbs, 1963; Dubbs & Kit, 1964) is of central importance in the action of nucleoside analogue drugs effective against the virus, being responsible for the initial phosphorylation and consequent activation of these compounds in infected cells (for review, see Larder & Darby, 1984). Such drugs presumably interact with the HSV TK enzyme in a similar way to the natural nucleoside substrates thymidine and deoxycytidine (Jamieson et al., 1974; Jamieson & Subak-Sharpe, 1974) during the course of their phosphorylation. Our aim is to define some of the features of the nucleoside binding site of TK, as an understanding of the structure of this site and the nature of the interactions between the amino acid side chains of the enzyme and elements of the substrate would be invaluable in the design of further anti-herpesvirus nucleoside analogue drugs.

The HSV-1 TK probably exists as a dimer (Chen & Prusoff, 1978), comprising two identical subunits, each of 376 amino acids, the sequence of which has been deduced for several strains (McKnight, 1980; Wagner et al., 1981; Darby et al., 1986). The nucleoside binding site of the enzyme has not yet been defined but we have recently postulated that a central region of the polypeptide, spanning residues 168 to 176 may be involved. This hypothesis was based on results from a recent study in which we examined the genes for three mutant HSV-1 TK enzymes, known to display altered interactions with nucleoside substrates. We found that in each case a single amino acid substitution in the polypeptide chain was responsible for the altered properties (Darby et al., 1986). Two of the lesions (alanine → threonine at amino acid 168 in mutant B3 and arginine → glutamine at position 176 in mutant Tr7), both of which specifically affect nucleoside binding to the enzyme, were situated close together in the primary sequence of the polypeptide in a region which is highly conserved between the TKs of HSV-1 and HSV-2. We therefore postulated that this region of the polypeptide may constitute part of the nucleoside-binding site of the enzyme and we are concentrating our initial investigations on this region of the protein sequence.

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Our interest was focused first of all on the cysteine residue at position 171 (Fig. 1), lying between the sites of the lesions mentioned above. Apart from the possibility that this might have a direct role in the catalytic activity of the enzyme, there was an alternative possibility that it might be important for the structural integrity of the enzyme. This latter possibility was considered in view of the finding that the third mutant in our previous study (Darby et al., 1986), SC16 S1, had its cysteine 336 residue replaced with tyrosine. This substitution caused wide disruption to the functioning of the protein, affecting both ATP and nucleoside binding and also reducing the thermal stability of the enzyme (Larder & Darby, 1982; Larder et al., 1983). If this were due to the loss of a disulphide bridge then cysteine 171 would be a good candidate for the other half of such a bridge as this is the only other cysteine residue in the polypeptide which is conserved in the TKs of four strains of HSV (see Fig. 2 of Darby et al., 1986).

In order to gain insight into the importance of the cysteine 171 side chain in enzyme activity we have carried out site-directed mutagenesis experiments on the TK gene of HSV-1 (strain SC16) after cloning this into an M13 bacteriophage vector which allows its expression in bacteria. By this means we have generated mutant TKs containing either serine or glycine at position 171 in place of cysteine. Analysis of the activities of these enzymes suggests that the presence of a cysteine side chain at residue 171 is not a critical requirement for enzyme activity.

METHODS

Phage and bacteria. Bacteriophage M13 mp8 (Messing & Vieira, 1982) and derivatives were grown in Escherichia coli (strain TG1) cells.

Construction of M13/TK expression vector. The 2 kb PvuII fragment of HSV-1 strain SC16 (Hill et al., 1975) DNA, which contains the TK gene, was subjected to partial digestion with restriction endonuclease RsaI. Fragments of approximately 1.7 kb were recovered (Vogelstein & Gillespie, 1979) following agarose gel electrophoresis of the digest and these were incubated overnight at room temperature with SmaI-digested, alkaline phosphatase-treated M13 mp8 replicative form DNA and T4 DNA ligase. The products of this ligation mix were subsequently introduced into E. coli cells by transformation (Hanahan, 1983). Progeny insert-positive M13 clones were screened by restriction endonuclease and nucleotide sequencing analyses to identify the required construction (Fig. 2).

Nucleotide sequence determination. The dideoxynucleotide chain termination method of Sanger et al. (1977) was used to determine the nucleotide sequence of the TK gene in M13 vectors. Bacteriophage genome DNA was used as template with a set of six oligonucleotides (kindly provided by Dr D. Snary, Wellcome Biotechnology) designed to hybridize to the TK insert at approximately 190 nucleotide intervals, as specific primers. These allowed us to determine the entire sequence of TK in the vector without the need for subcloning.

Preparation of bacterial extracts for TK assay. Crude extracts of infected bacteria were prepared essentially as described by Winter et al. (1982). Stationary cultures of E. coli were diluted 100-fold into fresh medium containing 100 µg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) and inoculated with bacteriophage at high multiplicity. Cultures were grown for 6 h at 37 °C with shaking, after which the cells in 1.5 ml samples were collected by centrifugation, washed once in 10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, and resuspended in 200 µl of 50 mM-Tris-HCl pH 7.8, 1 mM-EDTA, 5 mM-mercaptoethanol, 0.1 mM-PMSF. Cells were lysed by three cycles of freezing at −70 °C and thawing, followed by 20 s sonication with a probe sonicator. Lysates were centrifuged for 2 min in a microcentrifuge and the supernatants were retained and stored in aliquots at −70 °C.

Preparation of HSV-infected cell extracts for TK assay. Confluent monolayers of 5-bromo-2'-deoxyuridine (BUdR)-resistant BHK cells (BU-BHK), which express no cellular TK, were infected with 10 p.f.u./cell HSV-1 strain SC16 and incubated at 37 °C. Cells were harvested after 18 h and extracts were prepared as previously described (Larder & Darby, 1982).

Enzyme assays. TK activity was assayed essentially as described by Klemperer et al. (1967). Unless otherwise
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Specified [14C]thymidine at a concentration of 30 μM (sp. act. 52 mCi/mmol) was used as substrate. Deoxycytidine kinase activity was assayed in the same way with 50 μM-[^3]H]deoxycytidine (19 Ci/mmol) as substrate.

**Site-directed mutagenesis.** Specific mutations were introduced into the TK gene in M13/TK using the method described by Zoller & Smith (1983). Oligonucleotide 5' GGTAGCTCAGGAGGGC 3' (kindly provided by Dr J. McCauley) was employed in the generation of vector M13/TK(ser 171) and oligonucleotide 5' GGTAGCCAGGGG 3' (provided by Dr D. Snary) was used to produce vector M13/TK(gly 171). Mutants tentatively identified in the first instance by the hybridization screening procedure of Wallace et al. (1981) as described by Zoller & Smith (1983), were positively designated only after nucleotide sequence determination of the entire TK gene.

**RESULTS**

**Construction and characterization of M13/TK expression vector**

In order to facilitate site-directed mutagenesis experiments we first cloned the HSV-1 TK coding sequence into M13 mp8 in such a way that functional TK could be expressed in bacteria infected with the recombinant phage. The structure of the expression vector, M13/TK, is shown in Fig. 2(a). The predicted protein expressed from the lac promoter of this construction starts with seven amino acids derived from the N terminus of β-galactosidase followed by the entire TK sequence from amino acid 5 onwards (Fig. 2b).

To test whether this fusion protein retained TK activity crude extracts made from cultures of E. coli infected with the recombinant M13/TK phage in the presence of the lac inducer IPTG were assayed. As shown in Fig. 3(a) high levels of TK activity were present in bacteria infected with M13/TK whereas cultures infected with M13 mp8 without an insert contained negligible amounts of TK activity. To confirm that the TK activity in M13/TK-infected bacteria was HSV-specific a serum inactivation experiment was carried out (Fig. 3b). The enzyme in the infected bacterial extract was inactivated by pre-incubation with anti-HSV-1 antiserum but not by pre-immune serum, indicating that the TK activity induced in M13/TK-infected bacteria was indeed HSV-1-specific.

**Generation of vectors expressing mutant TKs TK(ser 171) and TK(gly 171)**

We next introduced single nucleotide changes into the TK gene in M13/TK by oligonucleotide site-directed mutagenesis using oligonucleotides designed to change the TGC codon for cysteine 171 in native wild-type (wt) TK to AGC, which specifies serine, or GGC, specifying glycine. Mutants were identified initially by dot blot hybridization screening using the mutagenic oligonucleotide as probe, but were subsequently confirmed by direct nucleotide sequence determination of the entire TK coding sequence.

For each of the two required mutants we selected, for our investigations into enzyme activity, two independently isolated clones, each containing a TK gene which differed from wt only in that it contained the single predicted nucleotide change. Vectors M13/TK(ser 171)B and M13/TK(ser 171)C were selected to direct the expression of a TK enzyme with serine instead of cysteine in the position corresponding to residue 171 in native TK. Vectors M13/TK(gly 171)A and M13/TK(gly 171)B directed the expression of a TK enzyme with glycine in this position.

**Analysis of the activities of mutants TKs TK(ser 171) and TK(gly 171)**

Extracts prepared from cultures of E. coli infected with M13/TK(ser 171) and M13/TK(gly 171) vectors in the presence of IPTG were assayed for TK activity. Both classes of vector induced TK in the infected bacteria (Table 1). The amount of activity detected in bacteria infected with the mutant vectors as compared with the wt vector, M13/TK, was somewhat variable between experiments but was generally reduced and of the order of 20 to 40% in both cases. It was apparent however, from the finding that neither amino acid substitution destroys TK activity, that the cysteine side chain at residue 171 of HSV-1 TK was not an essential requirement for the ability of the enzyme to phosphorylate thymidine.

As wt HSV-1 TK also displays deoxycytidine kinase activity (Jamieson et al., 1974; Jamieson & Subak-Sharpe, 1974) we tested the ability of the TK(ser 171) mutant enzyme to phosphorylate deoxycytidine (Table 2). Deoxycytidine kinase activity was found to be present in extracts of
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(a) (b)

\[ \text{Sma I / Rsa I} \quad \text{Pvu I / Sma I} \]

HSV-1

\[ \text{B-Galactosidase} \]

\[ \text{Thr Met Ile Thr Asn Ser Arg Gly} \]

\[ \text{ATG ACC ATG ATT ACG AAT TCC CGG GGA} \]

![Diagram](image)

\[ \text{Smal} \]

TK

\[ \text{Met Ala Ser Tyr Pro} \]

\[ \text{ATG GCT TCG TAC CCC} \]

Predicted \( \beta \)-galactosidase/TK fusion

\[ \text{Thr Met Ile Asn Ser His Pro} \]

\[ \text{ATG ACC ATG ATT ACG ATC TCC CAC CCC} \]

Fig. 2. (a) Structure of M13/TK expression vector. M13/TK contains a RsaI/PvuII fragment of HSV-1 DNA inserted into the SmaI cloning site near the start of the lacZ' gene in M13 mp8; OP, lac operator. This HSV-1 DNA fragment contains all but the first 10 nucleotides of the TK coding sequence. (b) N-terminal sequences of \( \beta \)-galactosidase (the lacZ' gene product), native TK and the predicted fusion protein expressed from the lac promoter in M13/TK.

![Graphs](image)

Fig. 3. (a) Assay of TK activity in extracts prepared from cultures of E. coli infected with M13 (○) or M13/TK (●). (b) Serum inactivation of TK activity in M13/TK-infected E. coli. Aliquots of extract were incubated with equal volumes of buffer (○), antiserum raised against HSV-1-infected cells (■) or pre-immune serum (●) from the same rabbit for 2 h at 4 °C prior to assay for TK activity.

bacteria infected with the M13/TK and M13/TK(ser 171) vectors and the relative amounts of activity were similar to the relative amounts of TK activity measured in the same extracts, suggesting that cysteine 171 was also not essential for this enzyme function.

The reduced amounts of enzyme activity induced by the mutant vectors were possibly due to increased lability of the mutant enzymes compared with wt in the bacterial extracts. We therefore compared the stability of wt TK and TK(ser 171) at 43 °C by incubating the relevant bacterial extracts at this temperature for various times before assay. The results from this experiment (Fig. 4) indicated that both wt and mutant enzymes in these extracts were relatively unstable at 43 °C, with half-lives of approximately 7 min and 20 min respectively. The ser 171 mutant enzyme however appeared to be somewhat more stable than the wt TK.
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Fig. 4. Thermal inactivation of TK activity in extracts of E. coli infected with M13/TK (○) or M13/TK(ser 171)B (●). Extracts were incubated at 43 °C for various times prior to TK assay. Activities were expressed as a percentage of the TK activity measured without exposure to 43 °C.

Table 1. Assay of TK(ser 171) and TK(gly 171) activities

<table>
<thead>
<tr>
<th>Bacteriophage*</th>
<th>Thymidine phosphorylated (pmol/10 min reaction)</th>
<th>Activity (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mp8</td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>M13/TK</td>
<td>1047</td>
<td>100</td>
</tr>
<tr>
<td>M13/TK(ser 171)B</td>
<td>256</td>
<td>24</td>
</tr>
<tr>
<td>M13/TK(ser 171)C</td>
<td>237</td>
<td>23</td>
</tr>
<tr>
<td>M13/TK(gly 171)A</td>
<td>331</td>
<td>32</td>
</tr>
<tr>
<td>M13/TK(gly 171)B</td>
<td>370</td>
<td>35</td>
</tr>
</tbody>
</table>

* Extracts prepared from cultures of E. coli infected with the bacteriophage indicated were assayed for TK activity.
† TK activity expressed as a percentage of the activity in M13/TK-infected cells.

Table 2. Assay of thymidine and deoxycytidine kinase activities in bacterial extracts

<table>
<thead>
<tr>
<th>Bacteriophage*</th>
<th>Thymidine phosphorylated (pmol/20 min reaction)</th>
<th>Activity (%)†</th>
<th>Deoxycytidine phosphorylated (pmol/20 min reaction)</th>
<th>Deoxycytidine kinase activity (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mp8</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>M13/TK</td>
<td>2165</td>
<td>100</td>
<td>506</td>
<td>100</td>
</tr>
<tr>
<td>M13/TK(ser 171)B</td>
<td>798</td>
<td>37</td>
<td>127</td>
<td>25</td>
</tr>
<tr>
<td>M13/TK(ser 171)C</td>
<td>385</td>
<td>18</td>
<td>68</td>
<td>13</td>
</tr>
</tbody>
</table>

* Extracts were prepared from cultures of E. coli infected with the bacteriophage indicated.
† Enzyme activities expressed as a percentage of the activity in M13/TK-infected cells.
‡ Enzyme activities expressed as a percentage of the activity in M13/TK-infected cells.

To investigate further the interaction of the mutant TKs with nucleoside substrates we next examined the sensitivity of the TK(ser 171) enzyme to inhibition by the nucleoside analogue drugs E-5-bromovinyl-2'-deoxyuridine (BVdU) and 5-bromo-2'-deoxyuridine (B UdR) compared with that of the wt enzyme. The results shown in Table 3 demonstrated first that the wt enzyme expressed from the M13/TK vector in bacteria displayed very similar sensitivity to the drugs as the native enzyme expressed in HSV-1-infected tissue culture cells. Furthermore the
Fig. 5. Reaction kinetics of thymidine phosphorylation by extracts of HSV-1-infected BU-BHK cells (O) or of E. coli cells infected with M13/TK (●), M13/TK(ser 171)B (□), M13/TK(ser 171)C (■), M13/TK(gly 171)A (▲) and M13/TK(gly 171)B (▲). Initial reaction rates over 5 min were measured in the presence of different thymidine concentrations and the Lineweaver–Burk plots obtained are shown. \( V \) represents pmol thymidine phosphorylated per min per µl enzyme.

Table 3. Inhibition of wt TK and TK(ser 171) by BVdU and BUdR

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>BVdU</th>
<th>BUdR</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1-infected BU-BHK</td>
<td>47</td>
<td>73</td>
</tr>
<tr>
<td>M13/TK-infected E. coli</td>
<td>52</td>
<td>74</td>
</tr>
<tr>
<td>M13/TK(ser 171)B-infected E. coli</td>
<td>44</td>
<td>69</td>
</tr>
</tbody>
</table>

* TK activities measured in the presence of 10 µM-BVdU or -BUdR expressed as a percentage of activity in the absence of drug.

Table 4. \( K_m \) values of TKs for thymidine

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>( K_m ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1-infected BU-BHK</td>
<td>0.3</td>
</tr>
<tr>
<td>M13/TK-infected E. coli</td>
<td>0.3</td>
</tr>
<tr>
<td>M13/TK(ser 171)B-infected E. coli</td>
<td>0.4</td>
</tr>
<tr>
<td>M13/TK(ser 171)C-infected E. coli</td>
<td>0.2</td>
</tr>
<tr>
<td>M13/TK(gly 171)A-infected E. coli</td>
<td>0.4</td>
</tr>
<tr>
<td>M13/TK(gly 171)B-infected E. coli</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* \( K_m \) values were calculated from the Lineweaver–Burk plots shown in Fig. 5.

sensitivity of the TK(ser 171) enzyme in bacterial extracts was not significantly different from that of the wt enzyme.

Finally we have measured the Michaelis–Menten constants (\( K_m \) values) for thymidine for the wt and mutant TK enzymes in the bacterial extracts to provide an indication of the relative binding affinities of the enzymes for thymidine. The data, in the form of Lineweaver–Burk plots, are shown in Fig. 5 and the \( K_m \) values obtained are summarized in Table 4. Once again the wt enzyme in the bacterial extracts was found to be similar to the native enzyme in extracts of HSV-1-infected BU-BHK cells, as the two enzymes were found to have the same affinity for thymidine. The \( K_m \) values of the mutant enzymes TK(ser 171) and TK(gly 171) for thymidine were also not significantly different from that of wt TK, indicating that the side chain of cysteine 171 is not involved in the binding of thymidine to the enzyme.

DISCUSSION

The expression vector M13/TK (Fig. 2) was constructed to provide a suitable system for mutagenesis experiments. As indicated in Fig. 2(b) the protein expressed from the lac promoter
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of M13/TK would be predicted to be a fusion protein of 379 amino acids, comprising seven amino acids derived from the N terminus of β-galactosidase followed by the entire TK sequence from amino acid 5 onwards. We considered it highly likely that this fusion protein would retain TK activity as there is evidence to suggest that active TK enzyme is expressed when translation starts at the second AUG triplet in the coding sequence of native TK, at codon 46, implying that the N-terminal amino acids are not critical for activity (Marsden et al., 1983; Smiley et al., 1983). In fact a high level of TK activity was induced in bacteria following infection with M13/TK and furthermore this activity was specifically inactivated by serum containing anti-HSV-1 antibodies. These findings indicate that the vector M13/TK can be used to express the HSV-1 TK enzyme in bacteria. Furthermore our evidence suggests that the modified N terminus of the enzyme synthesized in bacteria has little or no effect on the catalytic activity of the enzyme. Firstly the bacterially expressed enzyme displayed both thymidine and deoxycytidine kinase activity; secondly it showed the same degree of sensitivity to the nucleoside analogue drugs BVDU and BUdR as native TK; and finally it had an identical $K_m$ value for thymidine. Taken together these findings provide strong evidence that the TK enzyme expressed in bacteria from M13/TK functions in the same way as native HSV-1 TK in virus-infected cells. This bacteriophage expression vector therefore provides us with a convenient system for introducing mutations into the TK gene and rapidly assessing the effects of these mutations on the activity of the enzyme.

In this study we have investigated the effects on TK activity of substituting the cysteine residue at position 171 in native wt TK with either serine or glycine. The mutant enzymes TK(ser 171) and TK(gly 171) were expressed in bacteria infected with M13/TK vectors into which we had introduced appropriate single nucleotide changes by oligonucleotide-directed mutagenesis. Both mutant enzymes retained TK activity and appeared to have an unaltered affinity for the substrate, thymidine, as indicated by comparison of $K_m$ values obtained with mutant and wt enzyme extracts. Furthermore TK(ser 171) and wt TK were equally sensitive to inhibition by the nucleoside analogue drugs BVDU and BUdR, and both enzymes recognized deoxycytidine as a substrate for phosphorylation. Together these findings suggest that the side chain of cysteine 171 is not important for the activity of the enzyme and is not involved in thymidine binding. In particular this conclusion is supported by the observation that TK(gly 171) retains activity and has an unaltered affinity for thymidine as the glycine side chain is most unlikely to be able to substitute for the cysteine side chain in any functional capacity.

Although the mutant enzymes TK(ser 171) and TK(gly 171) were indistinguishable from the wt TK by any of the biochemical criteria discussed above, we generally observed reduced amounts of activity in bacterial extracts containing mutant enzymes compared to wt extracts. The reason for this is not known. The mutant vectors M13/TK(ser 171) and M13/TK(gly 171) appeared to grow in the cells just as well as the parental M13/TK vector (data not shown) and the thermostability of the TK(ser 171) enzyme was not increased; in fact this enzyme appeared somewhat more stable than wt at 43°C in these extracts. Furthermore it is unlikely that the acquisition of random promoter mutations, reducing the level of expression in individual clones, was responsible since similar results were obtained with two, separately isolated, mutant clones.

It is possible that the mutant enzymes are more vulnerable to forms of inactivation other than heat e.g. to degradation by bacterial proteases. Alternatively it may be that some aspect of enzyme activity other than nucleoside binding (e.g. the rate of phosphorylation of the bound substrate) is reduced in the absence of the cysteine 171 side chain. Measurement of absolute amounts of TK polypeptide in the extracts would help to discriminate between these alternatives.

It is apparent however that cysteine 171 does not have a key functional role to play in the catalytic mechanism of the enzyme. In addition cysteine 171 cannot be involved in the formation of a disulphide bridge required to hold the enzyme in the appropriate conformation.

In conclusion therefore we have demonstrated the use of a bacterial expression system based on M13 to generate specific mutations in the TK gene of HSV-1 and to analyse the effects of such mutations on enzyme activity. Loss of the cysteine side chain from residue 171 had little effect on enzyme activity indicating that this is not involved in nucleoside interaction and is not essential.
for enzyme function. Studies are now in progress to investigate the roles of other amino acids in this region of the polypeptide and also in the region around cysteine residue 336.

REFERENCES


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