Biochemical Studies on the Herpes Simplex Virus-specified Deoxypyrimidine Kinase Activity

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SUMMARY

The response to various stimuli of herpes simplex virus-specified deoxypyrimidine kinase activity differs significantly from that of the thymidine kinase and deoxycytidine kinase of mammalian cells. However, the two phosphorylating activities of the HSV deoxypyrimidine kinase are themselves quite distinct in their biochemical behaviour.

It has been impossible to separate the HSV-coded thymidine and deoxycytidine phosphorylating activities by their sedimentation, electrophoretic or size characteristics. The evidence suggests that both deoxypyrimidine nucleosides are phosphorylated at the same active site.

INTRODUCTION

Thymidine kinase and deoxycytidine kinase are two enzymes able to form deoxypyrimidine nucleotides from corresponding preformed nucleosides and both are generally considered 'salvage pathway' enzymes. Their role in cell metabolism is thought to be the synthesis of deoxypyrimidine nucleotides from preformed nucleosides (released through cell death or catabolic activity) as well as transport of these molecules across the cell membrane (Roy-Burman, 1970; Cleaver, 1971).

There is induction of a new thymidine kinase activity following infection of mammalian cells with vaccinia (Kit, Pieraiski & Dubbs, 1963; McAuslan, 1963; Kit & Dubbs, 1965) or herpes simplex virus (HSV) (Dubbs & Kit, 1964; Klemperer et al. 1967). HSV also induces a new deoxycytidine kinase activity (Hay et al. 1970; Perera & Morrison, 1970). Genetic evidence has been provided that the HSV induction of thymidine kinase activity cannot be separated from that of deoxycytidine kinase activity (Jamieson, Gentry & Subak-Sharpe, 1974).

Several enzymes involved in deoxynucleotide biosynthesis are known to accept more than one substrate, while others have a more rigid specificity. Calf thymus deoxycytidine kinase has been shown to phosphorylate deoxyadenosine, deoxyguanosine as well as deoxycytidine (Durham & Ives, 1970), while thymidine kinase from the same source is specific for thymidine and unable to utilize deoxycytidine (Bresnick et al. 1970).

So far as we know the HSV coded enzyme which will accept both thymidine and deoxycytidine as substrate is unique.

The experiments in this paper were designed to characterize the two enzymic activities, and to obtain evidence whether the HSV deoxypyrimidine kinase contains one or more active sites.
METHODS

Cells. BHK C13 cells and PyY/TG/CAR/BUDR cells were propagated as described previously (Jamieson et al. 1974).

Virus. Herpes simplex virus type 1 strain 17 syn was used throughout.

Preparation of enzyme extracts. Cells (either BHK or PyY/TG/CAR/BUDR as required) previously infected at an input multiplicity of 10 p.f.u./cell were harvested 8 h post-infection by scraping them into the medium. The cells were then pelleted at 2000 g for 5 min, washed twice in 0.14 M-NaCl and once in a 50 mM-tris, 5 mM-mercaptoethanol, 5 μM-thymidine buffer, pH 7.5 (TMT buffer). The cells were then resuspended in a small vol. of TMT buffer and disrupted by ultrasonic vibration (4 × 60 s at 60 mA in a Dawe Soniclean Generator). The disrupted cells were then centrifuged for 1 h at 100000 g and the supernatant fluid removed and used as the enzyme fraction. All the above steps were carried out between 0 and 4 °C. Enzyme extracts prepared in this manner generally contained 2 to 4 mg per ml. The enzyme extract was either used immediately or stored at −70 °C.

Enzyme assays

Thymidine kinase. The reaction mixture contained (final concentrations) 10 μM-[5-3H]-thymidine (10 μCi/μmol), 10 mM-ATP, 10 mM-MgCl₂, 0.02 M-phosphate buffer, pH 6, plus 100 μg enzyme protein in a total vol. of 100 μl. The reaction mixture was incubated at 37 °C for 15 min and the reaction stopped by immersing the mixtures in a boiling water bath, followed by cooling in ice.

Deoxycytidine kinase. The reaction mixture was identical to that for thymidine kinase except that the buffer was 50 mM-tris, pH 7, and 10 μM-[5-3H]-deoxycytidine was used instead of thymidine.

Separation of reaction products

Disc method. The method routinely used to separate the products of the reaction was to spot 10 μl on to Whatman DE81 paper discs and wash these discs twice in 4 mM-ammonium formate containing 5 μM-thymidine at 37 °C, once in distilled water. They were then rinsed in absolute alcohol and dried prior to estimating the retained radioactivity by scintillation counting. This method effectively removes all unconverted nucleosides from the paper, leaving the nucleotides attached to the paper.

Descending paper chromatography. Samples (50 μl) of the reaction mixtures were separated by descending chromatography on Whatman No. 1 paper using one of the following solvents: (a) butanol/glacial acetic acid/water in a v/v ratio of 2:1:1 for most kinase assays; (b) pyridine/methanol/water/glacial acetic acid in a v/v ratio of 6:6:4:1 to separated deoxycytidine di- and trinucleotides. The chromatograms were run for 16 h at room temperature and dried in a fume cupboard. The chromatograms were then cut into 1 cm strips transverse to the direction of running, and these strips counted, as described below.

Protein estimations. The amount of protein in a preparation was determined by the method of Lowry et al. (1951).

Detection of radioactivity. Paper strips, discs and glass coverslips were air dried after an alcohol rinse and placed in glass scintillation vials containing 5 ml toluene based scintillant; 2.51 toluene, 12.5 g PPO and 0.125 g POPOP. The vials were then counted in an Intertechnique SL40 Scintillation Counter with an efficiency of 25% for [3H], 75% for [14C] and 90% for [32P].

Sucrose gradients sedimentation. 200 μl of enzyme solution (400 μg) were layered on to
Properties of HSV deoxypyrrimidine kinase

I. Materials and Methods

1. Enzyme Induction

HSV-1 induced thymidine and deoxycytidine kinase activities show similar patterns of induction (Fig. 1). Both activities begin to rise between 2 and 4 h after infection, and continue to increase in parallel until 10 h post-infection, when both activities reach their plateau level. At this time the thymidine kinase and deoxycytidine kinase activities have increased approximately ten- and fivefold, respectively.

Fig. 1. Time course of induction of thymidine and deoxycytidine kinase activities by HSV-1 in RK13 cells (input multiplicity 5 p.f.u./cell). Activity expressed as nmol nucleoside phosphorylated/10 min/μg protein. ▲—▲, thymidine kinase; △—△, deoxycytidine kinase.

2. Biochemical Differences

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RESULTS

Enzyme induction

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Biochemical differences between virus induced thymidine and deoxycytidine kinase activities

When the effect of pH on enzyme activity was measured, the infected cell thymidine kinase exhibited optimal activity at pH 6, while the deoxycytidine kinase exhibited optimal...
Fig. 2. Effect of the pH on HSV-1 induced and BHK C13 thymidine and deoxycytidine kinase activities. ●——●, HSV thymidine kinase; △——△, HSV deoxycytidine kinase; ○——○, BHK C13 thymidine kinase; ▲——▲, BHK C13 deoxycytidine kinase.

activity at pH 7. In contrast, thymidine kinase and deoxycytidine kinase activities from uninfected BHK C13 cells had pH optima of 8 and 7.5, respectively (Fig. 2).

In cell-free extracts from HSV-1 infected PyY/TG/CAR/BUdR cells the thymidine kinase activity was saturated at lower substrate concentrations than the deoxycytidine kinase activity. Fig. 3 shows that the $K_m$ for the thymidine substrate was about 0.4 μM (pH 6) while the $K_m$ for the deoxycytidine substrate was 6.4 μM (pH 7). Both these values differ significantly from those obtained with enzyme from uninfected BHK C13 cells, the respective $K_m$ values being: for thymidine 2.3 μM and for deoxycytidine 9 μM.

HSV induced thymidine kinase activity is known to be more resistant to end product inhibition by TTP than the host activity (Klemperer et al. 1967). Fig. 4(a) confirms this and shows that the virus activity is significantly stimulated by the presence of dCTP, while the host thymidine kinase is inhibited by this triphosphate though much less than by TTP. Fig. 4(b) shows that both host and virus deoxycytidine kinases are inhibited by TTP.

When undiluted enzyme extract was heated at 45 °C the virus thymidine kinase activity was much more stable than the virus deoxycytidine kinase activity (Fig. 5), but both virus activities are more stable than the cellular enzymes. Prior incubation of the virus enzyme at 45 °C in the absence of substrate alters the response of the thymidine kinase activity to dCTP stimulation and TTP inhibition: both disappear coincidentally (Table 1). After incubation for 5 min the stimulation caused by dCTP is reduced by 80% while a further 5 min incubation prevents this stimulation entirely. Five min at 45 °C gives a 55% reduction and 10 min prevents end product inhibition by TTP.

Fig. 6(a) shows that the virus thymidine kinase activity is unaffected by the presence of deoxycytidine in the reaction mixture, up to a concentration of $5 \times 10^{-8}$ M (which is five
Properties of HSV deoxypyrimidine kinase

Fig. 3. Increase of reaction velocity with substrate concentration. Double reciprocal plots of velocity ($V = \text{nmol thymidylate or deoxycytidylate synthesized per 100 \mu l in 5 or 10 min}$) against substrate concentration ($S = \mu M$-thymidine or deoxycytidine). (a) HSV thymidine kinase activity (100 \mu g of protein per 100 \mu l) incubated for 5 min in the presence of $[^3H]$-thymidine, ranging from 0.05 to 2 \mu M. Intercept = 2.2, $K_m = 0.45$ \mu M. Other experiments gave $K_m$ values 0.32 to 0.5 \mu M. (b) $[^3H]$-deoxycytidine ranging from 0.5 to 50 \mu M. Intercept = 0.16, $K_m = 6.4$ \mu M. Other experiments gave $K_m$ values 6 to 7.5 \mu M.

Sedimentation analysis

Crude extract from uninfected cells exhibits one discrete peak of thymidine kinase activity, and one of deoxycytidine kinase activity, on sedimentation through a 5 to 20% sucrose gradient (Fig. 8a), with thymidine kinase migrating approximately twice as far as the deoxycytidine kinase activity. This pattern agrees well with published data on the mol. wt. of these enzymes from mammalian sources; thymidine kinase has a mol. wt. of 100000 (Bresnick, 50% in the presence of $5 \times 10^{-6}$ M-thymidine (which is half the substrate concentration). Neither of the cellular activities are affected by the presence of the other pyrimidine deoxyribonucleosides in the reaction mixture (Fig. 6b).

Analysis of the Lineweaver–Burke plots for deoxycytidine kinase in the presence and absence of thymidine shows that thymidine is a competitive inhibitor of deoxycytidine (Fig. 7a).
Fig. 4. Effect of deoxypyriridine triphosphates on HSV and BHK C13 thymidine and deoxycytidine kinase activities. (a) HSV and BHK C13 thymidine kinase activity. TTP or dCTP was added at the concentration shown to the normal reaction mixture. ■—■, BHK C13 thymidine kinase + TTP; □—□, BHK C13 thymidine kinase + dCTP; ▼—▼, HSV thymidine kinase + TTP; ▼—▼, HSV thymidine kinase + dCTP. (b) HSV and BHK C13 deoxycytidine kinase activity. TTP or dCTP was added at the concentration shown to the normal reaction mixture. ■—■, BHK C13 deoxycytidine kinase + TTP; □—□, BHK C13 deoxycytidine kinase + dCTP; ▲—▲, HSV deoxycytidine kinase + TTP; ▲—▲, HSV deoxycytidine kinase + dCTP.

Fig. 5. Enzyme inactivation at 45 °C. Supernatant fluids from uninfected and infected cells were heated at 45 °C in the presence of 50 mM-tris, pH 7.5. Samples were withdrawn at intervals for determination of thymidine and deoxycytidine kinase activities. ■—■, BHK C13 thymidine kinase activity; □—□, BHK C13 deoxycytidine kinase activity; ▲—▲, HSV thymidine kinase activity; △—△, HSV deoxycytidine kinase activity.
Table 1. Thermal inactivation of TK allosteric sites

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<th>Time of incubation (min)</th>
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<th>Residual activity in the presence of 10^{-4} M-dCTP</th>
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<tr>
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Fig. 6. Substrate inhibition of thymidine and deoxycytidine kinase activities. Deoxycytidine of thymidine was added to the standard reaction mixture, i.e. thymidine kinase: 10 mM-ATP, 10 mM-MgCl₂, 0.02 M-phosphate buffer, pH 6, 10 μM-[³H]-TdR + unlabelled CdR; deoxycytidine kinase: 10 mM-ATP, 10 mM-MgCl₂, 0.02 M-phosphate buffer, pH 7, 10 μM-[³H]-CdR and unlabelled TdR. (a) HSV enzyme activities: ●, thymidine kinase; ○, deoxycytidine kinase. (b) BHK C13 enzyme activities: ●, thymidine kinase; ○, deoxycytidine kinase.
Fig. 7. (a) Increase of reaction velocity with substrate concentration (deoxycytidine) in the presence (Δ——Δ) and absence (●——●) of 5 × 10⁻⁵ M-thymidine. Double reciprocal plots of velocity ($V = \text{nmol of deoxycytidylate synthesized per 100 } \mu\text{l in 10 min}$) against substrate concentration ($S, \mu\text{M}$). Intercepts no deoxycytidine, Δ——Δ $K_m = 6.3 \mu\text{M}, 5 \times 10⁻⁵ \text{M-thymidine} = \circ$; $K_m = 10 \mu\text{M}$. (b) Increase of reaction velocity with substrate concentration (thymidine) in the presence (○——○) and absence (▼——▼) of 5 × 10⁻⁴ M-deoxycytidine. Double reciprocal plots of velocity ($V = \text{nmol of thymidylate synthesized per 100 } \mu\text{l in 5 min}$) against substrate concentrations ($S = \mu\text{M}$). Intercept = −2.3; $K_m = 0.43 \mu\text{M}$.

Thompson & Lyman, 1966), while deoxycytidine kinase has a mol. wt. of 30000 (Durham & Ives, 1970). Cells resistant to BUdR and araC exhibit no trace of either peak (Fig. 8b). After infection of BHK C13 cells with herpes a much faster sedimenting additional peak of enzyme activity is observed, which contains both thymidine and deoxycytidine kinase activities (Fig. 8c), while BHK C13 cells infected with either a BUdR or an araC resistant HSV mutant only exhibit the same slow-running discrete activities found in uninfected cells. The virus enzyme activities are also not resolved by G2000 Sephadex. In extracts from HSV-1 infected BHK C13 cells, the virus activities are excluded in the void vol. while the cell thymidine kinase and the cell deoxycytidine kinase activities are retained on the column and then separately eluted by tris buffer as expected.
Fig. 8. Sucrose gradient sedimentation of cellular enzymes. (a) 200 μl of enzyme extract from BHK C13 cells was layered on top of a 4-8 ml 5 to 20% sucrose gradient. The gradients were centrifuged at 39000 g in a SW39 rotor in a Spinco model L ultracentrifuge at 4 °C for 10 h. 8-drop fractions were collected and assayed for both thymidine and deoxycytidine kinase activity. (b) PyY/TG/CAR/BUDR cell extracts similarly prepared and analysed. △—△, thymidine kinase activity; •—•, deoxycytidine kinase activity. (c) BHK C13 cells infected with 17 syn harvested 8 h after infection. (d) BHK C13 cells infected with a mutant of herpes simplex 17 syn selected for resistance to 25 μg/ml araC harvested 8 h after infection. □—□, thymidine kinase activity; ○—○, deoxycytidine kinase activity.

**Gel electrophoresis**

Herpes virus thymidine kinase activity can be located separately from the cellular enzyme on non-SDS discontinuous polyacrylamide gels (Munyon et al. 1971).

The cell thymidine kinase activity migrates relatively slowly with an Rf value of 0.45 while the deoxycytidine kinase is located just behind the marker dye with an Rf of nearly 1.0. A second small peak of thymidine kinase activity is sometimes found with an Rf of 0.85, although this finding is not a consistent one (Fig. 9a). After infection of BHK C13 cells with wild type virus, a large thymidine kinase peak is found which runs behind the cellular enzyme and moreover this peak also has deoxycytidine kinase activity (Fig. 9b). PyY/TG/CAR/BUDR cells lack both thymidine and deoxycytidine kinase activities and show no detectable activity peaks (Fig. 9c). But when PyY/TG/CAR/BUDR cells are infected with wild type HSV, a peak is observed having both thymidine and deoxycytidine kinase activities and migrating in the position of the large peak found previously only in Fig. 9(b).

**DISCUSSION**

The results presented show both by sucrose gradient sedimentation and by gel electrophoresis that the HSV coded thymidine and deoxycytidine kinase activities cannot be separated from each other but can be separated from the respective cell enzymes. The
Fig. 9. Gel electrophoresis of enzyme extracts. Longitudinal gel slices were placed on strips of Whatman DE 81 paper, saturated with 10 mM-ATP, 10 mM-MgCl₂, 10 µM-[3H]-thymidine or deoxycytidine and 0.02 M-phosphate buffer, pH 6, or 50 mM-tris-HCl, pH 7.5. The strips were incubated at 37 °C in a CO₂ incubator for 1 h, the gel slices were then removed and strips washed and dried before cutting up for counting. The results are plotted as enzyme activity ct/min x 10⁻² per 2 mm strip of paper against distance migrated in gel. The bromophenol blue marker dye taken as the reference point. (a) BHK C13 cell extract. (b) HSV 17 syn infected BHK C13 cell extract. (c) PyY/TG/CAR/BUDR cell extract. (d) HSV 17 syn infected PyY/TG/CAR/BUDR cell extract. []—[], thymidine kinase activity; ●—●, deoxycytidine kinase activity. Note the apparent doublessness of the virus thymidine kinase activity peak in B is not a consistent finding.
Properties of HSV deoxypyrimidine kinase

sedimentation results together with the G200 column chromatography provide an indication that the HSV enzyme may well exist in a multimeric form. We have obtained neither genetic nor biochemical evidence of the existence of subunits containing only one activity: indeed all our results suggest a multimer of identical subunits. Recently obtained extensive genetic evidence of intragenic complementation (A. T. Jamieson & J. H. Subak-Sharpe, unpublished observations) also supports the model of a multimeric enzyme, built up of normally identical monomers.

Our results from kinetic studies show that HSV induces thymidine and deoxycytidine kinase activities synchronously and in parallel. Detailed investigation divulges that these two activities exhibit diverse biochemical properties. Both virus enzyme activities have a lower optimum pH than the respective host activities and like the thymidine kinases induced by vaccinia (McAuslan, 1963) and polyoma viruses (Sheinin, 1966) their $K_m$ is lower than that of the respective host enzyme. Thus the virus activities have higher affinity for their substrates than the respective host specified activities. The deoxycytidine kinase activities induced by HSV are more heat stable than the respective cell enzymes. (Similar findings have previously been reported for vaccinia virus (Kit et al. 1965) and polyoma virus (Sheinin, 1966) induced thymidine kinases). But again, when compared with one another, the HSV thymidine kinase is much more stable than the HSV deoxycytidine kinase activity.

The two HSV induced activities also differ in their response to the addition of deoxy- pyrimidine triphosphates. The virus thymidine kinase activity is more resistant than the cell enzyme to feedback inhibition by TTP, which could reflect the higher substrate affinity of the virus activity, but whereas the cell enzyme is inhibited, the virus thymidine kinase activity is actually stimulated by dCTP.

In contrast the virus deoxycytidine kinase activity is inhibited by both TTP and dCTP just like the cell enzyme, though the inhibition of the cell enzyme activity is weaker than that of the HSV induced activity.

The regulatory effects of both triphosphates on the HSV induced thymidine kinase activity are equally and co-ordinately labile to heat, which may indicate either a common allosteric site or different sites with nearly identical heat sensitivity. As they stand these results suggest that the HSV induced thymidine and deoxycytidine kinase activities are separate functions albeit residing in the same molecule. However, the substrate inhibition studies show that thymidine is a competitive inhibitor for deoxycytidine while the converse could not be demonstrated. There is no hint of competitive inhibition in our experimental results given in Fig. 6(b).

We conclude that herpes virus induces two enzyme activities which are genetically linked and which as yet cannot be physically separated. The two deoxynucleoside substrates appear to be competing for the same active site, but with characteristically different affinities, while the regulatory effects exerted by the two deoxypyrimidine triphosphates are quite diverse.

The enzyme is visualized as having one substrate binding site and two allosteric sites for binding triphosphates. The effects of interacting triphosphates, pH conditions, heat, etc., all would be expected to alter the affinity of the active site, but with greater consequence to deoxycytidine binding than to that of thymidine.

It must be emphasized that our studies were carried out on relatively crude enzyme preparations which could have contained unidentified molecules that affected the competitive substrate inhibitions found.

We consider that the results presented here, together with the genetic evidence (Jamieson et al. 1974) are consistent with the conclusion that HSV codes for a single enzyme, a
deoxypyrimidine kinase, which phosphorylates both thymidine and deoxycytidine at the same active site, but which may contain more than one allosteric site.

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REFERENCES


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