Adenylic Acid: Deoxythymidine 5'-Phosphotransferase: Evidence for the Existence of a Novel Herpes Simplex Virus-induced Enzyme

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SUMMARY

BHK (dPyK-) cells infected with herpes simplex virus type 1 (HSV-1) contain a virus-induced deoxythymidine (dThd)-phosphorylating enzyme. This enzyme uses AMP as phosphate donor and is called AMP:deoxythymidine 5'-phosphotransferase (or kinase). The enzyme was purified over 1300-fold and was found to be specific for an AMP substrate. It can thus be distinguished from virus-specific deoxypyrimidine kinase (dPyK). It was shown that the two substrates AMP and dThd participate in the reaction at a 1:1 molar ratio; the $K_m$ for AMP was 2.3 µM and for dThd it was 2.1 µM. The mol. wt. of the enzyme was estimated to be between 110000 (by glycerol gradient centrifugation) and 90000 (by gel filtration). For optimum activity, the phosphotransferase required an alkaline pH, and 37 °C; the activation energy of the reaction was 18450 cal/mol. The appearance of the enzyme after infection parallels that of viral DNA synthesis-related functions.

INTRODUCTION

Nucleoside kinase was the first enzyme found to be coded by herpes simplex virus type 1 (HSV-1) (Kit & Dubbs, 1963; Kit et al., 1974a). This enzyme activity catalyses the phosphorylation of deoxythymidine (dThd) to TMP and deoxycytidine (dCyd) and was therefore termed deoxypyrimidine kinase (dPyK) (Hay et al., 1970; Jamieson et al., 1974). The HSV-induced enzyme differs from the host cell thymidine kinases with respect to mol. wt. (Kit et al., 1974b; Ogino et al., 1973), substrate affinity (Jamieson & Subak-Sharpe, 1974) and immunogenicity (Thouless, 1972; Thouless & Wildy, 1975; Ogino et al., 1973). Thymidine kinase, a dPyK, is a salvage enzyme of DNA synthesis in uninfected (Bollum & Potter, 1959) as well as in HSV-infected cells (Jamieson et al., 1974). It was shown (Jamieson et al., 1974) that HSV-specific dPyK is indispensable for virus growth in serum-starved cells, but not in actively growing cells.

Both mitochondrial- and HSV-induced thymidine kinase use ATP, GTP, UTP and CTP as phosphate donors, while host-cell cytoplasmic thymidine kinase can only efficiently utilize ATP as phosphate donor (Kit et al., 1974b). In addition, it was reported by Jamieson et al. (1976) that in crude extracts from HSV-infected cells, a dThd-phosphorylating kinase activity is present which also uses AMP as phosphate donor. This novel enzyme was called adenylic acid:deoxythymidine 5' phosphotransferase (or AMP:deoxythymidine kinase). In the present study we have shown that the phosphotransferase activity and the dPyK activity are functions of different enzyme complexes, although these complexes may contain common polypeptides.
METHODS

Materials. Bromodeoxyuridine (BrdUrd) and other nucleosides and nucleotides, as well as streptomycin sulphate, were obtained from Boehringer, Mannheim; 2-14C-dThd (sp. act. 50 mCi/mmol), methyl-3H-dThd (sp. act. 25 Ci/mmol) and 32P-AMP (sp. act. 1.56 Ci/mmol) from The Radiochemical Centre, Amersham; Sephadex G-150, dextran blue and AMP-Sepharose from Deutsche Parmacia, Freiburg, West Germany; haemoglobin (human) and lactate dehydrogenase (ox heart) from Serva, Heidelberg, West Germany.

Cells and viruses. BHK-21 cells grown in Eagle's medium containing 5% newborn calf serum (Just et al., 1975) were used for all studies. The cells were devoid of mycoplasma. Cells lacking the cytosol deoxypyrimidine kinase (BHK-TK-; CIID) were obtained by a selection procedure for BrdUrd resistance described by Jamieson et al. (1974). At the beginning of the experiments the cells were in the G0 phase (confluency). The BHK-TK- cell monolayers were infected with HSV-1 strain Lennette (dPyK+) at an m.o.i of 5 to 10 p.f.u./cell. After an adsorption period of 2 h, the cells were washed twice and incubated in new growth medium at 37 °C for 0 to 24 h and then harvested. Time zero was considered to be at the end of the adsorption period. After different incubation periods the cells were scraped off the culture flask and homogenized in a Dounce homogenizer in 50 mM-tris-HCl buffer pH 7.8 containing 2 mM-dithiothreitol, and stored at -70 °C.

Enzyme assays. All reactions were carried out in a final vol. of 450 μl at 37 °C for 30 min. The dPyK reaction mixture contained 20 mM-tris-HCl pH 7.8, 2 mM-dithiothreitol, 10 mM-MgCl2, 6 mM-ATP, 40 μM-14C-dThd (sp. act. 110 d/min/pmol) and 50 μl enzyme preparation. The reaction was terminated by heating the reaction tubes in boiling water. The reaction product (dTMP) was isolated as described by Dundaroff & Falke (1972). The radioactivity was counted in a mark III (Searle) liquid scintillation spectrometer with automatic quench correction. The standard AMP:dThd kinase assay contained in a final vol. of 450 μl: 25 mM-HEPES buffer pH 7.6, 2 mM-dithiothreitol, 10 mM-MgCl2, 3 mM-AMP, 20 mM-NaF, 4 μM-3H-dThd (sp. act. 55 × 103 d/min/pmol) and 50 or 150 μl enzyme preparations. After incubation for 30 min at 37 °C the amount of dTMP formed was determined (Dundaroff & Falke, 1972). In some experiments 32P-AMP at a concentration of 13 μM (sp. act. 3 × 103 d/min/pmol) was used as tracer; 100 μM-dThd was added to the assays as acceptor. The ADP-dependent dThd kinase assay was identical to the AMP:dThd kinase reaction mixture, except that 3 mM-ADP was added instead of AMP.

The assays were linear with time for at least 60 min. One unit of enzyme is defined as catalysing the formation of 1 pmol dTMP/min. The specific activities of the kinases in HSV-infected cells as well as in uninfected cells are expressed in pmol dTMP formed during the 30 min incubation period/100 μg cell DNA; this amount of DNA is equivalent to 1.1 × 10⁷ cells (Müller et al., 1978).

Analytical methods. Protein concentration was determined either colorimetrically (Lowry et al., 1951) or optically (Kalb & Bernlohr, 1977). DNA was measured according to Burton (1956). The mol. wt. of the enzymes in the glycerol gradient were determined according to Martin & Ames (1961). Calibration of a Sephadex G-150 column for mol. wt. determinations was performed as described earlier (Schröder et al., 1980) using the following marker proteins: phosphorylase a, bovine serum albumin, egg-white albumin and cytochrome c. The elution data of these proteins were included in the calculations, which are summarized in the following equation: log M = 6.124-0.912 (Ve/Vo). The Michaelis constant (Km) and the maximum reaction velocity (Vmax) was determined according to Lineweaver & Burk (1934).

RESULTS

Changes in the kinase activities during infection

The activities of dPyK, as well as of AMP : and ADP : kinase, changed characteristically in HSV-infected cells during the period following infection (Fig. 1). Immediately after infection
Fig. 1. Changes in the kinase activities in extracts from HSV-infected as well as from uninfected cells. At different times, cells were extracted and the activities of the three different kinases were determined. HSV-infected cells: dPyK (○), AMP:dThd kinase (●), ADP:dThd kinase (×); uninfected cells: dPyK (□), AMP:dThd kinase (■) and ADP:dThd kinase (△). The enzyme activities are expressed in pmol TMP formed/100 µg cell DNA.

Table 1. Purification of AMP:deoxythymidine kinase from HSV-1-infected BHK-TK− cells

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Total enzyme activity (units)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>52.2</td>
<td>0.032</td>
<td>1.67</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>II. Streptomycin sulphate</td>
<td>50.3</td>
<td>0.029</td>
<td>1.46</td>
<td>87.4</td>
<td>0.9</td>
</tr>
<tr>
<td>III. Ammonium sulphate</td>
<td>14.2</td>
<td>0.077</td>
<td>0.94</td>
<td>65.3</td>
<td>2.4</td>
</tr>
<tr>
<td>IV. Affinity column chromatography</td>
<td>0.017</td>
<td>42.2</td>
<td>0.70</td>
<td>41.7</td>
<td>1320.3</td>
</tr>
</tbody>
</table>

none of the kinases could be detected under the assay conditions used. However, 6 h post-infection the activities of the three enzymes increased dramatically; at 12 h post-infection the amounts of extractable enzyme activities were as follows: dPyK, 118 pmol TMP formed/100 µg cell DNA (= 10.7 pmol TMP/10⁶ cells); AMP:dThd kinase, 0.12 pmol TMP/100 µg (= 0.01 pmol TMP/10⁶ cells); and ADP:dThd kinase, 0.05 pmol TMP/100 µg (= 0.005 pmol TMP/10⁶ cells). In control cells (TK−), which have not been infected, no kinase activity was detected.

**Purification of the enzyme**

All procedures were carried out at 0 to 4 °C unless stated otherwise; a summary of the steps is given in Table 1.

**Step I: crude extract**

About 3 × 10⁸ cells were harvested 12 h post-infection, suspended in 11 ml of a 50 mM-tris–HCl buffer pH 7.8 containing 2 mM-dithiothreitol, and sonicated for 15 s in a Branson sonifier B15. The homogenate was centrifuged at 120000 g for 30 min. The supernatant was collected (fraction I).

**Step II: streptomycin sulphate**

To remove DNA, fraction I was stirred while streptomycin sulphate solution was added to a final concentration of 1%. After standing for 15 min, the precipitate formed was removed
Fig. 2. Purification of AMP:dThd kinase by affinity chromatography on AMP-Sepharose. A column (0.5 x 4 cm) was equilibrated with 50 mM-tris-HCl buffer pH 7.8 containing 2 mM-dithiothreitol and 10% (v/v) glycerol, and loaded with 0.7 ml of the dialysed fraction III. After washing with 5 ml of the equilibration buffer at a flow rate of 15 ml/h, elution was continued with the same buffer, containing 50 mM-AMP (arrow). Fractions of 1 ml were collected and 150 μl amounts were assayed for AMP:dThd kinase activity (●), as well as for dPyK activity (○); [2, protein content.

by centrifugation (3000 g, 10 min). The supernatant contained 87·4% of the enzyme activity (fraction II).

**Step III: ammonium sulphate**

Fraction II was dialysed for 12 h against 1 l 50 mM-tris-HCl buffer pH 7·8 containing 2 mM-dithiothreitol and 50% saturated (NH₄)₂SO₄. The precipitate formed was collected by centrifugation (3000 g, 15 min) and suspended in 0.7 ml 50 mM-tris-HCl buffer pH 7·8 containing 2 mM-dithiothreitol and 10% (v/v) glycerol, and subsequently dialysed against 1 l of the same buffer (fraction III).

**Step IV: affinity column chromatography**

Fraction III was applied to a column of Sepharose 4B-conjugated AMP. The kinases were eluted with 50 mM-AMP (Fig. 2); while the AMP:dThd kinase appeared as a sharp peak, the dPyK eluted as a broader band. The fractions containing the AMP:dThd kinase (numbers 9 to 12) were pooled and dialysed (24 h) against 50 mM-tris–HCl buffer pH 7·8, 2 mM-dithiothreitol, 10% (v/v) glycerol. Fraction IV was free of DNA. The specific activity of AMP:dThd kinase was determined to be 42·2 units/mg (0·175 units/ml). The protein concentration of this fraction was determined optically.

**Associated kinase activities**

The 1300-fold-enriched AMP:dThd kinase preparation contained 54369 units dPyK/mg (224·3 units/ml) and 11·4 units of ADP-dependent dThd kinase/mg (0·047 units/ml). The dPyK was enriched 1700-fold (yield: 48%) during the described procedure.

**Properties**

The following determinations were performed with fraction IV to elucidate whether the three kinase activities are associated with the same protein molecule.
**HSV-induced AMP: phosphotransferase**

Fig. 3. Sedimentation properties of the three kinase activities in a glycerol gradient. A linear glycerol gradient (10 to 20%, v/v) in 50 mM-tris-HCl buffer pH 7.8, 2 mM-dithiothreitol was loaded with 0.6 ml of fraction IV. Centrifugation at 4 °C for 24 h was carried out in an SW40 rotor (Beckman) at 35,000 rev/min (152,000 g). After centrifugation, 0.66 ml fractions were collected; the direction of sedimentation is to the right. The marker proteins are lactate dehydrogenase (LDH; mol. wt. 140,000) and haemoglobin (Hb; 64,500). A 150 µl amount of each fraction was assayed for AMP:dThd kinase (○), ADP:dThd kinase (x) and dPyK activity (○). No enzyme activities were detected between fractions 10 and 19 (bottom).

Fig. 4. Separation of AMP:dThd kinase from dPyK. A Sephadex G-150 column (0.9 x 90 cm) equilibrated with 50 mM-tris-HCl buffer pH 7.8, 2 mM-dithiothreitol, 10% (v/v) glycerol was loaded with 1 ml of fraction IV. Fractions of 0.6 ml were collected, and 150 µl of each fraction were assayed for AMP:dThd kinase activity (●) and dPyK activity (○). The arrow marks the position of dextran blue. For explanation of V_c/V_0 values, see Determann (1969).

Fig. 5. Double reciprocal plot of the initial velocity studies (Lineweaver & Burk, 1934) of AMP:dThd kinase. The enzyme studies were performed in the standard reaction mixture with either 3H-dThd (●) or 32P-AMP (○) as the variable substrate and 150 µl of fraction IV.
Molecular weight

The mol. wt. of the AMP:dThd kinase was determined by two independent methods; first, by determining the sedimentation behaviour in a linear glycerol gradient and second, by gel filtration. The distribution pattern of the kinase preparation after glycerol gradient centrifugation revealed (Fig. 3) that the AMP:dThd bands at a higher density compared to dPyK and ADP:dThd kinase. Although some overlapping exists the mean mol. wt. (± s.d.) of the enzymes differ and were determined as follows: AMP:dThd kinase, 111000 ± 27 500; ADP:dThd kinase, 75 200 ± 18 400; and dPyK, 74 100 ± 18 200. Fig. 4 depicts the elution pattern of the two enzymes AMP:dThd kinase and dPyK obtained by gel filtration on Sephadex G-150. The pattern shows two peaks; however, again the two enzyme activities could not be separated completely. The mean mol. wt. which were estimated on the calibrated Sephadex G-150 column were found to be 90 600 ± 13 200 for the AMP:dThd kinase and 78 100 ± 11 900 for the dPyK.

Kinetics

The kinetic properties of AMP:dThd kinase were determined by keeping one substrate (either 3 mM-ATP or 110 μM-dThd) at a fixed concentration and varying the concentration of the other substrate (0-67 to 11 μM-3H-dThd or 0-67 to 5 μM-32P-AMP); Fig. 5. From double reciprocal plots the $K_m$ of dThd was calculated to be $2.1 \pm 0.2 \mu M$ and the $K_m$ of AMP to be $2.3 \pm 0.2 \mu M$. The $V_{max}$ values were $1.57 \pm 0.28$ pmol dTMP formed/μg protein × 20 min in the assays with 3H-dThd as substrate and $1.74 \pm 0.31$ pmol/μg × 30 min in the experiments with 32P-AMP. The finding of identical kinetic constants for both substrates suggests that AMP is the only source of phosphate which is transferred to dThd.

Stoichiometry of reaction

To prove that two substrates AMP and dThd participate in the reaction at a 1:1 molar ratio, TMP synthesis was measured as a function of time with 3H-dThd or 32P-AMP as substrates, under otherwise identical incubation conditions (Fig. 6a). The results revealed identical rates of initial velocities of TMP formation irrespective of the nature of the radiolabelled tracer substrate used.
**Effect of enzyme concentration**

Under standard incubation conditions, the reaction velocity was proportional to the enzyme concentration (Fig. 6b).

**Effect of temperature**

The enzyme reaction was strongly dependent upon incubation temperature (Fig. 6c; 4.3 µg enzyme protein was used in the standard assay). The temperature optimum was determined to be 37 to 38 °C; the temperature coefficient $Q_{10}$ (Netter, 1959) for 37/27 °C is 9.1 and for 37/47 °C is 2.7. By plotting $\log_{10}$ of the initial reaction velocity against $1/T$ (Sizer, 1943), the activation energy was found to be 18 500 cal/mol.

**Effect of pH**

Maximum enzyme activity was attained in HEPES buffer at pH 7.6. At pH 6.3 (HEPES/HCl) the enzyme activity was determined to be 17% of the maximum activity and at pH 8.8 (HEPES/NaOH) only 32%.

**DISCUSSION**

From the experiments of Jamieson *et al.* (1974) it is apparent that under special physiological conditions the presence of thymidine-phosphorylating enzyme activity is a prerequisite for HSV replication. It is well documented that the induction of the HSV-induced thymidine kinase runs parallel with that of viral DNA synthesis (Kit & Dubbs, 1969). This virus-induced kinase is a multifunctional enzyme which carries the following activities: thymidine phosphorylation (= thymidine kinase activity) (Jamieson *et al.*, 1974); deoxycytidine phosphorylation (= deoxycytidine kinase activity) (Jamieson *et al.*, 1974); and thymidylate phosphorylation (= thymidylate kinase activity) (Chen & Prusoff, 1978). The phosphate donor in all these enzymic reactions is ATP.

HSV-infected cells contain an AMP-dependent phosphorylating enzyme activity in addition to the ATP-dependent kinases (Jamieson *et al.*, 1976). In the present paper it is demonstrated that in cells infected with HSV-1, the phosphorylating enzyme activity is associated with a protein which can be separated from the enzyme complex with the ATP-dependent kinase activities. This conclusion, based first on the enzyme elution pattern from an AMP-Sepharose affinity column, second on the elution from Sephadex G-150 and third on sedimentation in a glycerol density gradient, indicates that the AMP-dependent thymidine-phosphorylating activity can be separated from the ATP-dependent kinase reaction. Therefore, it is justified to designate the AMP-dependent thymidine-phosphorylating enzyme as a novel HSV-induced enzyme which we have termed AMP : deoxythymidine 5'-phosphotransferase, or AMP : deoxythymidine kinase. From previous studies it is known (Jamieson *et al.*, 1976) that the HSV-1-induced phosphotransferase preferentially phosphorylates thymidine, and with a lower affinity deoxycytidine and deoxyadenosine. In the present study it has been shown that the two substrates AMP and dThd participate in the reaction at a 1:1 molar ratio. This finding rules out the possibility that the observed AMP-dependent thymidine phosphorylation is mediated by a true phosphotransferase. The recently discovered deoxyribozyme triphosphatase (Wohlrab & Francke, 1980), which was found to be specific for HSV-1-infected cells, is not identical with the enzyme described here. Up to now with such activity has not been detected in uninfected vertebrate cells, but only in plants, e.g. radish seedlings (Delseny *et al.*, 1976) and carrot (Brunngraber & Chargaff, 1967). The mol. wt. of the HSV-induced phosphotransferase is between 90000 and 110000, depending on the method used, and it is thus higher than those of the HSV-induced dPyK (85000; Chen & Prusoff, 1978), cytoplasmic host cell thymidine kinase.
In a detailed study it was shown (Ogino et al., 1973) that the HSV-1-induced dPyK can form aggregates, depending on the buffer systems used. Therefore, one could argue that either the HSV-induced dPyK or the cellular thymidine kinase is modified during virus infection by a cofactor resulting in an addition of a new catalytic function. Although this possibility cannot be ruled out at present, the alternative is more likely. This is based upon the finding that analysis of the 1300-fold-enriched AMP:dThd kinase by gel filtration or by density-gradient centrifugation shows that a significant amount of AMP-dependent activity is separated from the ATP-dependent activity.

In the present study we have also described an ADP-dependent phosphotransferase activity. This enzyme has not been detected in earlier studies using HSV-1-infected BHK cells (Jamieson et al., 1976). Characterization of this enzyme activity has not been performed in the present study. The following data seem to eliminate the possibility that the AMP:dThd phosphotransferase is a host-cell enzyme and favour the assumption that it is encoded at least in part in the HSV DNA. Analyses using HSV-1 (dPyK+) -infected BHK cells which lack thymidine kinase and are devoid of mycoplasma revealed: (i) absence of this enzyme in uninfected cells; (ii) induction of the phosphotransferase after HSV-1 infection; (iii) that the kinetics of appearance of the enzyme resemble that of the HSV-induced DNA polymerase (Müller et al., 1978), HSV-induced alkaline DNase (Weissbach et al., 1973; Müller et al., 1978) and HSV DNA synthesis (Müller & Zahn, 1979). To substantiate further that this enzyme is virus-coded, thymidine kinase-positive BHK cells were infected with a naturally developed HSV-1 (dPyK−) variant (strain IES) and assayed for phosphotransferase activity (D. Falke et al., unpublished results). The results showed that at no time after infection was detectable activity of AMP:dThd phosphotransferase present in the cell extract.

Among the kinetic parameters determined, the figure calculated for the activation energy of the AMP:dThd phosphotransferase reaction is unusual. It is found that the high amount of energy, 18500 cal/mol, is required to bring the molecules at an incubation temperature of 37 °C to their activated state. This result helps to understand why the described phosphotransferase reaction, at a 1:1 molar ratio of substrates, is kinetically possible.

In summary, it is now documented that HSV-infected cells contain in addition to an ATP-dependent thymidine-phosphorylating enzyme an AMP- and very likely also an ADP-dependent phosphotransferase. This result further indicates that phosphorylation of thymidine is of physiological importance for HSV-infected cells both in culture, and what is more likely, for infected cells in animals or humans. At present, it is not possible to decide whether the AMP:dThd phosphotransferase represents an enzyme species which is different from the dPyK or whether both enzymes contain the same polypeptide. The latter possibility seems to be more likely because the findings of Jamieson et al. (1976) revealed that both enzyme activities were inactivated by anti-HSV serum with the same efficiency. The difference in mol. wt. of the two enzymes seems to support the suggestion from Jamieson et al. (1976) that host factors may be involved in the AMP:dThd phosphotransferase activity.

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REFERENCES


HSV-induced AMP: phosphotransferase


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