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# NOTES

## Altered Properties of Thymidine Kinase After Infection of Mouse Fibroblast Cells with Herpes Simplex Virus

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Previous studies have shown that, after infection by herpes simplex virus (HSV) or vaccinia virus, thymidine kinase activity was induced in either LM mouse fibroblast cells or LM(TK$^-$) cells, a mutant subline lacking this enzyme activity. At 5 to 7 hr after infection, the enzyme activities induced by these viruses in the LM(TK$^-$) cells were about three times as high as those found in exponentially growing cultures of noninfected LM cells. Mutant vaccinia virus and HSV strains have been isolated which lack enzyme-inducing activity (D. R. Dubbs and S. Kit, Virology **22**:214, 1964; Virology **22**:493, 1964). Partially defective HSV mutants have been obtained which fail to induce the enzyme at 37 C, but which induce about one-tenth the enzyme level attained with the wild-type virus at 31 C (D. R. Dubbs and S. Kit, Virology **25**:256, 1965). The thymidine kinase partially purified from vaccinia virus-infected LM(TK$^-$) cells differed from the enzyme from noninfected LM cells in thermal stability, serological properties, and in its Michaelis constant ($K_m$) for deoxyuridine (dU) (S. Kit and D. R. Dubbs, Virology **26**:16, 1965). The LM cell- and the HSV-induced enzymes, however, were thermally inactivated at about the same rate. Data are now presented demonstrating that the kinetic properties of the HSV-induced thymidine kinase are different from those of the enzyme from either noninfected or from vaccinia virus-infected cells.

Cultures of LM and LM(TK$^-$) cells were grown as described previously (S. Kit and D. R. Dubbs, Virology **18**:274, 1962). Confluent monolayer cultures were infected with these viruses at input multiplicities of 1 to 7 plaque-forming units (PFU) per cell. The cells were harvested 6 to 7 hr after infection. Cell extracts containing thymidine kinase activity were assayed with tritium-labeled deoxyuridine ($^3H$-dU) as nucleoside substrate. The assay was routinely performed at three enzyme concentrations, and activity was proportional to enzyme concentration under the conditions employed. The enzyme was partially purified by the procedure previously described (S. Kit et al., Virology **29**:69, 1966), except for the following modifications. (i) The centrifuged enzyme extracts (fraction S3) were first mixed for 8 min at 4 C with calcium phosphate gel (1.5 mg of gel per mg of protein in fraction S3). (ii) After centrifugation to remove the calcium phosphate gel, 0.14 g of ammonium sulfate was added slowly to the supernatant fluid. The precipitate was discarded, and an additional 0.14 g of ammonium sulfate was slowly added to precipitate the thymidine kinase activity (As 20–40 fraction). The enzyme was then dissolved in buffer solution [0.15 M KCl; 0.01 M tris(hydroxymethyl)-aminomethane (Tris chloride buffer), *p*H 8.0; 0.003 M 2-mercaptoethanol] in one-fourth the volume of the crude-enzyme extract at a concentration of 0.24 to 0.59 mg of protein per ml of enzyme. (iii) With enzyme extracts from vaccinia virus-infected cells, the centrifuged enzyme fraction (S3) was mixed with calcium phosphate gel equivalent to 0.5 mg of gel per mg of protein. The reduction in the amount of calcium phosphate gel was necessary because the vaccinia virus-induced enzyme differed from the LM cell- and the HSV-induced thymidine kinases in that it was tightly adsorbed by higher amounts of calcium phosphate.

By use of the modified procedure, approximately one-fifth to one-third of the total activity present in crude-enzyme extracts was recovered. The purified LM, herpes simplex-induced, and vaccinia-induced thymidine kinases, respectively, exhibited 4 to 18, 12 to 28, and 5 times greater specific activities than the crude-enzyme extracts. The specific activity of the purified vaccinia-induced enzyme was 64 [expressed as millimicromoles of deoxyuridine monophosphate (dUMP) formed per milligram of protein in 10 min at 38 C]. The specific activities of the herpes simplex-

TABLE 1. *Michaelis constants ($K_m$) for deoxyuridine of thymidine kinases partially purified from noninfected LM and from virus-infected LM(TK⁻) cells*

| Source of enzyme | $K_m$ ($\times 10^{-5}$ M) | No. of preparations assayed |
|---|---|---|
| LM mouse fibroblast cells | 3.2 ± 0.11 | 8 |
| Herpes simplex virus-infected LM(TK⁻) cells | 1.9 ± 0.15 | 7 |
| Vaccinia virus-infected LM(TK⁻) cells | 8.7 | 1 |
| LM mouse fibroblast cells[a] | 3.5 ± 0.4 | 14 |
| Vaccinia virus-infected LM(TK⁻) cells[a] | 7.2 ± 0.5 | 12 |

[a] Results of a previous study (S. Kit and D. R. Dubbs, Virology **26:** 16, 1965).

induced and LM cell enzymes, respectively, varied from 21 to 103 and 7 to 28.

From Lineweaver-Burk plots, the $K_m$ values at 38 C for dU were calculated for the enzyme from noninfected and from virus-infected cells (Table 1). In confirmation of previous studies (S. Kit and D. R. Dubbs, Virology **26:**16, 1965), in which thymidine kinase was purified by a different method, it was found that the $K_m$ value of the vaccinia virus-induced enzyme was considerably greater than that of the corresponding enzyme from noninfected LM cells. Table 1 shows, however, that the $K_m$ value of the HSV-induced enzyme was significantly smaller than that of the LM cell enzyme. An interesting feature revealed by the Lineweaver-Burk plots was the following. The plot of the reciprocal of activity versus the reciprocal of substrate concentration curved upwards at high dU concentrations ($1.3 \times 10^{-4}$ M) with the HSV-induced enzyme, but not with the LM or vaccinia virus-induced enzymes. Thus, inhibition by excess substrate was encountered with the HSV-induced enzyme, but not with the other enzymes.

The nucleoside 5-trifluoromethyl-2'-deoxyuridine ($F_3dT$) (P. Reyes and C. Heidelberger, Mol. Pharmacol. **1:**14, 1965) competetively inhibits the phosphorylation of $^3H$-dU by thymidine kinase (S. Kit, *in* J. Colter [ed.], Symposium on the Molecular Biology of Viruses, *in press*). Figure 1 shows that the enzyme reaction catalyzed by the HSV-induced thymidine kinase is considerably more susceptible to inhibition by $F_3dT$ than the corresponding enzyme from noninfected LM cells. Moreover, the phosphorylation of $^3H$-dU catalyzed by the HSV-induced enzyme was inhibited at lower $F_3dT$ concentrations than the vaccinia virus-induced enzyme. In the experiments of Fig. 1, the $^3H$-dU concentration was held constant at $6.6 \times 10^{-5}$ M. However, similar differences between the HSV-induced enzyme and the LM cell enzyme were found at $^3H$-dU concentrations varying from $10^{-5}$ to $1.3 \times 10^{-4}$ M.

In other studies, it has been found that the $K_m$ values (dU) for partially purified thymidine

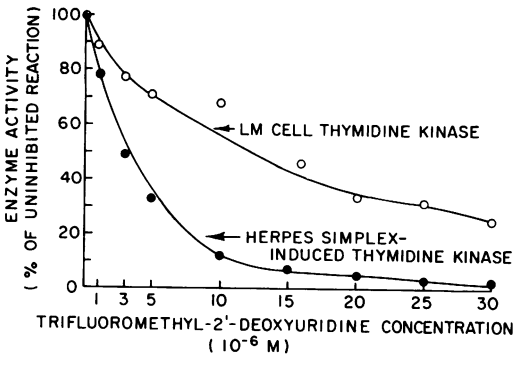


FIG. 1. *Effect of 5-trifluoromethyl-2'-deoxyuridine ($F_3dT$) on the phosphorylation of $^3H$-dU by thymidine kinases partially purified from noninfected LM and HSV-infected LM(TK⁻) cells. The reaction tubes contained in a total volume of 0.125 ml the following substances at the indicated final concentrations: $F_3dT$ (as shown); $^3H$-dU, 0.066 mM; ATP, 12 mM; 3-phosphoglycerate, 14.4 mM; $MgCl_2$, 16 mM; Tris chloride buffer, 96 mM; pH 8.0; and 7, 10.5, and 17.5 or 9, 13.5, and 22.5 μg of protein, respectively, of the purified LM enzyme and herpes simplex-induced enzyme fractions. The initial specific activity (100% activity) of the LM cell enzyme was 13.9 and that of the herpes simplex-induced enzyme was 103. The enzyme reaction was terminated after an incubation period of 10 min at 38 C by the addition of trichloroacetic acid.*

kinase preparations from noninfected LM mouse fibroblasts, monkey kidney (GMK or CV-1), and primary cultures of mouse kidney cells were all approximately the same. The $K_m$ values of the thymidine kinases from these cell lines were all increased two- to threefold after infection by vaccinia virus, a deoxyribonucleic acid (DNA)-containing poxvirus which replicates in the cytoplasm (S. Kit, *in* J. Colter [ed.], Symposium on the Molecular Biology of Viruses, *in press*). Papovavirus SV40 and adenovirus SV15 are DNA-containing viruses which replicate in the nuclei of cells. The $K_m$ values of thymidine kinases prepared from cells infected by the latter viruses were also elevated (S. Kit et al., Virology **29:**69,

TABLE 2. *Effect of adding dTTP ($10^{-4}$ M) to enzyme extraction buffer on the activity of LM cell thymidine kinase during partial purification*

| Enzyme fraction and source | Specific activity of enzyme extracted[a] | | Total amt (mg) of protein in fraction of enzyme extracted | | Total activity in fraction of enzyme extracted[b] | |
|---|---|---|---|---|---|---|
| | Without dTTP | With dTTP | Without dTTP | With dTTP | Without dTTP | With dTTP |
| Centrifuged extract (S3) | 2.3 | 0.4 | 25.4 | 25.4 | 58.4 | 10.2 |
| Calcium phosphate gel[c] | 5.9 | 3.2 | 7.7 | 7.3 | 45.4 | 23.4 |
| Second $(NH_4)_2SO_4$ precipitation[c] | 20.5 | 20.0 | 0.6 | 0.5 | 12.3 | 10.0 |

[a] Specific activity expressed as millimicromoles of dUMP formed per milligram of protein in 10 min at 38 C.

[b] Total activity of fraction: specific activity times the amount (milligrams) of protein in fraction.

[c] The S3 fraction was shaken with calcium phosphate gel (1.5 mg of gel per mg of enzyme protein) for 8 min at 4 C. After centrifugation to remove the calcium phosphate, 0.14 g of $(NH_4)_2SO_4$ per ml of supernatant fluid was added. The small precipitate which formed was discarded. An additional 0.14 g of $(NH_4)_2SO_4$ per ml was added to precipitate the thymidine kinase activity. The enzyme was dissolved in 0.15 M KCl, 0.003 M mercaptoethanol and 0.01 M Tris chloride buffer (*p*H 8), and was reprecipitated by the addition of 0.23 g of $(NH_4)_2SO_4$ per ml. The purified enzyme was dissolved in the buffer solution and assayed at dU concentrations varying from $10^{-5}$ to $1.3 \times 10^{-4}$ M to determine the $K_m$ values.

1966; J. Virol. **1**:10, 1967). It is therefore interesting that the $K_m$ value of the HSV-induced enzyme, another DNA-containing virus that replicates in the nucleus, had a reduced $K_m$ value, thus differing from the $K_m$ values of all of the other enzymes studied.

Thymidine triphosphate (dTTP) exerts a feedback inhibition on crude or purified thymidine kinase preparations from LM or vaccinia virus-infected LM($TK^-$) cells. The degree of inhibition is strongly modulated by the concentrations of the substrates, dU and adenosine triphosphate (ATP). The enzymes from LM and vaccinia virus-infected LM($TK^-$) cells were nearly equal in susceptibility to inhibition by dTTP at several ATP and dU concentrations (S. Kit and D. R. Dubbs, Virology **26**:16, 1965). To learn whether the LM- and the HSV-induced thymidine kinases differed in susceptibility to dTTP inhibition, these enzymes were studied at varying dTTP concentrations, and with dU and ATP concentrations of $6.6 \times 10^{-5}$ and $9.6 \times 10^{-3}$ M, respectively. Both enzymes were inhibited about 57% and 33 to 46%, respectively, by $5 \times 10^{-5}$ and $10^{-4}$ M dTTP.

The fact that the $K_m$ values of the LM, the vaccinia-induced, and the HSV-induced thymidine kinases are all different suggests that new and distinctive proteins are induced by vaccinia and HSV. The isolation of mutant virus strains as well as the demonstration of other distinctive properties support this conclusion. An alternative hypothesis is that host cells contain several allelic cistrons for thymidine kinase and that specific derepressors for these are generated by vaccinia and by HSV. One should also consider the possibility that the alterations in $K_m$ values are attributable to conformational changes in the enzymes; these conformational changes could perhaps be ascribed to variations in the intracellular dTTP concentrations after infection. To study this last possibility, the LM cell enzyme was extracted with buffer containing inhibitory concentrations of dTTP ($10^{-4}$ M) and then purified. Table 2 shows that the centrifuged enzyme fraction (S3) which contained dTTP was indeed inhibited. However, the dTTP was very loosely bound to the enzyme; calcium phosphate gel treatment and two ammonium sulfate precipitations removed the dTTP, so that the specific activity of the enzyme extracted in buffer containing dTTP was about the same as that of the enzyme extracted without dTTP. Moreover, the $K_m$ values of the two enzyme preparations were indistinguishable. The present experiments and previous studies (S. Kit, *in* J. Colter [ed.], Symposium on the Molecular Biology of Viruses, *in press*) demonstrate that changes in intracellular pools of loosely bound metabolites do not account for the observed differences between $K_m$ values of host-cell and virus-induced enzymes. They do not, however, rule out the possibility that unknown products of virus infection are tightly bound to the enzymes in infected cells and change the conformations of the latter and, hence, their kinetic properties.

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