Vaccinia Virus-Induced Ribonucleotide Reductase Can Be Distinguished from Host Cell Activity

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Increased ribonucleotide reductase activity has been detected in vaccinia virus-infected BSC-40 cells. We have studied certain biochemical and kinetic properties of CDP reduction in extracts from infected and uninfected cells. ATP inhibited reductase activity in crude extracts by rapid and extensive substrate phosphorylation. Substitution of adenylylimido-diphosphate (AMP-PNP), a noncleavable analog that functions as positive activator for reductase, but inhibits phosphorylation and cleavage of substrate, allowed us to reliably measure reductase activity. In the presence of AMP-PNP, CDP reduction by extracts from infected or uninfected cells was linear with time for 60 min and with enzyme concentration, except at very low enzyme levels. Activities from both sources were optimally active at pH 8.1. Variation of AMP-PNP and Mg^{2+} concentrations revealed, however, that in the absence of exogenous Mg^{2+} , AMP-PNP strongly stimulated virus-induced CDP reduction, but inhibited endogenous CDP reduction. In the presence of the activator, increasing Mg^{2+} concentrations progressively inhibited the induced activity, but stimulated the endogenous activity up to a 1:2 Mg^{2+} /activator molar ratio. The vaccinia virus-induced activity was highly dependent on AMP-PNP and was not detectable over underlying cellular activity in its absence. Determination of substrate kinetics with respect to CDP revealed a threefold-lower K_m for the virus-induced enzyme as compared with the cellular enzyme. These data suggest, but do not prove, that a novel ribonucleotide reductase is expressed on infection by vaccinia virus.

We have shown in the accompanying paper (16) that a marked induction of ribonucleotide reductase (ribonucleoside-diphosphate reductase, EC 1.17.4.1) activity occurs after the infection of BSC-40 cells with vaccinia virus. Although the time course of the increased activity resembles that of other well-characterized products of early viral transcription (8), determination of the viral or cellular origin of the induced activity will require genetic evidence such as has been recently presented for thymidine kinase (6, 20) and DNA polymerase (15, 17). The complex allosteric regulation to which ribonucleotide reductases are subject has allowed investigators to study virus-induced reductases even in the presence of endogenous host cell activity, since conditions can sometimes be found which preferentially suppress or activate one or the other enzyme (7, 12). We have used this approach to delineate some characteristics of the vaccinia virus-induced activity in cell-free extracts.

In most instances, ribonucleotide reductase is an essential enzyme for replication, since it provides the only source of de novo-synthesized deoxyribonucleotide precursors for DNA synthesis. In both bacteria and eucaryotes, reduction of the four ribonucleoside diphosphate substrates is accomplished by a single enzyme, which is differentially directed toward substrates by the relative concentrations of dATP, dTTP, and dGTP. Overall activity is regulated by binding of ATP (activation) or dATP (inhibition) to a site separate from that determining substrate specificity (5, 19).

The reductases encoded by bacteriophage T4 and by the herpesviruses deviate from this general scheme in that they are insensitive to dATP inhibition (1, 2, 7, 12, 13). The herpesvirus enzyme, in addition, is fully active in the absence of ATP activation and fails to respond to allosteric regulation by deoxyribonucleoside triphosphates. Indeed, free ATP and also free Mg^{2+} are apparently inhibitory (1). Since these are the only viral reductases characterized to

date, one might suspect that relaxation of regulatory restraints on ribonucleotide reduction may be advantageous to the viral lifestyle in that it may facilitate a rapid rate of DNA synthesis. The rate-limiting role of cellular ribonucleotide reductase, which maintains precursor pools sufficient to support replication for only a brief period, is exemplified by the effectiveness of reductase inhibitors such as hydroxyurea and thymidine in blocking DNA synthesis.

Experiments reported in the accompanying paper established that the enhanced CDP reduction in vaccinia virus-infected cells resembled the endogenous activity in sensitivity to dATP and dTTP (16). However, in the course of early experiments we noticed that the induced activity seemed more responsive to ATP activators and less sensitive to low substrate concentrations, as compared with the activity in uninfected cells. A systematic study of activator and Mg^{2+} requirements has clarified and extended these observations. We report that the vaccinia virus-induced activity is distinguishable from host cell reductase in activator and Mg^{2+} requirements and in affinity for at least one of its four substrates.

MATERIALS AND METHODS

The sources of radiochemicals, reagents, and supplies were as described in the accompanying paper, as were maintenance of host cells and vaccinia virus and the assay of ribonucleotide reductase activity (16).

Determination of substrate diversion. Ribonucleotide reductase reactions were scaled up to 120 μ l and incubated at 37°C as described in the accompanying paper (16). At intervals (0, 5, 15, and 30 min), 5- μ l samples from each reaction were added to 10 μ l of ice-cold 10% trichloroacetic acid. After 15 min, precipitated macromolecules were removed by centrifugation, and 10 μ l was spotted onto polyethyleneimine-cellulose plastic-backed thin-layer chromatography plates that had been prespotted with the appropriate markers. Plates were developed with 1 M LiCl, and

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FIG. 1. Chromatographic separation of ribonucleotide reductase reaction products and substrate on cellulose thin-layer chromatography. Reactions containing extract from vaccinia virus-infected cells (10 PFU/cell) were terminated after incubation at 37°C for 0 min (○) or 60 min (●). Chromatograms were spotted and run as described in the text, except that the 1.5-cm-wide lanes were sectioned into 1-cm pieces for counting. The inset shows the dCMP-dUMP region with an expanded ordinate scale.

the radioactivity migrating as CTP, CDP, and CMP was quantitated by scintillation counting as described in the accompanying paper for the reductase reaction (16).

RESULTS

Detection of reaction products. To reliably assay CDP reduction in crude extracts, it is desirable to use a deoxyribonucleotide detection methodology that allows quantitation of various side reactions. The separation of reaction products on cellulose-coated thin-layer chromatography sheets as described above provides a sensitive, convenient, and relatively rapid assay for CDP and UDP reduction which has certain advantages over other methods commonly used for assay of ribonucleotide reductase. Figure 1 shows that the products (dCMP and dUMP) are completely separated from the unreacted substrate (CMP) and from cytosine. Cytidine migrates to a position between dUMP and cytosine. The presence of cytosine in commercial preparations of radioactive CDP (commonly 3 to 5% in our experience), or its generation during incubations, interferes with analysis with borate affinity columns to separate ribo- and deoxyribonucleosides because cytosine elutes as false-positive radioactivity (1). Chromatography of acid-hydrolyzed reaction products on cellulose allows the use of [³H]CDP without further purification and quantitation of any cytosine or cytidine that may be generated without resort to double column procedures (9, 14).

Substrate diversion. The assay of ribonucleotide reductase in crude extracts is complicated by the presence of substrate-diverting activities. Extracts prepared from both infected and uninfected BSC-40 cells harbor hydrolytic enzymes that dephosphorylate CDP to CMP and thence to cytidine and kinase activities that phosphorylate the substrate to CTP. To assess the extent to which competing reactions deplete the substrate under various assay conditions, we sampled standard reactions containing either ATP or the ATP analog, adenylylimido-diphosphate (AMP-PNP), at 0, 5, 15, and 30 min, and quantitated the proportion of the added [³H]CDP label that migrated as CMP, CDP, and CTP on polyethyleneimine-cellulose thin-layer chromatograms.

The cleavage pathway was greatly favored in the absence of exogenous ATP or Mg²⁺, whereas addition of ATP to the reaction mixtures stimulated the kinase activity (Fig. 2). In all cases, only a fraction of the added substrate remained at the diphosphate level. These reactions occurred very rapidly, and the steady-state distribution of nucleotides shown was achieved within 5 to 15 min at 37°C.

The noncleavable ATP analog AMP-PNP was effective at minimizing substrate diversion. In the presence of this compound, added substrate was maintained as 55 to 65% CDP. Small differences in the extent of substrate diversion between extracts from uninfected and infected cells, although consistently observed, were judged not to have significant effects on relative rates of ribonucleotide reduction.

Linearity of reaction with time and enzyme concentration. Under standard reaction conditions employing AMP-PNP as the activator, the rate of CDP reduction by extracts from either uninfected or infected cells was linear with time for at least 60 min (Fig. 3A). For the determination of kinetic parameters, a reaction time of 15 min was used to ensure that an insignificant fraction (<5%) of the substrate was consumed.

In standard reactions, cell-free extract was present at a final concentration of 2.5×10^7 to 5×10^7 cell equivalents per ml (approximately 1 to 2 mg of soluble protein per ml). The rate of CDP reduction was linear with enzyme concentration in this range (Fig. 3B). However, we observed nonlinearity at the lowest enzyme concentrations tested, with infected as well as uninfected cell extracts.

pH optima. A broad pH optimum of 7.8 to 8.4 was determined in 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-sodium buffer for the vaccinia virus-induced ribonucleotide reductase (Fig. 4). The activity at pH 8.1 was fourfold that observed at pH 7.2.

Activation of CDP reduction by ATP and AMP-PNP. Stud-

FIG. 2. Effects of ATP (10 mM) and AMP-PNP (10 mM) on conversion of [³H]CDP to [³H]CMP and [³H]CTP. Reductase reaction mixtures containing 50 μM [³H]CDP (100 cpm/pmol), with or without the indicated concentrations of ATP activator, were sampled at 0, 5, 15, and 30 min. The bars represent the steady-state distribution of radioactivity in phosphorylated cytidine nucleotides achieved within 15 min at 37°C in the presence of infected (A) or uninfected (B) cell extracts.

ies of purified ribonucleotide reductase have demonstrated that CDP reduction by the mammalian enzyme is dependent on positive activation by ATP and is stimulated by, but does not require, Mg^{2+} (5). The reductase induced by herpesviruses, on the other hand, is independent of ATP (1, 13). It was of interest, therefore, to investigate the positive activator requirements of the vaccinia virus-induced enzyme. Increasing concentrations of ATP progressively inhibited reduction by both uninfected and infected cell extracts, although activity in infected samples was always higher than the corresponding control (Fig. 5). In light of substrate diversion studies, we attribute the apparent inhibitory effect of ATP to conversion of CDP to CTP in this crude enzyme system. The use of AMP-PNP, however, revealed a clear-cut difference between host cell- and virus-induced CDP reduction in that the induced activity was markedly stimulated by increasing AMP-PNP concentrations, whereas host cell activity was inhibited (Fig. 6).

Effects of AMP-PNP and Mg^{2+} on endogenous and induced CDP reductase. The finding that endogenous and induced reductase responded differently to AMP-PNP led us to investigate in more detail the activator and Mg^{2+} requirements of these activities. The results of these experiments (Fig. 6) confirmed that the virus-induced and host activities differ markedly in their responses to these modulators.

To investigate the requirements of the vaccinia virus-induced enzyme, we carried out reductase assays under three sets of conditions: (i) increasing concentrations of AMP-PNP in the absence of added Mg^{2+} , (ii) increasing concentrations of AMP-PNP in the presence of constant Mg^{2+} concentration, and (iii) increasing concentrations of Mg^{2+} in the presence of constant AMP-PNP concentration. The results are plotted both as absolute activities (Fig. 6A, B, and C) and as percentages of maximal activity under a given set of conditions (Fig. 6D, E, and F).

Figure 6A and B show that the induced activity in crude

FIG. 3. Dependence of CDP reduction on reaction time (A) and on the amount of extract added to reactions (B) of BSC-40 cell extract (●) or vaccinia virus-infected (10 PFU/cell) cell extract (○). The assays in the experiment in A contained 20×10^5 cell equivalents per reaction.

FIG. 4. Effect of pH on ribonucleotide reductase activity in extracts from uninfected (●) and virus-infected (○) BSC-40 cells.

cell-free extracts was more dependent upon exogenous activation than was the host enzyme, since no enhancement of CDP reduction was observed in infected cell extracts in the absence of AMP-PNP, even at high multiplicities of infection (10 to 20 PFU/cell). Extracts from uninfected cells, on the other hand, displayed low, but easily detected, levels of activity under these conditions. Increasing concentrations of AMP-PNP strongly activated the vaccinia virus-induced activity, an effect that was observed whether or not exogenous Mg^{2+} was present. The endogenous enzyme displayed a differential response to the activator, depending on the relative Mg^{2+} concentration. In the absence of the cation, the activity was progressively inhibited (Fig. 6A and D), whereas in its presence, CDP reduction was stimulated as much as fivefold by AMP-PNP (Fig. 6B and E). In the inverse experiment, Mg^{2+} produced a twofold stimulation of endogenous activity, but progressive inhibition of virus-induced activity (Fig. 6C and F).

To control for differential substrate diversion in these experiments, the relative proportion of input [³H]CDP present as mono-, di-, and triphosphate at the end of the reactions was monitored (data not shown). In general, increasing Mg^{2+} concentrations favored the cleavage of the substrate to CMP, whereas increasing AMP-PNP concentrations favored the retention of the diphosphate. CTP was less than 10% in all cases. Consideration of these effects on actual substrate concentration led to the conclusion that in reactions containing Mg^{2+} , but no AMP-PNP, low CDP levels due to unopposed, Mg^{2+} -stimulated cleavage reactions may have contributed to the exaggeration of the apparent dependence of both endogenous and induced activities on AMP-PNP. However, in no case do the activity curves correlate with the actual CDP concentration deduced from diversion data.

Substrate kinetics with respect to CDP. Using standard assay conditions (5 mM AMP-PNP, 2 mM magnesium acetate), we determined the effects of substrate concentration

FIG. 5. Effects of ATP on ribonucleotide reductase activity in uninfected (●) and virus-infected (○) cells. BSC-40 monolayers were infected at 10 PFU/cell and harvested 5 h postinfection. Magnesium acetate was omitted from the reaction mixtures.

on reaction rate for the vaccinia virus-induced ribonucleotide reductase. The actual substrate concentration in individual reactions was estimated by correcting for changes due to substrate diversion during the 15-min reaction period, as monitored by subjecting 2- μ l samples of the reaction mixture to polyethyleneimine-cellulose chromatography. A preliminary experiment established that the proportion of CDP converted to CMP and CTP under conditions of the experiment did not vary with the absolute CDP concentration and was 30 to 32% in all cases (data not shown).

A K_m of 11 μ M was determined for the induced activity by computer analysis of the data shown in Fig. 7 with the programs of Cleland (3). The K_m for CDP for the endogenous enzyme determined concurrently (30 μ M) is in agreement with published values for the mammalian reductase (5).

DISCUSSION

In the accompanying paper (16), we showed that the large DNA virus, vaccinia virus, strongly induced ribonucleotide reductase activity in BSC-40 cells. The time course after infection was coincident with the wave of viral DNA synthesis, with activity peaking at 4 to 5 h and declining to background levels by 8 h. The induced enzyme required iron and dithiothreitol for full activity, suggesting that, like most known reductases, it is an iron-binding, sulfhydryl protein. Susceptibility to hydroxyurea inhibition implicates free radical involvement in the reductive reaction. Sensitivity to the negative allosteric effectors dATP and dTTP distinguished the vaccinia virus-induced reductase from the herpesvirus enzyme, which is reported to lack positive or negative regulation by nucleoside triphosphates (1, 7, 12, 13).

In this report, we show that the activity induced in vaccinia virus-infected cells and the endogenous cellular reductase exhibit differential responses to both positive activators and Mg^{2+} . We also report that the induced enzyme has a threefold lower K_m for CDP than does the cellular enzyme. These results support the notion that the vaccinia virus-induced reductase, like that induced by bacteriophage T4 in *Escherichia coli* and by herpesviruses in mammalian cells, is a novel enzyme distinct from that of the host cell.

The assay of ribonucleotide reductase with crude extracts as the enzyme source is complicated by the presence of competing activities that rapidly deplete the ribonucleoside diphosphate substrate (1, 10). Consequently, studies of ribonucleotide reduction are best carried out on purified, or at least partially purified, enzymes. Early in the course of this work, however, it became apparent that the vaccinia virus-induced activity was extremely unstable, losing more than 50% of the initial activity in a few hours at 4°C and over 95% of the initial activity on storage of infected cells or cell-

FIG. 6. Effects of AMP-PNP and Mg^{2+} concentrations on ribonucleotide reductase activity in extracts from uninfected (●) and virus-infected (○) BSC-40 cells. Results are shown as the actual activity measured (A, B, and C) and as the percentage of maximal activity observed under a given set of conditions (D, E, and F). Panels A and D show increasing AMP-PNP concentration in absence of exogenous Mg acetate; panels B and E show increasing AMP-PNP concentration in the presence of 3 mM exogenous Mg acetate; and panels C and F show increasing Mg^{2+} concentration in the presence of 6 mM AMP-PNP. The multiplicity of infection was 20 PFU/cell in A and 40 PFU/cell in B and C.

free extracts for 1 week at -20°C . Reductase activity in extracts of uninfected BSC-40 HeLa cells displayed no such lability. We elected, therefore, to utilize only freshly prepared crude extracts in these studies.

Increasing ATP concentrations inhibited CDP reduction by extracts from both infected and uninfected BSC-40 cells, presumably a consequence of the phosphorylation of $>70\%$ of substrate to CTP. However, reduction in infected cell extract not only was greater than in uninfected cell extract at all ATP concentrations tested, but also appeared to be more resistant to inhibition. In light of subsequent results (below), we attribute this "resistance" to a greater sensitivity of the induced enzyme to activator and to the lower K_m for CDP. Omission of ATP from reactions, on the other hand, favored extensive cleavage of the substrate to the monophosphate level and subsequent accumulation of cytidine due to phosphatase activities. Under these conditions, only 12 to 13% of input $[^3\text{H}]\text{CDP}$ remained at the diphosphate level after 30 min, and very little vaccinia virus-specific reduction was detected.

The noncleavable ATP analog AMP-PNP, which has been shown to activate L-cell ribonucleotide reductase (10), inhibited both phosphorylation and cleavage of CDP. In the presence of the analog, the major part of the input $[^3\text{H}]\text{CDP}$ remained at the diphosphate level after a 30-min incubation, and reductase activity was easily detected in both uninfected and infected cell-free extracts. Virus infection did not significantly alter the kinetics or extent of substrate diversion, although we did note that in the presence of AMP-PNP infected cell extracts consistently maintained a slightly higher proportion of radioactivity as $[^3\text{H}]\text{CDP}$. The cleavage of cytidine nucleotides to cytosine by nucleotide phosphorylase, reported to be enhanced in herpesvirus-infected cells (1), was not detected.

The validity of a standard assay employing AMP-PNP as the activator was confirmed by the linearity of CDP reduction with respect to time and also with respect to the amount of enzyme added to the reactions, except at the lowest extract levels tested. The latter result is characteristic of most reductases that have been studied and is thought to be due to concentration-dependent dissociation of the nucleotide-binding regulatory subunit from the free radical-containing catalytic subunit (4). The activities from both infected and uninfected cells were stimulated fourfold by an increase in buffer pH from 7.2 to 8.1, but the induced activity displayed a broader pH optimum similar to that reported for the reductase induced by herpesvirus (1).

Increasing the AMP-PNP concentration from 0 to 6 mM enhanced the CDP reducing activity of infected cell extracts 26-fold, whereas CDP reduction by control cell extracts increased only 5-fold (in the presence of Mg^{2+}), leading to the tentative conclusion that the induced reductase is more responsive to activator than is the endogenous cellular reductase. The host reductase was activated by AMP-PNP only in the presence of exogenous Mg^{2+} . Although unknown amounts of endogenous ATP and Mg^{2+} present in the cell-free extracts preclude precise determination of the optimal activator/ Mg^{2+} ratio, an apparent optimum at a 2:1 molar ratio was observed in several independent experiments, suggesting an Mg^{2+} -AMP-PNP complex as the active agent. Endogenous contributions should be minor since the cellular contents were diluted at least 10-fold in the final reaction mixtures.

Magnesium ions are commonly included in assays of ribonucleotide reductase, although their role is not well

FIG. 7. Determination of K_m for CDP in extracts of vaccinia virus-infected cells. The indicated substrate concentrations represent the actual CDP added to standard reactions corrected for conversion of substrate to CMP and CTP during the reaction period. The reaction velocity is expressed as picomoles per 15 min per 3×10^5 cell equivalents. The multiplicity of infection was 40 PFU/cell.

defined. The enzyme from *E. coli* is active in the presence of Mg^{2+} , which is apparently required for subunit association (18). The reductases of algae and yeasts, however, which also dissociate readily, exhibit full activity without exogenous divalent cations (11), and the purified mammalian enzyme has no absolute Mg^{2+} requirement, but is nonetheless stimulated twofold by its addition (4). Characterization of a partially purified preparation of the reductase induced in herpesvirus-infected cells indicates that this enzyme also lacks an Mg^{2+} requirement and is in fact inhibited by free Mg^{2+} (1). With respect to Mg^{2+} , then, our results suggest that the vaccinia virus-induced enzyme resembles herpes virus reductase, and the behavior of the BSC-40 activity is in agreement with published studies of mammalian ribonucleotide reductase. However, unlike the herpesvirus enzyme, which is fully active in the absence of positive activator, the vaccinia virus reductase displayed an absolute requirement for activation.

Determination of the K_m for CDP reduction yielded values of 11 μM for the vaccinia virus-induced enzyme and 30 μM for the endogenous enzyme. The published values for the mammalian reductase are 30, 50, 40, and 100 μM for CDP, GDP, ADP, and UDP, respectively (5). Allosteric regulation by deoxyribonucleoside triphosphates directs the enzyme to one or another of the four substrates so that an adequate pool of each DNA precursor is maintained. The reductase induced by herpesvirus has remarkably low K_m values for CDP (0.65 μM) and GDP (1.2 μM), but retains relatively typical values for UDP (80 μM) and ADP (12 μM) (1). In the absence of allosteric activation of the enzyme toward different substrates (1), intrinsic kinetic factors may be instrumental in providing appropriate precursor pools for synthesis of the G+C-rich (70%) herpesvirus DNA. The K_m for CDP determined in this study of the vaccinia virus-induced reductase is intermediate between those of mammalian and herpesvirus enzymes. Whether the kinetic parameters of this reductase toward the other three substrates resemble those of the herpes simplex virus or mammalian enzyme remains to be determined. Such studies await stabilization and purification of the induced enzyme.

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