Herpes Simplex Virus Ribonucleotide Reductase Induced in Infected BHK-21/C13 Cells: Biochemical Evidence for the Existence of Two Non-identical Subunits, H1 and H2

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SUMMARY

In nearly all systems studied, ribonucleotide reductase consists of two non-identical subunits. We present here the results of our study on herpes simplex virus (HSV) ribonucleotide reductase in favour of the existence of two subunits, H1 and H2, different from the mammalian subunits, M1 and M2. First, although the viral subunits could not be separated by Blue Sepharose chromatography (unlike mammalian subunits), they seemed to dissociate at very low protein concentration as suggested by the non-linear relationship between activity and low protein concentration. Second, pyridoxal phosphate (Pyr.P)-NaBH₄ treatment and 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ) treatment of partially purified extract of mammalian ribonucleotide reductase which inactivated M1 and M2 respectively also inhibited the HSV ribonucleotide reductase. This activity could be restored by mixing Pyr.P-NaBH₄-treated extracts with MAIQ-treated extracts of viral ribonucleotide reductase, suggesting that each treated extract contains one active subunit. Moreover, the addition of exogenous M1 or M2 subunits to one or the other of these two treated extracts did not produce any detectable reductase activity. Our interpretation of these results is that the two subunits H1 and H2 which could dissociate upon treatment did not form enzymically active hybrids with the mammalian subunits. Also, the higher degree of resistance to heat inactivation and to hydroxyurea of the viral reductase as compared to the mammalian enzyme suggests that H1 differs from M1 and H2 from M2.

INTRODUCTION

DNA replication requires a balanced supply of the four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP and dTTP). Ribonucleotide reductase (EC 1.17.4.1), which catalyses the reduction of the four ribonucleoside diphosphates to the corresponding deoxynucleoside diphosphates, is a key enzyme in the pathway leading to the formation of DNA precursors for prokaryotic and eukaryotic cells (Thelander & Reichard, 1979).

The enzyme from Escherichia coli and mammalian species has been shown to consist of two non-identical subunits (Hooper, 1978; Cory et al., 1978; Chang & Cheng, 1979; Thelander et al., 1980). The best purified preparations from mammalian sources were obtained with the calf thymus ribonucleotide reductase (Thelander et al., 1980). Subunit 1 (M1) was shown to bind the allosteric effectors of the enzyme whereas subunit 2 (M2) contained non-haem iron and a tyrosine free radical necessary for activity (Engström et al., 1979; Thelander et al., 1980; Graslund et al., 1982). The two subunits which participate in the formation of the catalytic site can be separated by Blue Sepharose affinity chromatography (Cory et al., 1978; Thelander et al., 1980; Gudas et al., 1981).

Many compounds are known to be inhibitors of mammalian ribonucleotide reductase. Among them, pyridoxal phosphate (Pyr.P) has been shown to inhibit this enzyme reversibly,
possibly by interaction with an allosteric site or the catalytic site on M1 (Cory & Mansell, 1975). Hydroxyurea (HU), guanazole, pyrogallol and 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ) are four compounds which are known to interrupt DNA synthesis by inhibiting ribonucleotide reductase (Brockman et al., 1970; Elford et al., 1979; Engström et al., 1979; Akerblom et al., 1981). Recent studies have shown that, in vitro, HU, guanazole and MAIQ interact with the M2 subunit, scavenging the tyrosine free radical (Larsen et al., 1982; Thelander & Graslund, 1983). However, the radical can be regenerated after HU or guanazole treatment by the addition of dithiothreitol in the presence of oxygen and iron, explaining the fact that these compounds produce a reversible inactivation of the enzyme activity (Graslund et al., 1982).

Moreover, Cory & Fleischer (1979) have found that each subunit, either separated or not, could be irreversibly inactivated by two types of treatment. First, the interaction of Pyr.P with the M1 subunit can be made irreversible by NaBH₄ reduction. Second, the destruction of the tyrosine free radical produced by MAIQ treatment is to a large extent irreversible in the absence of added iron (Thelander & Graslund, 1983).

Many viruses of the herpes group, including herpes simplex virus (HSV) (Cohen, 1972; Langelier et al., 1978; Huszar & Bacchetti, 1981; Langelier & Buttin, 1981), equine herpesvirus (EHV) (Cohen et al., 1977; Allen et al., 1978), Epstein–Barr virus (EBV) (Henry et al., 1978) and pseudorabies virus (Lankinen et al., 1982) have been reported to induce a new ribonucleotide reductase with altered allosteric properties, such as insensitivity to inhibition by dTTP and dATP. Moreover, CDP reduction by the HSV- or pseudorabies-induced enzymes does not require ATP as a positive effector (Ponce de Leon et al., 1977; Lankinen et al., 1982; Averett et al., 1983).

These enzymes differ also from the mammalian reductase in their requirement for Mg²⁺ (Huszar & Bacchetti, 1981; Lankinen et al., 1982; Averett et al., 1983).

The recent finding by Dutia (1983) of an HSV-1 temperature-sensitive mutant (tsG) that induces both in vivo and in vitro a thermolabile ribonucleotide reductase demonstrated that at least one component of the enzyme is virus-coded. The ts mutation has been mapped recently within a DNA fragment encoding a polypeptide of 140000 (140K) (Preston et al., 1984). Moreover, immunoprecipitation studies with monoclonal antibodies that specifically inhibit or neutralize the HSV ribonucleotide reductase activity have always revealed the presence in the immunoprecipitates of a 38K peptide in addition to the 140K peptide (Huszar et al., 1983; Bacchetti et al., 1984). As the two peptides do not seem to have homologous amino acid sequences (McLauchlan & Clements, 1983), the best explanation for the fact that a monoclonal antibody directed against one protein could immunoprecipitate two polypeptides is that the two peptides are tightly associated.

As these results suggested that the HSV ribonucleotide reductase consists of two subunits different from M1 and M2, we attempted to separate them by Blue Sepharose chromatography. Although this procedure efficiently separates cellular M1 and M2, we were unable to separate the HSV reductase into two inactive fractions; as described below, we consider this to be the expected outcome given the properties of the enzyme.

However, using Pyr.P-NaBH₄ and MAIQ treatments which efficiently inactivate M1 and M2 respectively, we have demonstrated that the viral reductase was also irreversibly inactivated by each of these treatments and that the viral activity (insensitive to dATP) could be restored when the two types of treated extracts were mixed. Moreover, addition of separated M1 or M2 to the treated viral extracts did not restore the activity. The results of these experiments are indirect evidence for the existence of two viral subunits, H1 and H2, which do not form enzymically active hybrids with M1 and M2 subunits.

**METHODS**

Cell and viruses. BHK-21/C13 cells, obtained from the American Type Culture Collection, were grown at 37 °C in α modified Eagle’s medium supplemented with 10% foetal bovine serum and antibiotics (α 1000). Exponentially growing cells were prepared by seeding 4 × 10⁶ cells in 850 cm² plastic roller bottles (Corning) and harvesting them 24 h later. Using the same initial density, confluent cells were obtained after 4 days of culture. 96-V-2(600), a cell line resistant to 7.8 mM-HU and derived from V-79/V6, was kindly provided by W. H. Lewis (Lewis & Srinivasan, 1983). The cells were grown in α medium supplemented with 5% horse serum and 2% foetal
bovine serum. They were maintained in the presence of 3.75 mM-HU until one passage before the mass production for ribonucleotide reductase extraction. HSV-1 (strain F) and HSV-2 (strain HG-52) stocks were prepared using a low multiplicity of infection and titrated as previously described (Langelier et al., 1978). The absence of mycoplasma was carefully verified not only for the cell line but also for the virus stocks by the sensitive Hoechst staining technique described by Chen (1977).

Cell infection and extract preparation. Confluent BHK-21/C13 cells were infected at an input multiplicity of 10 to 20 p.f.u./cell, and the virus was allowed to adsorb at 37 °C for 1 h. The medium containing unattached virus was then replaced by 2% FCS and the incubation was continued at 37 °C for 6 to 7 h.

Washing and harvesting of exponentially growing or infected cells were done as previously described (Langelier et al., 1978) except that the washed cells were suspended in 50 mM-HEPES pH 7.8, 2 mM-dithiothreitol (DTT), 1 mM-MgCl2 (buffer A). The cells were kept frozen at −80 °C until extraction, which was done by sonication with an MSE 150 W ultrasonic disintegrator at 8/10 of maximal power for 30 s. The suspension was then centrifuged at 12000 g for 10 min at 4 °C and the supernatant was used as the crude extract.

Partial purification was carried out essentially as described by Huszar & Bacchetti (1981). Briefly, streptomycin sulphate (5% in buffer A) was added dropwise to the crude extract to a final concentration of 1%. Having been stirred for 20 min at 4 °C, the suspension was centrifuged at 12000 g for 20 min. The supernatant was collected and brought to 60% saturation by addition, with constant stirring, of a solution of (NH4)2SO4 at 90% saturation in buffer A. After 20 min of stirring at 4 °C and centrifugation as above, the pellet was dissolved in a minimal volume of buffer A and dialysed overnight against two changes of 1 litre of the same buffer. The precipitate was removed by centrifugation and the supernatant was stored at −80 °C. Protein concentration was measured by the Coomassie Brilliant Blue staining method of Bradford (1976) using serum albumin as standard.

Blue Sepharose affinity chromatography. Affinity chromatography on Blue Sepharose was performed following the conditions described by Eriksson & Martin (1981) for the separation of M1 and M2 subunits of ribonucleotide reductase extracted from lymphoma cells. Briefly, the (NH4)2SO4 precipitate was suspended and dialysed against 50 mM-HEPES pH 7.8, 2 mM-DTT (buffer B). The desalted protein extract (6 ml at 10 mg protein/ml) was loaded onto a Blue Sepharose column (0.9 × 4 cm) equilibrated with buffer B. The flow-through (10 ml) was collected and, when necessary to eliminate residual M2 subunit, it was applied to a second Blue Sepharose column (0.9 × 11 cm) equilibrated with buffer B. Twelve ml of eluate from the second column were collected. The first Blue Sepharose column was washed with 20 ml of buffer B and the bound proteins were eluted with 5 ml 1 M-KCl in buffer B. The bound fraction was desalted by dialysis against buffer B. Proteins in each fraction were concentrated by ultrafiltration (Amicon PM-10) to approximately 1 ml.

Pyr P–NaBH4 and MAIQ treatments. These treatments were done as described by Cory et al. (1978; Cory & Fleischer, 1979). Briefly, for Pyr P–NaBH4 treatment, samples (0.5 ml) of mammalian or HSV-1 enzymes purified by (NH4)2SO4 precipitation and suspended in buffer B were incubated on ice for 30 min in the presence or absence of 2.5 mM-Pyr P, NaBH4 (50 µl of a solution 40 mM in 0.05 M-HEPES pH 7.8) was added to each tube and the reaction was allowed to proceed for 1 h. The samples were then dialysed overnight against two changes of 2 litres of buffer B. Samples treated in parallel with NaBH4 alone were used as controls.

For MAIQ treatment, similar samples (0.5 ml) of mammalian or viral enzymes were incubated on ice with 50 µM-MAIQ for 1 h. The samples were then dialysed overnight against two changes of 2 litres of buffer B. The MAIQ was dissolved in 25% DMSO. The final concentration of DMSO in the samples was 0.5%. Samples treated in parallel with 0.5% DMSO were used as controls.

Heat inactivation. Samples of the mammalian and viral enzymes in buffer A containing equal protein concentrations (20 mg/ml) were pre-incubated at 50 °C in a water-bath. Samples of separated subunits were diluted in buffer B at 8 mg/ml. At the appropriate time, aliquots were removed from the water-bath, supplemented with the standard reaction mixture and assayed for enzyme activity using the standard assay conditions at 37 °C. When the inactivated M1 or M2 subunits were assayed, excess of the unactivated complementary subunit were also included.

Ribonucleotide reductase assays

CDP reduction. The standard reaction mixture contained in a final volume of 60 µl: 50 mM-HEPES pH 7.8, 4 mM-MgCl2, 4 mM-ATP, 4 mM-NaF, 6 mM-DTT, 54 µM-CDP and 0.25 µCi [3H]CDP. For the viral activity, 2 mM-ATP was used.

ADP reduction. As for CDP reduction except that ATP was replaced by 1 mM-dGTP as activator and CDP by 54 µM-ADP and 0.25 µCi [3H]ADP as substrate.

GDP reduction. As for CDP reduction except that 500 µM-dTTP and 2 mM-ATP were used as activators and 54 µM-GDP and 0.25 µCi [3H]GDP as substrate.

UDP reduction. As for CDP reduction except that 54 µM-UDP and 0.25 µCi [3H]UDP were used as substrate.

After 45 min of incubation at 37 °C, the reaction was stopped by immersing the tubes in boiling water for 4 min and the precipitate was removed by centrifugation. Nucleotides in the supernatant were converted to nucleosides by enzymic hydrolysis with Crotalus adamanteus snake venom as previously described (Bradley et al., 1982). The deoxyribonucleosides were subsequently separated from the ribonucleosides by chromatography on polyethylene-
imine (PEI)-cellulose plastic sheets which had been converted to the borate form as described by Schrecker et al. (1968). To a 10 μl sample, appropriate unlabelled ribonucleosides and deoxynucleosides (0.5 μmol each) were added as markers and the mixture was spotted on PEI-cellulose. The chromatogram was developed by ascending irrigation with ethanol-20 mM-ammonium formate (1:1, v/v) for 2 h. After localization by u.v. illumination, the appropriate spots were cut out and placed in scintillation vials. Then, the radioactive nucleosides were solubilized with 125 μl of water and 800 μl of NCS solubilizer. After 30 min, 10 ml of OCS scintillation solution was added and the radioactivity counted in a Beckman liquid scintillation spectrometer.

All the assays have been performed in duplicate at two protein concentrations in the range of linearity (200 to 600 μg/reaction for the mammalian enzyme and 150 to 500 for the viral enzyme). One unit (U) of ribonucleotide reductase is defined as the amount of enzyme generating 1 nmol of dC per h under the standard assay conditions. The limit of sensitivity of the assay was 0.05 U/mg.

In some experiments, the identity of [3H]dC detected as the product of viral or mammalian reductase activities was confirmed by analysis of the radioactivity eluted from the dC spot using HPLC analysis on a Partisil PXS 10/25 SAX Whatman column. Samples were eluted with methanol:0-01 m-NH₄H₂PO₄ (6:94) at a flow rate of 1.5 ml/min at 20 °C.

Nucleoside diphosphate kinase was measured as described by Averett et al. (1983).

Chemicals and radiochemicals. All the ribo- and deoxynucleotides were obtained from P-L Biochemicals. [5-3H]CDP, [5-3H]ADP and [5-3H]GDP, obtained from Amersham, were repurified by ion-exchange chromatography. NCS and OCS were also obtained from Amersham. Blue Sepharose CL-6B was provided by Pharmacia. HU and Crotalus adamanteus venom were purchased from Sigma and pyrogallol from Aldrich. Guanazole and MAIQ (NSC 246112) were obtained from the Drug and Synthesis Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A. through the kind assistance of Dr L. H. Kedda. Polygram CEL 300 PEI/UV 254 plastic sheets for thin-layer chromatography were purchased from Macherey-Nagel, Düren. F.R.G.

RESULTS

Separation of protein M1 and M2 from BHK-21/C13 cells on Blue Sepharose

As reported by several laboratories (Cory et al., 1978; Thelander et al., 1980; Gudas et al., 1981), the mammalian ribonucleotide reductase can be separated into two non-identical subunits (M1 and M2) by affinity chromatography on Blue Sepharose: M1 binds to the column, whereas M2 does not. When an (NH₄)₂SO₄-purified extract from BHK-21/C13 cells was applied onto a Blue Sepharose column, no ribonucleotide reductase activity could be detected either in the flow-through or in the bound fraction eluted with 1 M-KCl (Table 1). Upon recombination of these two fractions, the activity was recovered. It was sometimes necessary to load the flow-through onto a second Blue Sepharose column to obtain a preparation of M2 completely free of M1. Different preparations of M1 and M2, assayed in the presence of an excess of the complementary subunit, had specific activities ranging respectively between 10 and 15 U/mg and between 3 and 6 U/mg. The lower specific activity of M2 did not seem to be due to the presence of a lower amount of this subunit in exponentially growing BHK-21/C13 cells but to a higher loss of active subunits in the chromatography procedure (to be reported elsewhere). We also purified M2 from the 96-V-2(600) cell line resistant to HU isolated by Lewis & Srinivasan (1983). As expected from the fact that the high level of resistance to HU has been correlated to an overproduction of M2, we obtained preparations of M2 with higher specific activities (10 to 15 U/mg) when assayed in the presence of excess M1 from BHK-21/C13 cells.

Blue Sepharose chromatography of HSV-infected BHK-21/C13 cell extract

HSV ribonucleotide reductase was purified from BHK-21/C13 cells infected 4 days after confluence with HSV-1 (strain F; 10 to 20 p.f.u./cell) for 7 to 8 h. The specific activities obtained after (NH₄)₂SO₄ precipitation were between 3 and 7 U/mg protein. As mock-infected cells prepared in parallel gave preparations with specific activities under 0.2 U/mg, and as 1 mM-dATP or -dTTP never inhibited this activity by more than 5%, we estimated that these preparations of HSV ribonucleotide reductase did not contain more than 5% of mammalian isozyme.

When HSV ribonucleotide reductase was applied onto a Blue Sepharose column, this activity (insensitive to 1 mM-dATP) was found in the flow-through (Table 1). After a second column, the activity was also recovered in the flow-through with, however, a poor yield. No increase in the specific activity could be observed after these two columns. Nevertheless, the passage of the extract on one column permitted complete removal of residual M1 subunit which assured that the
Table 1. Blue Sepharose chromatography of (NH₄)₂SO₄-purified extract from exponentially growing BHK-21/C13 cells and HSV-infected confluent BHK-21/C13 cells

<table>
<thead>
<tr>
<th>Resistance to 1 mM-dATP (%)</th>
<th>BHK-21/C13</th>
<th>BHK-21/C13 + HSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (mg)</td>
<td>Total activity (U)</td>
</tr>
<tr>
<td>Infected cell extract after (NH₄)₂SO₄ precipitation</td>
<td>60.0</td>
<td>354.0</td>
</tr>
<tr>
<td>First column</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow-through</td>
<td>57.0</td>
<td>0</td>
</tr>
<tr>
<td>Bound fraction (eluted with 1 M-KCl)</td>
<td>7.5</td>
<td>110.3</td>
</tr>
<tr>
<td>Bound fraction (M1) + M2</td>
<td>8.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Second column</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow-through</td>
<td>23.0</td>
<td>0</td>
</tr>
<tr>
<td>Flow-through + M1*</td>
<td>23.0</td>
<td>73.6</td>
</tr>
<tr>
<td>M2 [96-V-2(600)] + M1 (BHK)↑</td>
<td>8.8</td>
<td>92.4</td>
</tr>
<tr>
<td>* The flow-through was assayed for M2 activity by adding an excess of protein M1.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>† M2 fraction was purified from the mutant line 96-V-2(600) overproducing the M2 subunit.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>‡ The value represents the specific activity for protein M1 assayed in the presence of an excess of protein M2.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>§ ND, Not determined.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
preparations were completely free of mammalian reductase activity. Also, as previously reported by Cheng & Domin (1978), this chromatography retained more than 95% of nucleoside diphosphate kinase activity and reduced the otherwise extensive conversion of diphosphate substrates to their triphosphate forms (data not shown).

The Blue Sepharose-purified HSV enzyme exhibited the same specific properties that we and others have observed with (NH₄)₂SO₄- or ATP-agarose-purified preparations (Charron et al., 1981; Huszar & Bacchetti, 1981; Averett et al., 1983). ATP and MgCl₂, which are essential for the mammalian isozyme, were both inhibitory. However, in the presence of 2 mM-ATP and 4 mM-MgCl₂ (our standard assay conditions) the activity was only slightly reduced (25%) probably due to the formation of ATP-Mg²⁺ complexes as suggested by the work of Averett et al. (1983). Nevertheless, for the purpose of comparison with our previous results on (NH₄)₂SO₄-purified HSV reductase (Charron et al., 1981), we did not modify our standard assay conditions.

The relationship between the activity and the amount of protein was not linear at the lowest protein concentration. This has been reported for HSV as well as other ribonucleotide reductases (Hooper, 1978; Huszar & Bacchetti, 1981; Averett et al., 1983) and could be explained by the existence of two subunits which have to bind to form an active complex. The Blue Sepharose-purified enzyme reduced all four substrates and these reductions were insensitive to inhibition by 1 mM-dTTP, -dGTP or -dATP.

**Heat inactivation of ribonucleotide reductase enzymes**

As the HSV thymidine kinase has been reported to be more resistant to heat treatment than the mammalian cytoplasmic isozyme (Jamieson & Subak-Sharpe, 1974), it was interesting to see whether such a difference existed for the ribonucleotide reductase. The effects of preincubation of the extracts at 50 °C can be seen in Fig. 1. The mammalian enzyme was inactivated much more rapidly than the viral isozyme. After 20 min of heat treatment, the mammalian enzyme retained only 2% of its activity whereas the viral one was still 90% active. Also presented in Fig. 1 are the results of experiments done to determine whether either M₁ or M₂, or both, was (were) responsible for the heat sensitivity of the mammalian reductase. For this purpose, we inactivated M₁ and M₂ separately. After the treatment, each subunit was assayed in the presence of untreated complementary subunits. We observed that the rate of heat inactivation of M₁ was almost identical to that of the holoenzyme. On the other hand, M₂ is almost completely resistant to the 20 min heat treatment. Therefore, the higher heat sensitivity of mammalian reductase as compared to the viral seems to be due almost exclusively to the sensitivity of the M₁ subunit.

**Effects of specific inhibitors of M₁ and M₂ subunits on the viral ribonucleotide reductase**

The effect of Pyr.P was measured on the two (NH₄)₂SO₄-purified enzyme activities since this compound has been shown to inhibit mammalian ribonucleotide reductase reversibly, presumably by interacting with subunit M₁ of the enzyme (Cory & Mansell, 1975). As observed by Huszar & Bacchetti (1981), we found that the two enzymes exhibited a similar sensitivity to inhibition by Pyr.P (data not shown). In both cases, 50% inhibition was attained at 150 µM-Pyr.P, whereas 100% inhibition was reached at 1 mM.

HU, guanazole, pyrogallol and MAIQ are known to be specific inhibitors of ribonucleotide reductase and interact with M₂ (Timson, 1975; Cory & Fleischer, 1979; Larsen et al., 1982). Previously, we reported that the ribonucleotide reductase activities from crude extracts of infected or uninfected cells showed a similar sensitivity to inhibition by HU with a 50% inhibition at about 0-5 mM (Langelier & Buttin, 1981). However, with (NH₄)₂SO₄-purified preparations and a more accurate assay, significant differences were observed between the two enzymes in their sensitivity to inhibition not only by HU but also by the three other inhibitors of M₂ that we have tested: guanazole, pyrogallol and MAIQ (Fig. 2; data not shown for pyrogallol and MAIQ). A concentration of inhibitor at least twofold higher was necessary to obtain 50% inhibition of the viral enzyme (respectively, 0-65 mM, 4-0 mM, 68 µM, 0-3 µM) compared to the mammalian enzyme (0-3 mM, 2-1 mM, 35 µM, 0-1 µM). The higher degree of HU resistance of viral reductase was also observed with Blue Sepharose-purified extract.
HSV ribonucleotide reductase

Fig. 1. Heat inactivation of CDP reductase activity. Aliquots were preincubated at 50 °C for the indicated period of time and assayed immediately thereafter. •, Blue Sepharose-purified HSV ribonucleotide reductase; △, ammonium sulphate-purified cellular enzyme; ▲, inactivated protein M1 assayed in the presence of an excess of protein M2; ■, inactivated protein M2 assayed in presence of an excess of protein M1.

Fig. 2. Inhibition by HU (■, ●) and guanazole (□, ○) of ribonucleotide reductase activity in partially purified extracts from HSV-infected confluent cells (■, ○) and exponentially growing uninfected cells (□, □). Points are averages of eight and six determinations for HU and guanazole, respectively.

Table 2. Addition of exogenous M1 or M2 to (NH₄)₂SO₄-purified extracts of ribonucleotide reductase

<table>
<thead>
<tr>
<th>BHK-21/C13 (µg)</th>
<th>BHK-21/C13 + HSV</th>
<th>M1 (µg)</th>
<th>M2* (µg)</th>
<th>Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>115</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>115</td>
<td>100</td>
<td>1.06</td>
</tr>
<tr>
<td>140</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.52</td>
</tr>
<tr>
<td>140</td>
<td>0</td>
<td>115</td>
<td>0</td>
<td>1.51</td>
</tr>
<tr>
<td>140</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>1.09</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>0</td>
<td>0</td>
<td>1.12</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>115</td>
<td>0</td>
<td>1.08</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>0</td>
<td>100</td>
<td>1.19</td>
</tr>
</tbody>
</table>

* M2 was purified from the mutant line 96-V-2(600) overproducing this subunit.

Addition of exogenous M1 or M2 to (NH₄)₂SO₄-purified extracts of mammalian and viral ribonucleotide reductases

As our attempts to separate viral reductase into two inactive fractions were unsuccessful, we sought indirect evidence for the existence of two viral subunits. We first examined the effects of adding separated M1 or M2 to (NH₄)₂SO₄-purified extracts of the two reductases (Table 2). The addition of M1 or M2 to an extract of mammalian ribonucleotide reductase caused respectively a fourfold and twofold increase in enzyme activity, whereas the addition of the same amounts of subunits did not significantly increase the viral activity present in a preparation of viral reductase. In other experiments with lower amounts of viral enzyme (50 µg) and higher quantities of subunits, we never observed an increase of reductase activity. These results suggested that the peptide(s) of the viral enzyme do(es) not associate with M1 or M2 to form an enzymically active hybrid. However, another explanation could be that the subunits of the viral reductase are so tightly associated that they are not available for an association with the cellular subunits.
Table 3. Inactivation of reductase activity by Pyr.P–NaBH₄

<table>
<thead>
<tr>
<th>BHK-21/C13 (μg)</th>
<th>M₁ (μg)</th>
<th>M₂ (μg)</th>
<th>Activity (U)</th>
<th>BHK-21/C13 + HSV (μg)</th>
<th>M₁ (μg)</th>
<th>M₂ (μg)</th>
<th>Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄ extract</td>
<td>400</td>
<td>0</td>
<td>0</td>
<td>1·25</td>
<td>(NH₄)₂SO₄ extract</td>
<td>300</td>
<td>0</td>
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<tr>
<td>NaBH₄-treated</td>
<td>400</td>
<td>0</td>
<td>0</td>
<td>1·2</td>
<td>NaBH₄-treated</td>
<td>270</td>
<td>0</td>
</tr>
<tr>
<td>Pyr.P–NaBH₄*</td>
<td>315</td>
<td>0</td>
<td>0</td>
<td>0·7</td>
<td>Pyr.P–NaBH₄</td>
<td>260</td>
<td>0</td>
</tr>
<tr>
<td>Pyr.P–NaBH₄</td>
<td>315</td>
<td>160</td>
<td>0</td>
<td>0·17</td>
<td>Pyr.P–NaBH₄</td>
<td>260</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>175</td>
<td>0</td>
<td>0</td>
<td></td>
<td>160</td>
<td>175</td>
</tr>
</tbody>
</table>

* Pyr.P–NaBH₄, fraction treated with pyridoxal phosphate and NaBH₄.

Table 4. Inactivation of HSV reductase activity by MAIQ

<table>
<thead>
<tr>
<th>BHK-21/C13 + HSV (μg)</th>
<th>M₁ (μg)</th>
<th>M₂* (μg)</th>
<th>Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄ extract</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMSO-treated</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAIQ-treated</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAIQ-treated</td>
<td>300</td>
<td>115</td>
<td>0</td>
</tr>
<tr>
<td>MAIQ-treated</td>
<td>300</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>115</td>
<td>100</td>
<td>1·06</td>
</tr>
</tbody>
</table>

* M₂ was purified from the mutant line 96-V-2(6000) overproducing this subunit.

Irreversible inactivation of the two ribonucleotide reductases

Cory et al. (1978) and Cory & Fleischer (1979) have shown that treatments of partially purified extracts of mammalian reductase with Pyr.P followed by NaBH₄, or with MAIQ, resulted in irreversible inactivation of M₁ or M₂ respectively. We reasoned that similar treatments of viral reductase extracts might be useful to test for the existence of two viral subunits and to determine whether they can dissociate.

Pyridoxal phosphate–NaBH₄ inactivation

As shown in Table 3, our experiments confirmed that the Pyr.P–NaBH₄ treatment irreversibly inhibited the mammalian reductase by inactivating the M₁ subunit. When the separated subunits were added to the treated extract, only M₁ restored the reductase activity (58% of the control value). The HSV ribonucleotide reductase was also irreversibly inactivated by treatment with Pyr.P–NaBH₄ (Table 3). However, even if the inactivation was not complete, the addition of M₁ or M₂ subunits to the treated extracts did not increase the residual viral activity (as defined by activity insensitive to 1 mM-dATP). In other experiments with complete inactivation of viral reductase, addition of M₁ or M₂ did not produce any detectable reductase activity (results not shown).

MAIQ inactivation

(NH₄)₂SO₄ extracts of mammalian ribonucleotide reductase were incubated in the presence or absence of MAIQ (50 μM). After treatment, the samples were extensively dialysed to eliminate MAIQ (which is inhibitory even at very low concentrations) and assayed for reductase activity in the presence or absence of exogenous M₁ or M₂. As previously described (Cory & Fleischer, 1979), only the addition of M₂ subunit restored the activity (data not shown). When MAIQ treatment was applied to an (NH₄)₂SO₄-purified extract of viral enzyme, the activity was also inhibited (Table 4). Even if a slight reactivation did occur after the removal of MAIQ (23% of control value), the addition of M₁ or M₂ did not increase the reductase activity.
Medical Research

Table 5. Combination of Pyr. P–NaBH₄-treated and MAIQ-treated extracts of HSV-1-infected confluent BHK-21/C13 cells

<table>
<thead>
<tr>
<th>Pyr. P–NaBH₄-treated*</th>
<th>MAIQ-treated*</th>
<th>Activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µg)</td>
<td>(µg)</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>600</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>300</td>
<td>0.12</td>
</tr>
<tr>
<td>600</td>
<td>600</td>
<td>0.22</td>
</tr>
<tr>
<td>300</td>
<td>300</td>
<td>0.41 (0.29)†</td>
</tr>
<tr>
<td>600</td>
<td>600</td>
<td>0.64 (0.41)</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>0.14 (0.06)</td>
</tr>
<tr>
<td>400</td>
<td>200</td>
<td>0.29 (0.21)</td>
</tr>
<tr>
<td>600</td>
<td>200</td>
<td>0.33 (0.25)</td>
</tr>
</tbody>
</table>

* The activity of the (NH₄)₂SO₄ extract treated with NaBH₄ without Pyr. P was 0.51 U. The activity of the extract treated with DMSO without MAIQ was 0.52 U. These values represent a 50% reduction in activity as compared to the extract without any treatment (1.0 U/mg). Dialysis alone was responsible for at least 50% of this reduction.
† The values in parentheses were obtained by subtracting the activity given by the MAIQ-treated fraction alone.

Combination of pyridoxal phosphate-treated and MAIQ-treated cell extracts of viral reductase

Finally, samples of the same (NH₄)₂SO₄-purified extract of viral reductase were treated in parallel with Pyr. P–NaBH₄ or MAIQ and were assayed alone or in combination for reductase activity. As shown in Table 5, the treatment with Pyr. P–NaBH₄ completely inactivated the reductase activity, whereas 23% of the reductase activity found in the control remained after treatment with MAIQ. This residual activity was increased by 3.4-fold when an equal protein amount of the Pyr. P–NaBH₄-treated extract was added. The resulting activity was insensitive to inhibition by dATP. When the activity of the MAIQ-treated extract alone was subtracted, the value obtained corresponded to 56% of the activities of the controls. This increased to 77% when the amount of MAIQ-treated extract was doubled. Similar increases in reductase activity were also observed with the addition of increasing amounts of Pyr. P-treated extract to a smaller amount of MAIQ-treated extract.

In control experiments, samples of Pyr. P-treated or MAIQ-treated extracts of mammalian reductase or of bovine serum albumin were added to one or other of these two treated extracts. In none of these samples did the reductase activity appear or increase. Our interpretation of these results is that Pyr. P–NaBH₄ treatment and MAIQ treatment inactivate different subunits of viral reductase, and that upon combination of the two treated extracts, the two unaffected subunits could associate to give reductase activity. Moreover, the active subunits present in these treated extracts did not form an enzymically active complex with M1 or M2.

DISCUSSION

Blue Sepharose chromatography has been widely used to purify numerous proteins which have nucleotide phosphate-binding sites. As the M1 subunit of ribonucleotide reductase possesses high-affinity binding sites for deoxynucleoside triphosphates, Blue Sepharose was successfully used to separate this subunit from the M2 subunit of partially purified ribonucleotide reductase from different species of mammalian cells (Cory et al., 1978; Gudas et al., 1981), including the BHK-21/C13 cells used in the present study. However, when we applied this procedure to partially purified HSV ribonucleotide reductase, we failed to separate the enzyme into two inactive fractions, all the activity being eluted in the flow-through. This finding was not too surprising, as many studies have demonstrated that nucleoside triphosphates do not regulate the HSV ribonucleotide reductase (Cohen, 1972; Langelier et al., 1978; Langelier & Buttin, 1981; Huszar & Bacchetti, 1981) suggesting that, in contrast to the mammalian enzyme, the viral reductase does not possess binding sites for allosteric effectors. Also, lack of binding of HSV ribo-
nucleotide reductase to an affinity column of ATP-agarose has been observed by Averett et al. (1983). Moreover, the ribonucleotide reductase induced by pseudorabies virus, which is also resistant to negative allosteric effectors, did not bind to a column of dATP-Sepharose (Lankinen et al., 1982), which is known to bind the mammalian enzyme (Engström et al., 1979). Therefore, affinity chromatography using nucleoside triphosphates as ligand, even if it can remove from the extract nucleoside diphosphate kinase, phosphatases (Averett et al., 1983) or subunit M1 of mammalian ribonucleotide reductase, does not appear to be very useful for purifying HSV ribonucleotide reductase.

Evidence that the HSV ribonucleotide reductase, like nearly all prokaryotic and eukaryotic isozymes studied, consists of two non-identical subunits can be found not only in our inhibitor studies (discussed below) but also in the fact that the plot of the activity against enzyme concentration exhibited an upward curvature at very high dilution of the enzyme. Inactivation of the enzyme in dilute solution, as an explanation for the phenomenon, is unlikely since the effect of dilution was reversible.

The degree of resistance to heat inactivation of the HSV-1 reductase also could be interpreted as an indication that at least one of the polypeptides of this enzyme differs from the polypeptides of the cellular isozyme. As the mammalian subunit 1 is much more sensitive to heat inactivation than M2, the difference in the heat sensitivity of the two enzymes might be due to differences in their subunit 1. A higher heat stability of M2 as compared to M1 has also been observed by Moore (1977) for rat ribonucleotide reductase. However, Huszar & Bacchetti (1981) have found a similar sensitivity to preincubation at 50 °C for the mammalian and the HSV-2 enzymes. It is unlikely that this discrepancy could be explained by the use of different strains of viruses, as we observed the same high level of heat resistance for one strain of HSV-1 (F) and one strain of HSV-2 (HG-52). Variation between the two procedures of partial purification could produce differences in the heat stability of the viral enzyme itself or modify its association with a non-enzymic peptide. In relation to the last hypothesis, the demonstration by Litteil et al. (1983) that the major DNA-binding protein (ICP8 for HSV-1) could play an important role in the heat stability of HSV DNA polymerase merits consideration in further experiments.

In view of the absence of allosteric binding sites on viral ribonucleotide reductase, our results showing that the viral and the mammalian enzymes exhibit the same sensitivity to Pyr.P were somewhat unexpected, as they suggested that the two enzymes had similar sites of action for this inhibitor. The specific inhibition of many enzymes by Pyr.P is known to be due to its binding to a specific lysyl residue in the catalytic or allosteric sites through Schiff base formation (Brown et al., 1972; Raetz & Auld, 1972; Venegas et al., 1973) and the inhibition of mammalian ribonucleotide reductase was supposed to be caused by the binding of this compound to the allosteric site of the enzyme (Cory & Mansell, 1975). However, this last hypothesis was based on results which, as mentioned by these authors themselves, did not rule out the possibility that the binding site of the inhibitor was in the catalytic site. Therefore the similarity in sensitivity of the two enzymes to Pyr.P indicates that this product probably interacts with the same residue in the catalytic site in each enzyme.

Our observation that the viral enzyme was significantly more resistant to HU, guanazole, pyrogallol and MAIQ, four compounds known to interact with the M2 subunit of mammalian enzyme, is in favour of the existence of a viral subunit 2 (H2) different from M2. It has been shown that these compounds act as radical scavengers and destroy the tyrosine free radical present on the subunit 2 of ribonucleotide reductase from E. coli, bacteriophage T4 and mammals (Larsen et al., 1982; Thelander & Graslund, 1983). The T4-coded enzyme is much more sensitive to HU inhibition than mammalian and E. coli enzymes, which show about the same sensitivity. These results have been interpreted as reflecting differences in the conformation of the protein around the free radical. Another likely explanation for the difference between the two enzymes in their sensitivity to subunit 2 inhibitors could be that the ratio M1/M2 differed from the ratio H1/H2, since it has been shown that variation in the ratio M1/M2 altered the degree of sensitivity to HU of the mammalian enzyme (Sato & Cory, 1981). Moreover, this could also be an explanation for the discrepancy between our previous results (Langelier & Buttin, 1981) and those reported here on HU sensitivity of the two enzymes. Differences between the procedures of purification could
have altered the yield of active subunits, thus changing the ratio for one of the two enzymes. The same argument could also be applied to other studies where similar or different sensitivities to HU were observed for viral and mammalian enzymes. For example, even though the pseudorabies virus reductase gave a new type of electron paramagnetic resonance signal in infected cells suggesting differences in the conformation of the subunit 2, it exhibited an extent of inhibition by HU similar to its mammalian isozyme (Lankinen et al., 1982). On the other hand, EBV and EHV whose replication has been found to be resistant to inhibition by HU (Cohen et al., 1975; Mele et al., 1974) exhibited a reductase activity more resistant to this inhibitor than the enzyme present in uninfected control cells (Cohen et al., 1977; Henry et al., 1978).

The irreversibility of the inactivation of the two enzymes by Pyr.P–NaBH₄ treatment was complete, as expected from the fact that a covalent bond is probably formed between Pyr.P and a lysyl residue on subunit 1, whereas the inactivation by MAIQ seems to be in part reversible. This slight reactivation has been previously observed (Cory & Fleischer, 1979) and the recent elucidation by Thelander & Graslund (1983) of the mechanism of action of this drug has given an explanation of this reactivation. The tyrosine free radical of M2 was destroyed by the drug in a reaction which requires oxygen. After removal of the drug, the radical could be regenerated in the presence of DTT, oxygen and iron. In the absence of iron, the regeneration was greatly reduced and was comparable to the level of activity that we observed here in our dialysed treated extracts (25% of the control).

Combination of Pyr.P–NaBH₄-treated and MAIQ-treated extracts of viral reductase resulted in restoration of viral activity (75% of the control values). These results argue strongly against the hypothesis that the sites of action of Pyr.P and MAIQ are present on the same peptide and the most likely explanation for them is that the non-inactivated subunit present in each of the two treated extracts (H₂ in the Pyr.P–NaBH₄-treated extract and H₁ in the MAIQ-treated extract) can associate to give reductase activity. This interpretation implies that the inactivated subunit can dissociate from the active one. The subunits could be either easily dissociable or the inactivation of one of the two subunits may cause the separation. From the results presented here, we cannot distinguish between these two possibilities. However, our preliminary experiments and a study by Averett et al. (1983) with gel filtration designed to separate polypeptides of 144K and 38K suggest that the two subunits are not easily dissociable. Therefore, we favour the hypothesis that the inactivation of one of the two subunits permits the dissociation.

An interesting parallel may be drawn with bacteriophage T4 ribonucleotide reductase. This viral enzyme, unlike the host E. coli enzyme which can easily dissociate into B₁ and B₂ subunits, remains as a tight complex under various chromatographic procedures (Thelander, 1973; Berglund, 1975). However, in the presence of 1 M-guanidine-HCl, the T4 holoenzyme dissociated into two subunits which could be subsequently separated by chromatography (Berglund, 1975). Interestingly, the subunits of T4 reductase do not form enzymically functional hybrids with the subunits of the E. coli enzyme. This parallels our observation that the active viral subunit present in the treated extracts of HSV reductase could not associate with M₁ or M₂ subunits to give activity. From our present data, the possibility of formation of an inactive hybrid between viral and cellular subunits cannot be excluded. More studies are needed to clarify this point, since such an association could play an important role in the inhibition of cellular DNA synthesis which accompanies HSV DNA replication (Roizman et al., 1965) and eventually in the mutagenic potential of this virus (Huszar & Bacchetti, 1983; Schleshofer & zur Hausen, 1982).

Further purification is needed to demonstrate unambiguously that the polypeptides of 144K and 38K form two subunits and to determine which polypeptide constitutes each subunit. As the mapping of the tsG mutation demonstrated that the 140K polypeptide (ICP6) must be a component of the viral ribonucleotide reductase (Preston et al., 1984), and as comparison of amino acid sequences of the B₂ subunit from E. coli (Carlson et al., 1984) with the 38K polypeptide of HSV-2 (Galloway & Swain, 1984) has revealed a short stretch of high homology (A. Seguin, E. Cohen & Y. Langelier, unpublished results), it is tempting to speculate that the 140K polypeptide forms the H₁ subunit and the 38K the H₂ subunit.
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REFERENCES


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