The Principal Hydrogen Donor for the Herpes Simplex Virus Type 1-encoded Ribonucleotide Reductase in Infected Cells Is a Cellular Thioredoxin

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SUMMARY

In this study herpes simplex virus type 1-encoded ribonucleotide reductase was shown to be able to utilize thioredoxin purified from the cyanobacterium Anabaena variabilis as a hydrogen donor for the enzyme. An assay has been developed to search for proteins which can function as a hydrogen donor for the viral ribonucleotide reductase. A protein has been identified and purified to homogeneity from infected cell extracts by a combination of fast protein liquid chromatography and gel filtration. This protein, which is also present in mock-infected cells, has been identified as a host cell thioredoxin by similarities in its physical characteristics with other thioredoxins. No evidence for the existence of a major virus-induced thioredoxin was obtained, suggesting that the host cell thioredoxin functions as the hydrogen donor for the herpes simplex virus type 1 ribonucleotide reductase in the infected cell.

INTRODUCTION

Deoxyribonucleotides, the precursors for DNA synthesis, are synthesized by the direct reduction of the corresponding ribonucleotides, the hydroxyl group at the 2' position of the ribose moiety being substituted by a hydrogen in a reaction catalysed by ribonucleotide reductase (EC 1.17.4.1) (Thelander & Reichard, 1979; Reichard & Ehrenberg, 1983). Thioredoxin, a small (M_r 12000) acidic protein was first purified and characterized as a physiological hydrogen donor for ribonucleotide reductase in Escherichia coli (Laurent et al., 1964). Electrons are transferred from NADPH in vivo via the flavoprotein thioredoxin reductase to an oxidation-reduction disulphide in thioredoxin which is situated on a unique protrusion of the three-dimensional protein structure (Holmgren et al., 1975). A second hydrogen donor protein for ribonucleotide reductase, glutaredoxin, has been identified in E. coli (Holmgren, 1976) and mammalian cells (Luthman & Holmgren, 1982). This protein shows no amino acid sequence homology with thioredoxin but has a very similar three-dimensional structure and also contains an oxidation-reduction disulphide which is reduced by NADPH via glutathione and glutathione reductase (Holmgren, 1979).

During ribonucleotide reduction, electrons are transferred from the oxidation-reduction disulphides on the hydrogen donor proteins to similar oxidation-reduction disulphides in the large subunit of ribonucleotide reductase (Thelander, 1974) which, along with the tyrosyl free radical in the small subunit, form the catalytic site of the enzyme (Thelander & Reichard, 1979; Reichard & Ehrenberg, 1983). Bacteriophage T4 encodes a new ribonucleotide reductase and also a new thioredoxin upon infection of E. coli cells (Berglund, 1969; Berglund & Sjöberg, 1970). The T4 thioredoxin is a specific hydrogen donor for the T4 ribonucleotide reductase (Berglund, 1972, 1975) and shows no activity with the E. coli ribonucleotide reductase. Conversely, T4 ribonucleotide reductase is not reduced by E. coli thioredoxin (Berglund, 1969). The T4 thioredoxin, like glutaredoxin, contains an oxidation-reduction disulphide and shows no amino acid sequence homology with other thioredoxins (Sjöberg & Holmgren, 1972) but X-ray
crystallography has revealed that the tertiary structure of all three proteins is similar (Hoog et al., 1983).

Several herpesviruses have been shown to encode or potentially encode a new ribonucleotide reductase, including herpes simplex virus type 1 (HSV-1) (Dutia, 1983; McLauchlan & Clements, 1983; Preston et al., 1984), herpes simplex virus type 2 (HSV-2) (Huszar & Bacchetti, 1983; Galloway & Swain, 1984; Swain & Galloway, 1986), varicella-zoster virus (VZV) (Davison & Scott, 1986), pseudorabies virus (Lankinen et al., 1982) and Epstein-Barr virus (Henry et al., 1978; Gibson et al., 1984). For VZV there is no corresponding biochemical evidence for the existence of a novel ribonucleotide reductase activity in infected cells although a protein thought to be the small subunit of the VZV ribonucleotide reductase has been detected in immunoprecipitation experiments (Dutia et al., 1986). Like the mammalian and E. coli ribonucleotide reductases which can utilize thioredoxin as a hydrogen donor (Thelander & Reichard, 1979) the HSV-1-encoded enzyme consists of two subunits (Cohen et al., 1985; Frame et al., 1985; Bacchetti et al., 1986). However, unlike the prokaryotic and eukaryotic enzymes, the viral enzyme is completely refractory to allosteric inhibition by dATP and dTTP and is not activated by ATP (Averett et al., 1983). The subunits of the viral enzyme are strongly bound together and do not dissociate easily (Bacchetti et al., 1986) whereas the binding of the subunits of other ribonucleotide reductases is much weaker and is dependent on the presence of magnesium (Thelander & Reichard, 1979).

To date there is no information available as to how these viral ribonucleotide reductases are reduced in vivo. In this paper evidence is presented for the role of host cell thioredoxin as the principal hydrogen donor for HSV-1 ribonucleotide reductase.

METHODS

Cells and virus. BHK-21 clone 13 cells (Macpherson & Stoker, 1962) were grown in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% calf serum. HSV-1 strain 17 (Brown et al., 1973) was used in all experiments at an m.o.i. of 10 p.f.u./cell.

Preparation of extracts for ribonucleotide reductase assays. Partially purified extracts containing HSV-1 ribonucleotide reductase with a high specific activity were prepared by streptomycin sulphate and ammonium sulphate treatment as described elsewhere (Dutia, 1983) and desalted on a 30 x 1 cm column of Sephadex G-75. Equilibration of the column and sample elution were accomplished with 50 mM-Tris-HCl buffer pH 8.0. Extracts were prepared 15 h post-infection at 31 °C.

Assay for thioredoxin. Thioredoxin was assayed for its ability to act as a hydrogen donor to HSV-1 ribonucleotide reductase in the presence of a limiting concentration of dithiothreitol (DTT). The assay mixture contained in addition to thioredoxin and HSV-1 ribonucleotide reductase with a high specific activity were prepared by streptomycin sulphate and ammonium sulphate treatment as described elsewhere (Dutia, 1983) and desalted on a 30 x 1 cm column of Sephadex G-75. Equilibration of the column and sample elution were accomplished with 50 mM-Tris-HCl buffer pH 8.0. Extracts were prepared 15 h post-infection at 31 °C.

Samples were then boiled at 100 °C for 2 min, cooled on ice and the product formation was then analysed by HPLC and by flow-through scintillation counting (Darling et al., 1988).

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out on 5% to 12.5% gradient gels cross-linked with N,N'-methylenebisacrylamide as described by Marsden et al. (1978). Isoelectric focusing was carried out on Ampholine PAG plates pH range 3.5 to 10 (LKB). Isoelectric point markers were purchased from Pharmacia Fine Chemicals.

Gels were stained and destained as described by Marsden et al. (1978).

RESULTS

Effect of Anabaena variabilis thioredoxin on HSV-1 ribonucleotide reductase activity

Dithiothreitol at high concentrations is commonly used to give maximum ribonucleotide reductase activity in assays in vitro, only low levels of ribonucleotide reductase activity being detected under limiting DTT concentrations (<10 mM). However, under such conditions ribonucleotide reductase activity was found to be stimulated by the addition of thioredoxin purified from the cyanobacterium A. variabilis (Fig. 1).
Thioredoxin and HSV-1 ribonucleotide reductase

Fig. 1. Effect of *A. variabilis* thioredoxin on HSV-1 ribonucleotide reductase activity. Various amounts of pure thioredoxin were added to an assay mixture containing 300 µg of partially purified HSV-1 ribonucleotide reductase (sp. act. 7.3 nmol dCDP formed/h/mg protein) and DTT to a final concentration of 1 mM. Ribonucleotide reductase activity due to DTT was subtracted from each point prior to the determination of the double reciprocal plot (see inset).

At a fixed low DTT concentration, viral ribonucleotide reductase activity increased with increasing thioredoxin concentration. The $K_m$ for this thioredoxin was calculated to be 1.9 µM (Fig. 1, inset). This experiment provides the first evidence for the potential role of thioredoxin as a hydrogen donor to HSV-1 ribonucleotide reductase. It is interesting that the viral ribonucleotide reductase is able to utilize thioredoxin purified from an unrelated source, in contrast to the T4-encoded ribonucleotide reductase, although a degree of cross-reactivity between thioredoxins and ribonucleotide reductases from other organisms has been observed (Luthman *et al.*, 1979).

**Purification and characterization of thioredoxin from HSV-1-infected cells**

In an attempt to identify the physiological hydrogen donor for the HSV-1 ribonucleotide reductase, infected cell extracts were fractionated and assayed for their ability to stimulate HSV-1 ribonucleotide reductase activity. The starting material for these experiments was the supernatant fraction remaining after the purification of the HSV ribonucleotide reductase (A. J. Darling & E. M. McKay, unpublished results) from $3 \times 10^{10}$ infected BHK cells.

The supernatant was treated with solid ammonium sulphate to 85% saturation and incubated at 4°C for 30 min. The precipitate formed was collected by centrifugation at 12,000 g for 20 min, resuspended in 10 ml of 50 mM-Tris–HCl buffer pH 8.0 (buffer A) and then dialysed extensively...
Fig. 2. Elution profile of partially purified HSV-1-infected cell-free extract from an 8 ml Mono Q column. The extract was applied to the column, and the column was washed with three column volumes of buffer A. Elution of proteins was accomplished with a 0 to 0.8 M linear gradient of NaCl (—) and the $A_{280}$ (——) was monitored continuously. After dialysis, fractions (5 ml) were assayed for thioredoxin activity (○) as described in Methods.

against the same buffer. The extract was then treated with DTT to 10 mM, incubated for 30 min at 4 °C, and then fractionated by fast protein liquid chromatography on an 8 ml Mono Q column which had previously been equilibrated with buffer A. Proteins were eluted from the column with a linear gradient of NaCl in buffer A. Fractions were dialysed extensively against buffer A and 50 µl of each fraction was assayed for the ability to stimulate HSV-1 ribonucleotide reductase activity (Fig. 2). Two major peaks of ribonucleotide reductase stimulation were seen. The first peak eluted in the void volume of the column and corresponded to DTT in the extract. The second peak eluted from the column at approximately 0.2 M-NaCl. No other fractions significantly affected the activity of ribonucleotide reductase even when strongly bound fractions eluted with 2 M-NaCl were tested (data not shown).

A similar pattern of ribonucleotide reductase stimulation was obtained when an uninfected cell extract was used (Fig. 3) suggesting that in the infected cell extract the stimulation of ribonucleotide reductase may be caused by a cellular protein, possibly by host cell thioredoxin. No evidence for a virus-induced thioredoxin was obtained.

To purify and characterize further the protein responsible for the observed stimulation of ribonucleotide reductase activity in infected cells, active fractions were pooled, concentrated to a minimal volume in an Amicon ultrafiltration cell fitted with a YM-5 membrane ($M_r$ 5000 cut-off), incubated with 10 mM-DTT for 30 min and applied to a column of Sephadex G-75 (100 × 1.5 cm). Equilibration and elution of proteins from the column were carried out with buffer A. Fractions were again assayed for their ability to stimulate ribonucleotide reductase activity (Fig. 4). A single major peak of activity was detected which eluted from the column consistent with an $M_r$ of approximately 13 100 (Fig. 5) which is similar to thioredoxins from most other sources (Holmgren, 1985). A second peak of activity corresponding to DTT in the extract was detected at later elution volumes (data not shown).

As a final purification step to remove minor contaminating proteins, use was made of the heat stability of many thioredoxins (Holmgren, 1985). The active fractions from the Sephadex G-75
Fig. 3. Elution profile of partially purified uninfected cell-free extract from an 8 ml Mono Q column. The conditions used were as described in Fig. 2, except that proteins were eluted with a 0 to 0.6 M linear NaCl gradient.

Fig. 4. Elution profile of thioredoxin activity from a column (100 cm x 16 mm) of Sephadex G-75. The eluate from the column was monitored continuously at 280 nm (---) and fractions (5 ml) were assayed (50 μl) for thioredoxin activity (•) as described in Methods.

Fig. 5. Determination of the Mr of thioredoxin by gel filtration on Sephadex G-75. The standards used were bovine serum albumin (67000), egg albumin (43000), trypsin (24700) and cytochrome c (12000). The arrow indicates the peak of thioredoxin activity.

column were pooled, heat-treated at 80 °C for 2 min and then centrifuged at 12000 g for 20 min to remove denatured proteins. The supernatant was then treated with DTT as before and applied to a 1 ml Mono Q column. The proteins were eluted as in Fig. 2 (data not shown).

After this purification step only a single protein species was detected on SDS–polyacrylamide gradient gel electrophoresis (Fig. 6). The migration of this polypeptide was consistent with an Mr of 13200 (Fig. 7). These data, in association with the Mr of 13100 estimated by gel filtration, suggest that the protein exists as a single polypeptide (Fig. 5 and 7). The isoelectric point of this protein was determined to be at pH 4.72 by isoelectric focusing (data not shown).
Fig. 6. SDS-gradient polyacrylamide gel electrophoresis of 0.8 μg (lane 1) 1.6 μg (lane 2) and 3.2 μg (lane 3) of purified thioredoxin. The gel was stained with Coomassie Brilliant Blue.

Fig. 7. Determination of the Mr of thioredoxin by SDS-gradient polyacrylamide gel electrophoresis. The standards used were myosin (205000), β-galactosidase (116000), phosphorylase B (97400), pepsin (34700), carbonic anhydrase (29000), trypsinogen (24000) β lactoglobulin (18400) and lysozyme (14300). The arrow indicates the migration of the pure thioredoxin band.

Therefore, this protein shares a number of characteristics with other thioredoxins including Mr, heat stability, isoelectric point and the ability to serve as a hydrogen donor for ribonucleotide reductase (Holmgren, 1985). Although glutaredoxin is similar in function to thioredoxin, it is a smaller protein (Mr 11000) and has an isoelectric point which lies above pH 7 (Luthman & Holmgren, 1982). In addition, glutaredoxin, unlike thioredoxin, can utilize reduced glutathione (GSH) as a hydrogen donor. No stimulation of the HSV-1 ribonucleotide reductase by protein purified in this study was observed when GSH was substituted for DTT in the assay mixture (data not shown). However, conclusive evidence that this protein is a thioredoxin will involve determining its amino acid sequence and identification of the conserved active site region -Cys-Gly-Pro-Cys- present in other thioredoxins.

Effect of thioredoxin purified from HSV-1-infected cells on HSV-1 ribonucleotide reductase activity

The thioredoxin purified above was added to HSV-1 ribonucleotide reductase to give a range of final concentrations, to determine its effect on enzyme activity (Fig. 8). At a fixed low DTT concentration (1 mM), ribonucleotide reductase activity increased with increasing thioredoxin concentration. The $K_m$ for this thioredoxin was calculated to be 1.5 μM from linear regression analysis of the data shown in the double reciprocal plot (Fig. 8, inset).

DISCUSSION

In this paper a reproducible procedure for the purification of a hydrogen donor protein for the HSV-1 ribonucleotide reductase from infected cells has been established. This protein, which has also been purified from uninfected cells, has been identified as a host cell thioredoxin. No
Thioredoxin and HSV-1 ribonucleotide reductase

Fig. 8. Effect of thioredoxin purified from HSV-1 17+-infected BHK-21 clone 13 cells on HSV-1 ribonucleotide reductase activity. Assay conditions were as described in Methods and in the legend to Fig. 1.

evidence for a new virus-induced hydrogen donor protein was observed. This is consistent with DNA sequencing studies where no homology to any known thioredoxin or glutaredoxin sequences has been identified in HSV-1 (D. J. McGeoch, personal communication) or in VZV (Davison & Scott, 1986). Since the HSV-1-encoded ribonucleotide reductase can utilize the host cell thioredoxin as a hydrogen donor, the lack of a virus-induced or encoded thioredoxin is perhaps not surprising as a new specific hydrogen donor protein is not essential. This is in contrast to the T4 ribonucleotide reductase which cannot utilize the host cell thioredoxin and where a specific T4 thioredoxin is encoded by the virus (Berglund, 1969).

Since the HSV-1 ribonucleotide reductase is capable of utilizing the host cell thioredoxin, it is possible that there is a potential competition for reducing power between the HSV-1 ribonucleotide reductase and the host cell ribonucleotide reductase. However, upon HSV-1 infection, deoxyribonucleoside triphosphate pool sizes dramatically increase in the cell (Jamieson & Bjursell, 1976; Roller & Cohen, 1976) and as a consequence the cellular ribonucleotide reductase, which is subject to allosteric control (Thelander & Reichard, 1979), may be inactivated. The HSV-1 ribonucleotide reductase, which lacks allosteric control sites, is unaffected by high dNTP levels (Cohen, 1972; Averett et al., 1983) and therefore all the reducing power could be diverted to the viral enzyme. Further experiments are under way to determine whether infection of cells with HSV-1 results in an increase in thioredoxin levels in the cell or a change in the redox state of this protein. Either modification could serve as a potential regulatory mechanism for HSV-1 ribonucleotide reductase activity.

Since the isolation of a temperature-sensitive mutant in ribonucleotide reductase, ts1207, which failed to grow at the non-permissive temperature (Dutia, 1983; Preston et al., 1984) there has been increasing interest in this enzyme as a target for antiviral therapy. Recent studies have shown that synthetic peptides that mimic regions on the enzyme can inhibit enzyme activity (Dutia et al., 1986; Cohen et al., 1986) possibly by disruption of the complex between the large and small subunits. The transfer of hydrogen between thioredoxin and ribonucleotide reductase
may prove to be a potential target to inhibit ribonucleotide reductase activity. The mechanism for the transfer of reducing power from thioredoxin to ribonucleotide reductase is thought to involve a transient binding of reduced thioredoxin to the large subunit of the ribonucleotide reductase, followed by reduction of an oxidation–reduction disulphide on this subunit and the subsequent removal of oxidized thioredoxin (Thelander, 1974). Any disruption in the formation of the transient complex would therefore result in the inability of the thioredoxin to transfer the hydrogen to the enzyme, resulting in the inhibition of ribonucleotide reductase activity. Similarly, synthetic peptides which mimic the active site on the large subunit of ribonucleotide reductase may prove useful in blocking the hydrogen transfer between the two proteins.

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REFERENCES


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