Characterization of heterosubunit complexes formed by the R1 and R2 subunits of herpes simplex virus 1 and equine herpes virus 4 ribonucleotide reductase

Yunming SUN and Joe CONNER¹

School of Biological and Biomedical Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA, U.K.

We report on the separate PCR cloning and subsequent expression and purification of the large (R1) and small (R2) subunits from equine herpes virus type 4 (EHV-4) ribonucleotide reductase. The EHV-4 R1 and R2 subunits reconstituted an active enzyme and their abilities to complement the R1 and R2 subunits from the closely related herpes simplex virus 1 (HSV-1) ribonucleotide reductase, with the use of subunit interaction and enzyme activity assays, were analysed. Both EHV-4 R1/HSV-1 R2 and HSV-1 R1/EHV-4 R2 were able to assemble heterosubunit complexes but, surprisingly, neither of these complexes was fully active in enzyme activity assays; the EHV-4 R1/HSV-1 R2 and HSV-1 R1/EHV-4 R2 enzymes had 50% and 5% of their respective wild-type activities. Site-directed mutagenesis was used to alter two non-conserved residues located within the

highly conserved and functionally important C-termini of the EHV-4 and HSV-1 R1 proteins. Mutation of Pro-737 to Lys and Lys-1084 to Pro in EHV-4 and HSV-1 R1 respectively had no effects on subunit assembly. Mutation of Pro-737 to Lys in EHV-4 R1 decreased enzyme activity by 50%; replacement of Lys-1084 by Pro in HSV-1 R1 had no effect on enzyme activity. Both alterations failed to restore full enzyme activities to the heterosubunit enzymes. Therefore probably neither of these amino acids has a direct role in catalysis. However, mutation of the highly conserved Tyr-1111 to Phe in HSV-1 R1 inactivated enzyme activity without affecting subunit interaction.

Key words: herpesvirus ribonucleotide reductase, heterologous subunit enzymes

INTRODUCTION

Ribonucleotide reductase (RR; EC 1.17.4.1) has an essential role in the synthesis of DNA *de novo* in all living organisms by catalysing the conversion of all four ribonucleoside diphosphates into the corresponding deoxyribonucleoside diphosphates (reviewed in [1,2]). Although three distinct classes of RR enzyme have been described, all are dependent on free radicals to achieve the chemically difficult replacement of the ribose 2'-OH in ribonucleotides with hydrogen in deoxyribonucleotides (reviewed in [3]). Class II RR enzymes are single-subunit or homodimeric proteins that use the cofactor adenosylcobalamin for the transient generation of radicals [4]. Class III enzymes are heterodimeric proteins that function under anaerobic conditions and generate a glycyl radical by using an *S*-adenosylmethionine cofactor [5]. The best-characterized RR enzymes are those in Class I, for which the *Escherichia coli* enzyme serves as a prototype. Class I enzymes are composed of two homodimeric proteins, termed R1 and R2, in an $\alpha_2\beta_2$ configuration; the separate three-dimensional structures of *E. coli* R1 and R2 homodimers, but not that of the complex, have been resolved [6,7]. Three redox-active cysteine residues form the catalytic centre in the large (R1) subunit; reduction of the bound ribonucleotide requires the transient formation of a thiyl radical in the active site [4]. The small subunit, protein R2, provides a stable tyrosyl radical, associated with a dinuclear iron centre, that contributes to the catalytic process by long-distance radical transfer to the active site in R1. The precise mechanism used to achieve radical transfer from R2 to R1, a total distance of 35 Å, is not fully understood but structural information and site-directed mutagenesis studies have

identified a potential hydrogen-bonding chain from the tyrosine radical in R2 to the active-site cysteine in R1. Six invariant residues in R2, Tyr-122 (the radical centre), Asp-84, His-118, Asp-237, Trp-48 and Tyr-356 (at the subunit interface), have been proposed for the transmission of radicals to the R2 surface. Two invariant R1 tyrosine residues, Tyr-731 and Tyr-730, are believed to complete the transfer to the active-site Cys-439 [8–10]. The long-distance radical transport is probably mediated by transfers of hydrogen atoms rather than electrons because the mutagenesis of Tyr-730 and Tyr-731 to Phe destroys activity [11]. Conclusive evidence for this transfer pathway, including the identification of transient amino acid radical intermediates, has yet to be demonstrated.

Many herpesviruses encode their own RR, which, like the mammalian cell enzyme, belongs to the Class I group; R1 and R2 counterparts have been identified in all members of the alpha- and gamma-herpesviruses sequenced so far (for molecular phylogenetic analysis see [12]). The most widely studied herpesvirus RR is the herpes simplex virus 1 (HSV-1) enzyme (reviewed in [13]). HSV-1 RR is essential for viral pathogenicity in animal models of primary infection [14,15] and is a target for antiviral chemotherapy. The nonapeptide Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu, corresponding to the extreme C-terminus of HSV-1 R2 and its site of interaction with R1, is a potent and specific inhibitor of HSV-1 RR [16,17]. The nonapeptide functions by binding to R1 [18], thus preventing normal R1/R2 subunit association. Rational drug design based on Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu has generated peptidomimetic antiviral compounds that are effective in animal models of infection [19,20] and HSV-1 RR has provided a model for the study of

Abbreviations used: EHV-4, equine herpes virus type 4; HSV-1, herpes simplex virus type 1; IPTG, isopropyl β -D-thiogalactoside; R1, large subunit of ribonucleotide reductase; R2, small subunit of ribonucleotide reductase; RR, ribonucleotide reductase; TAE, Tris/acetate/EDTA.

¹ To whom correspondence should be addressed (e-mail j.conner@gcal.ac.uk).

peptides that disrupt protein–protein interactions as a route for antiviral drug development. However, HSV-1 mutants that are resistant to peptidomimetic inhibitors develop in tissue culture [21] and there is a need to develop alternative inhibitors of this essential virus enzyme. In addition to its role in RR, HSV-1 R1 has another, as yet unidentified, function [22,23] that resides in a unique N-terminal extension of approx. 300 residues, which is not required for ribonucleotide reduction [24,25].

We report here on the separate cloning of equine herpes virus 4 (EHV-4) R1 and R2 subunits and the characterization of heterosubunit RR enzymes formed from the highly conserved HSV-1 and EHV-4 R1 and R2 that, surprisingly, either have decreased activity (EHV-4 R1/HSV-1 R2) or are almost inactive (HSV-1 R1/EHV-4 R2). Restoration of activity to the heterosubunit complexes was attempted by site-specific mutagenesis of several non-conserved residues within the R1 C-terminus but failed to create fully active enzymes.

EXPERIMENTAL

Proteins, peptides and DNA

The cloning, expression and purification of HSV-1 R2 have been described elsewhere [26]. The cloning, expression, purification and characterization of the N-terminal deletion of HSV-1 R1, dN245R1, which lacks the first 244 N-terminal amino acid residues, have been described previously [25]. Importantly, in terms of RR function, this polypeptide behaves exactly as full-length R1. Peptides Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu (pep1) and Tyr-Ser-Gly-Thr-Leu-Ile-Asn-Asp-Leu (pep2), representing the extreme C-termini of HSV-1 and EHV-4 R2 respectively, were synthesized by Dr Howard Marsden (Institute of Virology, Glasgow, U.K.). The extreme C-termini of EHV-4 and HSV-1 R2 differ by four amino acids; four additional peptides, Tyr-Ala-Gly-Thr-Leu-Ile-Asn-Asp-Leu (pep3), Tyr-Ser-Gly-Ala-Leu-Ile-Asn-Asp-Leu (pep4), Tyr-Ser-Gly-Thr-Val-Ile-Asn-Asp-Leu (pep5) and Tyr-Ser-Gly-Thr-Leu-Val-Asn-Asp-Leu (pep6), based on the EHV-4 R2 sequence but each incorporating one of these differences, were also synthesized by Dr Marsden. EHV-4 genomic DNA for PCR cloning of EHV-4 R1 and R2 was provided by Dr Lesley Nicolson (Department of Veterinary Pathology, Glasgow Veterinary School, Glasgow, U.K.).

PCR cloning of EHV-4 R1 and R2

Primers for the PCR amplification of the complete EHV-4 R1 and R2 open reading frames were based on the sequences published by Riggio and Onions [27]. A pair of primers, incorporating *Nco*I and *Bam*HI restriction sites (28-mer EHVR2.1, 5'-GTC GGATCC CTT ACA GAT CGT TTA TAA G-3'; 24-mer EHVR2.2, 5'-CAG CCATGG CCC TCG AAA ACT GAA-3'; restriction sites underlined) into the EHV-4 R2 DNA, were synthesized by Perkin-Elmer. Two primers that incorporated *Hind*III restriction sites (27-mer EHVR1.1, 5'-GAAGCTTCG ATG GCT TTG RAT TTY YTG-3'; 25-mer EHVR1.2, 5'-GAAGCTTAC TAC ARG CTG CAG CTR G-3'; restriction sites underlined) were designed in accordance with sequences of both EHV-1 and EHV-4 R1 genes and synthesized at Cruachem Ltd (Glasgow, U.K.). A *Pwo* PCR kit was purchased from Hybaid (Ashford, Kent, U.K.) and used to amplify EHV-4 R1 and R2 DNA. Each PCR reaction was performed in a 100 μ l reaction mix that contained 10 μ l of 10 $\times$ reaction buffer, the appropriate forward and reverse primers at 0.4 μ M, 1 ng of EHV-4 DNA, 400 μ M of dNTPs and 2 units of *Pwo* polymerase. PCR was performed on a Biometra Thermoblock, with the use of the following cycling profile: step

1, 2 min/94 °C; step 2, 30 s/94 °C; step 3, 30 s/55 °C; step 4, 3 min/68 °C; step 5, 15 min/68 °C. Steps 2–4 were repeated 25 times. Products were analysed with 1% (w/v) agarose/Tris/acetate/EDTA (agarose/TAE) gels.

The PCR product encoding EHV-4 R2 was ligated directly into an intermediate PCR cloning vector, pTag, to construct a recombinant plasmid called pTAE4R2. pTAE4R2 was cut with *Nco*I and *Bam*HI, the EHV-4 R2 DNA insert was purified from gel slices after electrophoresis and re-ligated into the expression vector pET28a to form the expression construct pEE4R2.

A similar strategy was applied to the cloning of EHV-4 R1. The PCR DNA encoding EHV-4 R1 was ligated directly to an intermediate PCR cloning vector, pGEM-T (Promega, Southampton, Hants., U.K.), to construct a recombinant plasmid, pGE4R1. pGE4R1 was cut with *Hind*III, the EHV DNA insert was purified from gel slices and re-ligated into the expression vector pET28a to generate the expression construct pEE4R1. The presence of EHV-4 R1 and R2 DNA in their respective plasmids was confirmed by DNA sequencing (MWG, Milton Keynes, Bucks., U.K.).

Site-directed mutagenesis of R1

Mutations were introduced into HSV-1 and EHV-4 R1 DNA by using the Altered Sites II *in vitro* mutagenesis system exactly as directed by the manufacturer (Promega). The replacement of Pro-737 by Lys in EHV-4 R1 used the mutagenesis oligonucleotide 5'-GTT TTC CGT CAG CCT TCT CAG TTA TAA AC-3'. Mutations that replaced Lys-1084 by Pro and Tyr-1111 by Phe were introduced into HSV-1 R1 separately by using the oligonucleotides 5'-GTC CCG TCC GCC GGC TCC GTG ACA TAC-3' and 5'-CTT GCA GTA GAA CAT CCC TG-3' respectively. HSV-1 and EHV-4 R1 amino acid numbers differ by approx. 350 because of the unique HSV-1 R1 N-terminal domain. The Altered Sites II system uses ampicillin selection to identify bacteria harbouring mutated plasmids. For each mutagenesis procedure, plasmids from at least five ampicillin resistant clones were sequenced (MWG) to confirm the presence of the altered R1 DNA.

EHV R1 and R2 expression

Expression plasmids were used to transform *E. coli* BL21 (DE3) cells. All culture media contained kanamycin at 50 μ g/ml. Transformed cells were grown overnight on Luria–Bertani plates, a single colony was selected and grown in 3 ml of 2 $\times$ YT broth until turbid and glycerol was added to 15% (v/v) for storage at –70 °C. A small amount of the stored cell suspension was streaked on a Luria–Bertani plate and incubated overnight to obtain single colonies, one of which was used to inoculate 5 ml of 2 $\times$ YT broth. After incubation at 37 °C with shaking until slightly turbid, the 5 ml culture was added to 300 ml of 2 $\times$ YT broth and incubated at 37 °C with shaking until a D_{600} of 0.5 was reached. Isopropyl β -D-thiogalactoside (IPTG), at 150 μ g/ml, was added and incubation continued at 30 °C, with shaking, for a further 2 h. Cells were pelleted at 4 °C by spinning at 4000 *g* for 15 min, then stored at –20 °C. To lyse the cells, 10 ml of cold Hepes buffer [50 mM Hepes (pH 7.6)/0.1 M KCl/2 mM dithiothreitol] was added to the pellet with 12 mg of lysozyme. The pelleted bacteria were left to thaw, then mixed well and incubated on ice for 30 min. Further lysis was achieved by ultrasonication on full power for 15 s. The supernatant was prepared by spinning at 20000 *g* for 20 min at 4 °C, to remove cell debris. Finely powdered $(\text{NH}_4)_2\text{SO}_4$ was then added to the supernatant to achieve 35% satn. After incubation on ice for 5 min, the protein

pellet was obtained by centrifugation at 15000 *g* for 20 min and resuspended in 1 ml of Hepes buffer.

Purification of EHV-4 R1 and R2

All purification procedures were performed at 20 °C. EHV-4 R2 purification, based on the HSV-1 R2 method of Lankinen et al. [26], was performed on a Bio-Rad Econo System at a flow rate of 1 ml/min with an Econo-Pac Q-cartridge anion-exchange column (Bio-Rad). Buffer A was 50 mM Hepes, pH 7.0; buffer B was 50 mM Hepes, pH 7.0, containing 1 M KCl. Before application to the column, R2 preparations were diluted 1:5 in buffer A; the column was then washed with 10 column vol. of buffer A and R2 was eluted in a 0–100 % gradient of buffer B developed over 20 ml.

Expression of EHV-4 R1 from pET28a incorporated a T7 tag to the N-terminus of the protein; this was exploited in its purification. EHV-4 R1 was purified from the 35 %-satd $(\text{NH}_4)_2\text{SO}_4$ fraction by using a T7-tag monoclonal antibody immobilized on an immunoaffinity column (Cambridge Biosciences, Cambridge, U.K.). For purification, the $(\text{NH}_4)_2\text{SO}_4$ pellet from a 300 ml culture was suspended in 2 ml of PBS, pH 7.3, with 0.1 % (v/v) Tween 20, and applied to a 1 ml T7-tag antibody affinity column equilibrated in the same buffer. Fractions of 1 ml were collected; after unbound material had been washed away, the column was washed extensively with 20 ml of PBS. Purified EHV-4 R1 was then eluted with 5 ml of 0.1 M citric acid, pH 2.2, and the low pH was neutralized immediately by 150 μl of 2 M Tris solution, pH 10.4, present in the collection tubes. The success of these purifications was monitored by SDS/PAGE; gels were stained with 0.25 % Coomassie Brilliant Blue R250. Protein estimations were performed with the Bio-Rad Coomassie Brilliant Blue G250 protein assay reagent with a BSA standard; 50 μl of sample was mixed with 1 ml of reagent and the A_{595} was determined.

R1/R2 ELISA-based binding assay

An ELISA-based method [24,25] was used to study R1/R2 interactions; 96-well Immulon 2 microtitre immunoassay plates (Dynex Technologies Ltd, Billingshurst, West Sussex, U.K.) were coated with 0.03 μg of R2 in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6) and incubated either overnight at 4 °C or for 1–2 h at room temperature. Excess liquid was poured off, 300 μl of blocking buffer (0.5 % Marvel dried milk in PBS) was added and the plate was incubated at room temperature for 1 h.

For binding studies, 50 μl of R1 $(\text{NH}_4)_2\text{SO}_4$ fractions was added to the wells along with 50 μl of blocking buffer containing 0.1 % (v/v) Nonidet P40. For peptide inhibition studies, 50 μl of peptides, at various concentrations, was added along with the R1. After incubation at room temperature for 1–2 h, plates were washed six times with washing buffer [PBS containing 0.1 % (v/v) Nonidet P40]; 100 μl of first antibody, at a 1:1000 dilution in PBS, was added. HSV-1 R1 binding was detected with polyclonal antibody 106, raised against the HSV-1 R1 truncation, dN245R1 [25]; EHV R1 binding was detected with a T7-tag monoclonal antibody (Cambridge Biosciences). After incubation at room temperature for 1–2 h, the first antibody was removed, plates were washed six times with washing buffer and 100 μl of diluted second antibody was added. Protein A–horseradish peroxidase conjugate and anti-(mouse IgG)–peroxidase conjugate, both at dilutions of 1:1000, were used for antibody 106 and the T7-tag monoclonal antibody respectively. After further washes, 100 μl of colour development solution [0.5 mg/ml 2,2'-azidobis-(3-ethylbenzothiazoline-6-sulphonic acid) in

0.1 M citrate/phosphate buffer, pH 4.0, containing 3 μl of H_2O_2 per 10 ml of buffer] was added. Plates were incubated at room temperature to allow colour development and A_{405} was read on a plate reader.

RR enzyme activity assay

The enzyme assay method of Jong et al. [28] was modified and used to measure RR activity. The assay system couples ribonucleotide reduction with a DNA polymerase reaction and measures the incorporation of [^3H]dCTP, converted from the [^3H]CDP substrate by the combined action of RR and a nucleoside diphosphate kinase, present in the bacterial 35 %-satd $(\text{NH}_4)_2\text{SO}_4$ fractions used in the assay as a source of R1 proteins, into newly synthesized DNA. For each assay, 20 μg of R1 and R2 were added.

The 35 %-satd $(\text{NH}_4)_2\text{SO}_4$ fraction containing R1 proteins was treated with RNase A, desalted through a PD-10 column and mixed with purified R2 in 60 mM Hepes buffer, pH 8.1, containing 8 mM dithiothreitol, 4 mM magnesium acetate, 2 mM ATP and 1 μl of [^3H]CDP (specific radioactivity 30 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) in a final volume of 60 μl . Samples were incubated at 37 °C for 30 min, inactivated by being heated at 90 °C for 10 min and left to cool on ice immediately. In the presence of an active RR, the cleared supernatant, obtained by centrifugation at 13000 *g* for 5 min, contains [^3H]dCTP from the combined RR/nucleoside diphosphate kinase reaction; this was then incorporated into DNA by using a polymerase reaction. Herring sperm DNA was digested by *Hpa*II to give fragments ranging in size from 0.2 to 3 kb. Repeated extraction with phenol/chloroform (1:1, v/v) was followed by precipitation with ethanol to obtain purified DNA. This DNA was mixed with a hexamer random primer (Amersham Pharmacia Biotech) at a final concentration of 150 ng/ μl and used as a template for DNA synthesis *de novo* in a labelling mixture comprising 90 mM Hepes buffer, pH 6.6, 10 mM MgCl_2 , 0.2 mM dNTPs and 4 units of the Klenow fragment of *E. coli* DNA polymerase I. The entire supernatant from the RR reaction was added and incubated at room temperature for 30 min. The labelled products were purified with GFX PCR purification columns (Amersham Pharmacia Biotech), transferred to a vial with 2 ml of scintillation fluid and counted in a liquid-scintillation counter.

RESULTS

Cloning and expression of EHV-4 R2

EHV-4 R2 DNA was amplified by PCR from viral genomic DNA; an amplified fragment of approx. 1000 bp, the correct size for the R2 open reading frame, was detected on a 1 % (w/v) agarose/TAE gel (Figure 1A). The purified DNA fragment was ligated to pTag, a vector specifically used for cloning PCR products; after this initial cloning, the R2 gene was excised by *Nco*I and *Bam*HI digests and then re-ligated into the corresponding sites of pET28a, an *E. coli* expression vector. The entire EHV-4 R2 insert was sequenced, which confirmed that the EHV-4 R2 gene was ligated between the *Nco*I and *Bam*HI sites of pET28a and that no PCR-induced mutations had been incorporated. Two methods were used to detect the expression of EHV-4 R2 in *E. coli*. First, a spectroscopic wavelength scan was used, because the presence of the tyrosyl radical in protein R2 results in a specific absorbance at 415 nm. A distinct absorbance profile with a peak at 415 nm was observed in crude bacterial extracts after induction with IPTG; this peak was absent from uninduced control extracts (results not shown). Secondly, West-

Figure 1 Cloning and purification of EHV-4 R2

(A) Gel [1% (w/v) agarose/TAE] showing PCR amplification of R2 DNA (lane 1; indicated at the right). Lane M, 100 bp ladder (sizes shown at the left). (B) SDS/PAGE demonstrating purification of EHV-4 R2 from IPTG-induced bacteria. Lane 1, $(\text{NH}_4)_2\text{SO}_4$ fraction applied to the column; lanes 2–9, R2-containing fractions eluted in a distinct peak that started at 75 mM KCl. Protein R2 is indicated at the right; the positions of molecular mass markers are indicated at the left.

Figure 2 Cloning and purification of EHV-4 R1

(A) Gel [1% (w/v) agarose/TAE] showing PCR amplification of R1 DNA (lane 1; indicated at the right). Lane M, 100 bp ladder (sizes shown at the left). (B) SDS/PAGE demonstrating immunoaffinity purification of EHV-4 R1. Lane 1, crude extract applied to the column; lanes 2 and 3, unbound fraction; lane 4, column wash; lanes 5 and 6, protein eluted with citric acid, pH 2.2. Protein R1 is indicated at the right; the positions of molecular mass markers are indicated at the left.

ern blotting with an antibody specific for the extreme C-terminus of HSV-1 R2 [16], a well-conserved region of the protein, also confirmed the expression of EHV-4 R2 (results not shown). Protein R2 was partly purified by 35% satd $(\text{NH}_4)_2\text{SO}_4$ precipitation and purified to homogeneity by ion-exchange chromatography (Figure 1B). After application of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction (Figure 1B, lane 1) to the column and extensive washing to remove unbound material, R2 was eluted from the Q-column as a distinct peak that started at 75 mM KCl (Figure 1B, lanes 2–9). The identity of the purified protein was confirmed by Western blotting with the C-terminus-specific antiserum (results not shown); approx. 1 mg of purified protein was obtained from 300 ml cultures.

Cloning and expression of EHV-4 R1

By using a similar approach, EHV-4 R1 DNA was amplified from the virus genome; a 2500 bp fragment, corresponding to the R1 open reading frame, was detected on 1% (w/v) agarose/TAE gels (Figure 2A). The R1 DNA was ligated into a pGEM-T vector, used for cloning PCR products, and the subsequently cloned plasmid was cut with *Hind*III. The purified R1 gene fragment was re-ligated into the *Hind*III site of pET28a. Restriction fragment mapping and DNA sequencing demonstrated that the EHV-4 R1 gene was inserted into the pET28a vector in

the correct orientation and that no PCR-induced mutations had been introduced (results not shown). Unlike EHV-4 R2, there were no HSV-1 R1 antibodies that cross-reacted with EHV-4 R1 (results not shown), making it difficult to confirm its expression. Fortunately, the expression of proteins from pET28a incorporates His₆ and T7 tags at the N-terminus of the polypeptide and specific monoclonal antibodies demonstrated the expression of the R1 protein in *E. coli* after induction with IPTG by Western blotting (results not shown, but see Figure 2B, lane 6). The protein was successfully purified with the T7-tag monoclonal antibody in an immunoaffinity column (Figure 2B). After application of the $(\text{NH}_4)_2\text{SO}_4$ fraction (Figure 2B, lane 1) to the immunoaffinity column and extensive washing, a single protein of molecular mass 90 kDa (the predicted size of pET28a-expressed EHV-4 R1) was eluted by citric acid at pH 2.2 (Figure 2B, lanes 5 and 6). The purified protein was confirmed as EHV-4 R1 through its specific interaction with EHV-4 R2 in the binding ELISA; approx. 100 µg of EHV-4 R1 was obtained from 300 ml of bacterial culture.

Subunit interactions of EHV-4 and HSV-1 RR

A well-characterized ELISA method was used to investigate the interactions between EHV-4 and HSV-1 R1 and R2 proteins [24,25]. ELISA plates were coated with purified EHV-4 R2; its

Figure 3 Interactions of EHV-4 and HSV-1 R1 with EHV-4 (A) and HSV-1 (B) R2

Readings from HSV-1 (□) and EHV-4 R1 (●) are shown. Each point is a mean for duplicate determinations. Abbreviations: OD405 nm, D_{405} ; ug, μ g.

Figure 4 Peptide inhibition results

An example of a peptide inhibition curve used to calculate the IC_{50} values in Table 1 is shown. Symbols: □, ■, results taken from EHV-4 R1/HSV-1 R2 interactions inhibited by increasing concentrations of pep1 (□) or pep2 (■); ○, ●, results taken from EHV-4 R1/EHV-4 R2 interactions inhibited by increasing concentrations of pep1 (○) or pep2 (●). Abbreviations: OD405nm, D_{405} ; uM, μ M.

interactions with EHV-4 and HSV-1 R1 are shown in Figure 3(A). Interactions of EHV-4 and HSV-1 R1 with purified HSV-1 R2 are shown in Figure 3(B). These preliminary comparisons suggested that heterosubunit complexes assembled as readily as their homosubunit counterparts; this was confirmed by peptide inhibition studies.

Peptide inhibition studies were performed for each of these homosubunit and heterosubunit complexes and IC_{50} values were calculated; the IC_{50} is the concentration of peptide required to decrease the absorbance by 50%. An example of a peptide inhibition analysis is shown in Figure 4 and the overall results are presented in Table 1. The IC_{50} values for inhibition of the HSV-1 R1/R2 complex by all six peptides were within the range 30–100 μ M previously reported for HSV-1 R1/R2 and peptide Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu [24,25]. The IC_{50} values

Table 1 IC_{50} values for peptide inhibition of EHV-4 and HSV-1 R1/R2 subunit interactions

The sequences of peptides 1–6 were: Tyr-Ala-Gly-Ala-Val-Val-Asn-Asp-Leu (pep1), Tyr-Ser-Gly-Thr-Leu-Ile-Asn-Asp-Leu (pep2), Tyr-Ala-Gly-Thr-Leu-Ile-Asn-Asp-Leu (pep3), Tyr-Ser-Gly-Ala-Leu-Ile-Asn-Asp-Leu (pep4), Tyr-Ser-Gly-Thr-Val-Ile-Asn-Asp-Leu (pep5) and Tyr-Ser-Gly-Thr-Leu-Val-Asn-Asp-Leu (pep6)

Peptide	IC_{50} (μ M)			
	EHV-4 R2		HSV-1 R2	
	EHV-4 R1	HSV-1 R1	EHV-4 R1	HSV-1 R1
Pep1	6	44	5	48
Pep2	3	78	5	90
Pep3	5	60	9	90
Pep4	12	53	11	95
Pep5	7	40	6	40
Pep6	9	63	13	88

for inhibition of the EHV-4 R1/R2 complex by all six peptides were lower than those obtained for HSV-1 R1/R2, suggesting that these subunits interact with a lower affinity. The IC_{50} values obtained for HSV-1 R1/EHV-4 R2 and for EHV-4 R1/HSV-1 R2 were approximately the same as those obtained for HSV-1 R1/R2 and EHV-4 R1/HSV-1 R2 respectively, demonstrating that the heterosubunit complexes assemble with approximately the same affinity as their homosubunit counterparts (Table 1).

Mutagenesis of HSV-1 and EHV-4 R1

The Altered Sites mutagenesis system was successful at introducing the desired mutations into HSV-1 and EHV-4 R1 DNA. DNA sequencing of several ampicillin-positive clones confirmed the presence of the HSV-1 Lys-1084 → Pro and Tyr-1111 → Phe and the EHV-4 R1 Pro-737 → Lys mutations and also indicated that these were the only mutations incorporated (results not shown). The mutated R1 DNA inserts were transferred into

Figure 5 Subunit interactions of mutant R1 proteins with R2

(A) Interaction of wild-type (■) and the Pro-737 → Lys mutated (●) EHV-4 R1 with EHV-2 R2. (B) Interaction of wild-type (■) and the Lys-1084 → Pro (●) and Tyr-1111 → Phe (▲) mutated HSV-1 R1 proteins with HSV-1 R2. (C) Inhibition of the interaction between Pro-737 → Lys EHV-4 R1 and EHV-4 R2 by increasing concentrations of pep1 (○) and inhibition of the interaction between Pro-737 → Lys EHV-4 R1 and HSV-1 R2 by increasing concentrations of pep2 (●). Abbreviations: OD_{405nm}; D_{405} ; μ g; μ M, μ M.

Table 2 Enzyme activities of homosubunit and heterosubunit EHV-4 and HSV-1 RR

Results are means $\pm$ S.D. and are expressed as pmol of [3 H]CDP incorporated/h per μ g of R1.

R1	R2	Activity (pmol/h per μ g)
HSV-1	—	1.41 $\pm$ 0.78
HSV-1	HSV-1	154.77 $\pm$ 30.41
HSV-1	EHV-4	11.95 $\pm$ 8.71
HSV-1 (Lys-1084 → Pro)	HSV-1	167.44 $\pm$ 70.68
HSV-1 (Lys-1084 → Pro)	EHV-4	12.50 $\pm$ 7.72
HSV-1 (Tyr-1111 → Phe)	HSV-1	2.23 $\pm$ 1.10
HSV-1 (Tyr-1111 → Phe)	EHV-4	2.59 $\pm$ 0.74
EHV-4	EHV-4	97.46 $\pm$ 28.21
EHV-4	HSV-1	60.70 $\pm$ 28.75
EHV-4 (Pro-737 → Lys)	EHV-4	44.94 $\pm$ 11.41
EHV-4 (Pro-737 → Lys)	HSV-1	34.36 $\pm$ 12.09
—	HSV-1	2.46 $\pm$ 1.19

pET28a and their expression was detected by Western blotting with the T7-tag antibody (results not shown). $(\text{NH}_4)_2\text{SO}_4$ fractions (35% satn) were prepared for each of the mutated proteins and these were used in the R2 subunit-interaction ELISA. Interactions of the mutated HSV-1 R1 with HSV-1 R2 and EHV-4 R1 with EHV-4 R2 were detected, confirming the successful expression of these proteins; the interactions were approximately the same as in their unmutated counterparts (Figures 5A and 5B). IC₅₀ values for all of the six peptides demonstrated that the mutations had no effects on the abilities of the mutated R1 proteins to form either homosubunit or heterosubunit complexes (overall results not shown but sample peptide inhibition curves are shown in Figure 5C).

RR activities of homosubunit and heterosubunit complexes

Purified EHV-4 and HSV-1 R1 proteins were used to calibrate the subunit-interaction ELISA and this allowed the accurate estimation of the amounts of R1 proteins that were present in the $(\text{NH}_4)_2\text{SO}_4$ fractions used for enzyme activity assays. A novel coupled RR–DNA polymerase assay was used to determine the RR activity of homosubunit and heterosubunit complexes. The presence of both R1 and R2 subunits was mandatory to achieve the incorporation of tritiated substrate into DNA because the levels of ^3H -DNA detected in the presence of R1 or R2 alone were similar to background (Table 2). Assays were performed with an excess of R2 and required R1 to be added to the 35%-satd $(\text{NH}_4)_2\text{SO}_4$ fraction, because no ^3H -DNA was detected with purified R1 proteins (results not shown). The lack of activity with purified R1 proteins was a result of the low-pH elution from the immunoaffinity column because a brief exposure of HSV-1 R1, purified by using heparin-affinity chromatography [25], to pH 2.5 resulted in a marked loss of activity (results not shown). The incorporation of tritiated substrate into DNA was linear over the 30 min incubation period and increased linearly with increasing amounts of R1 added, up to a maximum of 20 μ g (results not shown).

RR enzyme assays were performed with 20 μ g of wild-type or mutated R1 proteins in the presence of excess EHV-4 or HSV-1 R2; the results, in pmol of [3 H]CDP incorporated/h per μ g of R1, are presented in Table 2. The amounts of nucleoside diphosphate kinase present in each assay were presumably identical because the volumes of $(\text{NH}_4)_2\text{SO}_4$ extracts of R1 used were approximately the same. All assays were performed at least three times in separate experiments; means $\pm$ S.D. are reported

and, where appropriate, statistical comparisons were made with Student's paired *t* test. HSV-1 R1 and R2 reconstituted to give an active enzyme with 154.77 pmol of [³H]CDP incorporated/h per μ g of R1, compared with 1.41 pmol of [³H]CDP incorporated/h per μ g of R1 for the HSV-1 R1 subunit alone. Mutation of HSV-1 R1 Tyr-1111 to Phe reconstituted an RR enzyme that was devoid of any detectable activity (2.23 pmol of [³H]CDP incorporated/h per μ g of R1), whereas mutation of Lys-1084 to Pro reconstituted an enzyme with wild-type activity (167.44 pmol of [³H]CDP incorporated/h per μ g of R1; $P = 0.75$). Surprisingly, HSV-1 R1 was unable to reconstitute a fully active enzyme with EHV-4 R2; the mean activity value was 11.95 pmol of [³H]CDP incorporated/h per μ g of R1, which is approx. 1/15 that of the wild-type HSV-1 R1/R2 enzyme. In all experiments the activity of the HSV-1 R1/EHV-4 R2 enzyme was consistently greater than that of the inactive mutant, HSV-1 R1 (Tyr-1111 $\rightarrow$ Phe)/R2, suggesting that the heterosubunit enzyme possessed some activity, although the difference between these two was not significant ($P = 0.07$). The mutation Lys-1084 $\rightarrow$ Pro in HSV-1 R1 had no effect on the low level of activity detected with the heterosubunit enzyme (Table 2).

Reconstitution of wild-type EHV-4 R1 and R2 generated a functional RR enzyme with an activity of 97.46 pmol of [³H]CDP incorporated/h per μ g of R1. The mutation Pro-737 $\rightarrow$ Lys decreased the activity to approx. 50% of wild-type levels (44.94 pmol of [³H]CDP incorporated/h per μ g of R1); this difference was significant ($P = 0.01$). The activity of the heterosubunit enzyme, EHV-4 R1/HSV-1 R2, was also decreased by approx. 50% (60.7 pmol of [³H]CDP incorporated/h per μ g of R1) compared with wild-type EHV-4 RR but this difference was not significant ($P = 0.076$). The mutation Pro-737 $\rightarrow$ Lys in EHV-4 R1 decreased the activity of the heterosubunit enzyme by approximately the same percentage when compared with the wild-type heterosubunit enzyme; the level of activity (34.36 pmol of [³H]CDP incorporated/h per μ g of R1) was 57% of that obtained with the EHV-4 R1/HSV-1 R2 heterosubunit complex (60.7 pmol of [³H]CDP incorporated/h per μ g of R1) but this difference was not significant ($P = 0.11$).

DISCUSSION

RR is required by all living organisms to convert the four ribonucleoside diphosphates into the corresponding deoxyribonucleotides, which is essential for DNA synthesis *de novo*. All RR enzymes achieve this chemically difficult reduction by harnessing free radicals into the catalytic process [3]. Class I RRs, which include the mammalian cell and herpesviral enzymes and for which the *E. coli* enzyme serves as prototype, are tetramers comprising two non-identical homodimeric R1 and R2 subunits. The only apparent role of subunit R2 is to harbour a stable tyrosine free radical that, during catalysis, is transferred to a cysteine residue in the R1 active site. The putative radical transfer pathway comprises six invariant R2 residues (Tyr-122, Asp-84, His-118, Asp-237, Trp-48 and Tyr-356) and three invariant R1 residues (Tyr-730, Tyr-731 and Cys-439); their mutagenesis results in completely inactive enzymes [29–34]. However, conclusive evidence, such as the detection of short-lived radical intermediates, for the direct involvement of these residues in radical transfer has not yet been obtained and, indeed, other residues might be involved. Our results suggest that the heterosubunit complexes formed by closely related herpesvirus R1 and R2 subunits could provide a model system for investigating this further.

The R1 and R2 subunits of EHV-4 RR were readily amplified by PCR from viral DNA and sequencing confirmed that no

mutations were introduced by this process. The R1 and R2 DNA fragments were cloned in pET28a bacterial expression vectors, both proteins were readily purified from extracts of IPTG-induced *E. coli* and yields were similar to those reported by others [26,35].

The separately expressed EHV-4 R1 and R2 subunits reconstituted an active enzyme in a novel RR assay. EHV-4 and HSV-1 RR activities were determined by using an enzyme assay [28] coupling ribonucleotide reduction with DNA polymerase activity and relying on an *E. coli* nucleotide diphosphate kinase present in the 35%-satd $(\text{NH}_4)_2\text{SO}_4$ fractions that provided the R1 component. RR activity was detected only in this assay when both R1 and R2 subunits were present. Purified R1 preparations were not used for enzyme assays because the low pH used to elute the protein from the immunoaffinity column was detrimental to activity. The assay was convenient to use in comparison with the standard HPLC-based assay of Darling et al. [36], although there was considerable variation between assays, resulting in high standard deviations. However, all variations between assays were consistent. The activity of the EHV-4 RR enzyme was significantly lower than that of HSV-1 RR ($P = 0.015$) and this probably reflects a lower stability of the EHV-4 enzyme *in vitro*, which has been previously reported for EHV-1 RR [37].

Most surprisingly, neither of the heterosubunit complexes composed of EHV-4 R1/HSV-1 R2 or HSV-1 R1/EHV-4 R2 was able to form a fully active enzyme. The R1/R2 affinities of these heterosubunit complexes, determined with peptides known to disrupt specifically the herpesvirus RR subunit interaction, were approximately the same as those of the homosubunit complexes. Therefore, despite the ability of EHV-4 R1 to interact fully with HSV-1 R2, the complex possessed only 50% of EHV-4 RR activity. The HSV-1 R1/EHV-4 R2 complex had only minimal activity, approx. 5% of wild-type HSV-1 RR. Although the levels of activity detected with the HSV-1 R1/EHV-4 R2 complex were not significantly different from background, we believe that this complex possesses some basal RR activity because higher than background values were always observed and the lack of significance probably reflects the high variations between assays. Potentially, the decreased activities of the heterosubunit complexes are a consequence of an inefficient radical transfer from R2 to R1. The transfer process probably requires the precise arrangement of R1 and R2 amino acids at the subunit interface and, although the heterosubunits are able to assemble, key residues at the interface might be incorrectly aligned, resulting in inefficient radical transfer. This imprecise alignment would have a greater effect in the HSV-1 R1/EHV-4 R2 complex, resulting in an almost inactive enzyme.

The location of the R2 binding site was first mapped to the R1 C-terminus by using deletions of the HSV-1 R1 protein [25]. A structural determination of *E. coli* R1 confirmed this location [7] and identified a specific α -helical region that interacted with the *E. coli* R2 C-terminal peptide. Two HSV-1 R1 residues, Pro-1090 and Ala-1091, located within the proposed R2 interaction helix, were found to be mutated in viruses that developed resistance to peptidomimetic inhibitors of HSV-1 RR subunit interaction [21], providing direct evidence for the role of this R1 region in R2 binding. Amino acids in the C-termini of HSV-1 and EHV-4 R1 demonstrate an exceptionally high degree of conservation: there are only 17 non-conserved residues within the C-terminal 100 residues. Importantly, the R2 binding site, located within this C-terminal 100 residues, is highly conserved and, because the extreme C-termini of the R2 subunits are also highly conserved, our observations on EHV-4 and HSV-1 R1/R2 heterosubunit interactions were not unexpected. The decreased enzyme activity of the heterosubunit complexes was surprising; we suggest that

one (or several) of the non-conserved C-terminal R1 residues is (or are) responsible for this observed decrease.

We attempted to restore full RR activity to the heterosubunit enzymes by mutating one of the non-conserved amino acids in the R1 C-termini. The mutated residues, Lys-1084 → Pro in HSV-1 R1 and Pro-737 → Lys in EHV-4 R1, were selected for two reasons: first, they are immediately adjacent to the R2 binding site; secondly, unpublished studies had mapped the epitope of an inhibitory monoclonal antibody to this site. Importantly, this antibody, raised against HSV-1 R1, did not affect subunit assembly but was a potent activity inhibitor (J. Conner, unpublished work). Unfortunately, neither of these mutations had any major effects on the decreased activity of the heterosubunit enzymes, indicating that they probably have no direct role in catalysis. The mutation of Tyr-1111 to Phe in HSV-1 R1 destroyed enzyme activity, as reported for the mammalian cell [33] and *E. coli* [11] enzymes, confirming the importance of this highly conserved residue.

Interestingly, the Pro-737 → Lys mutation in EHV-4 R1 decreased the activity of EHV-4 RR by 50 %, suggesting that this residue could have some role in enzyme activity. Although not statistically significant, the activity of the EHV-4 R1 (Pro-737 → Lys)/HSV-1 R2 complex was also decreased by approx. 50 % compared with EHV-4 R1/HSV-1 R2, again indicating a possible role for this amino acid in catalysis. However, this residue might not be directly involved in activity because the insertion of a charged lysine in this region of the protein might have local structural effects that impinge on the EHV-4 enzyme activity. A localized structural effect seems most likely because the reverse mutation, replacement of Lys-1084 by Pro in HSV-1 R1, had no effect on HSV-1 RR activity. Detailed site-directed mutagenesis of the remaining 16 non-conserved residues in the herpesvirus R1 C-terminus and an analysis of their abilities to restore full activity to EHV-4/HSV-1 heterosubunit enzymes might identify key catalytic residues.

We thank Dr Lesley Nicolson for the kind gift of EHV-4 DNA, and Dr Howard Marsden for peptide synthesis.

REFERENCES

- Reichard, P. (1993) *Science* **260**, 1773–1777
- Fontecave, M. (1998) *Cell. Mol. Life Sci.* **54**, 684–695
- Stubbe, J. and Riggs-Gelasco, P. (1998) *Trends Biochem. Sci.* **23**, 438–443
- Licht, S., Gerfen, G. J. and Stubbe, J. (1996) *Science* **271**, 477–481
- Logan, D. T., Andersson, J., Sjöberg, B. M. and Nordlund, P. (1999) *Science* **283**, 1499–1504
- Nordlund, P., Sjöberg, B. M. and Eklund, H. (1990) *Nature (London)* **345**, 593–598
- Uhlén, U. and Eklund, H. (1994) *Nature (London)* **370**, 533–539
- Sjöberg, B. M. (1994) *Structure* **2**, 793–796
- Eriksson, M., Uhlén, U., Ramaswamy, S., Ekberg, M., Regnström, K., Sjöberg, B. M. and Eklund, H. (1997) *Structure* **5**, 1077–1092
- Ekberg, M., Potsch, S., Sandin, E., Thunnissen, M., Nordlund, P., Sahlin, M. and Sjöberg, B. M. (1998) *J. Biol. Chem.* **273**, 21003–21008
- Ekberg, M., Sahlin, M., Eriksson, M. and Sjöberg, B. M. (1996) *J. Biol. Chem.* **271**, 20655–20659
- Willoughby, K., Bennett, M., Williams, R. A., McCracken, C. and Gaskell, R. M. (1997) *Virus Genes* **15**, 203–218
- Conner, J., Marsden, H. and Clements, J. B. (1994) *Rev. Med. Virol.* **4**, 25–34
- Cameron, J. M., McDougall, I., Marsden, H. S., Preston, V. G., Ryan, D. M. and Subak-Sharpe, J. H. (1988) *J. Gen. Virol.* **69**, 2607–2612
- Jacobson, J. G., Leib, D. A., Goldstein, D. J., Bogard, C. L., Schaffer, P. A., Weller, S. K. and Coen, D. M. (1989) *Virology* **63**, 3769–3776
- Dutia, B. M., Frame, M. C., Subak-Sharpe, J. H., Clark, W. N. and Marsden, H. S. (1986) *Nature (London)* **321**, 439–441
- Cohen, E. A., Gaudreau, P., Brazeau, P. and Langelier, Y. (1986) *Nature (London)* **321**, 441–443
- Paradis, H., Gaudreau, P., Brazeau, P. and Langelier, Y. (1988) *J. Biol. Chem.* **263**, 16045–16050
- Liuzzi, M., Deziel, R., Moss, N., Beaulieu, P., Bonneau, A. M., Bousquet, C., Chafouleas, J. G., Garneau, M., Jaramillo, J., Krogsrud, R. L. et al. (1994) *Nature (London)* **372**, 695–698
- Moss, N., Beaulieu, P., Duceppe, J. S., Ferland, J. M., Garneau, M., Gauthier, J., Ghire, E., Goulet, S., Guse, L., Jaramillo, J. et al. (1996) *J. Med. Chem.* **39**, 4173–4180
- Bonneau, A. M., Kibler, P., White, P., Bousquet, C., Dansereau, N. and Cordingley, M. G. (1996) *J. Virol.* **70**, 787–793
- Conner, J., Murray, J., Cross, A., Clements, J. B. and Marsden, H. (1995) *Virology* **213**, 615–623
- Smith, C. C., Peng, T., Kulka, M. and Aurelian, L. (1998) *J. Virol.* **72**, 9131–9141
- Conner, J., Macfarlane, J., Lankinen, H. and Marsden, H. S. (1992) *J. Gen. Virol.* **73**, 103–112
- Conner, J., Furlong, J., Murray, J., Meighan, M., Cross, A., Marsden, H. and Clements, J. B. (1993) *Biochemistry* **32**, 13673–13680
- Lankinen, H., McLauchlan, J., Weir, M., Furlong, J., Conner, J., McGarrity, A., Mistry, A., Clements, J. B. and Marsden, H. S. (1991) *J. Gen. Virol.* **72**, 1383–1392
- Riggio, M. P. and Onions, D. E. (1994) *Gene* **143**, 217–222
- Jong, A. Y., Yu, K., Zhou, B., Frgala, T., Reynolds, C. P. and Yen, Y. (1998) *J. Biomed. Sci.* **5**, 62–68
- Larsson, A. and Sjöberg, B. M. (1986) *EMBO J.* **5**, 2037–2040
- Aberg, A., Hahne, H., Karlsson, M., Larsson, A., Orno, M., Ahgren, A. and Sjöberg, B. M. (1989) *J. Biol. Chem.* **264**, 12249–12252
- Mao, S. S., Holler, T. P., Yu, G. X., Bollinger, J. M., Booker, S., Johnston, M. I. and Stubbe, J. (1992) *Biochemistry* **31**, 9733–9743
- Climent, I., Sjöberg, B. M. and Huang, C. Y. (1992) *Biochemistry* **31**, 4801–4807
- Rova, U., Goodtzova, K., Ingemarsson, R., Behravan, G., Graslund, A. and Thelander, L. (1995) *Biochemistry* **34**, 4267–4275
- Persson, B. O., Karlsson, M., Climent, I., Ling, J. S., Sanders Loehr, J., Sahlin, M. and Sjöberg, B. M. (1996) *J. Biol. Inorg. Chem.* **1**, 247–256
- Furlong, J., Conner, J., McLauchlan, J., Lankinen, H., Galt, C., Marsden, H. S. and Clements, J. B. (1991) *Virology* **182**, 846–851
- Darling, A. J., Dutia, B. M. and Marsden, H. S. (1987) *J. Virol. Methods* **180**, 281–290
- Telford, E., Lankinen, H. and Marsden, H. (1990) *J. Gen. Virol.* **71**, 1373–1378

Received 20 October 1999/8 December 1999; accepted 11 January 2000