Production of the R2 subunit of ribonucleotide reductase from herpes simplex virus with prokaryotic and eukaryotic expression systems: higher activity of R2 produced by eukaryotic cells related to higher iron-binding capacity

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The R2 subunit of ribonucleotide reductase from herpes simplex virus type 2 was overproduced with prokaryotic and eukaryotic expression systems. The recombinant R2 purified by a two-step procedure exhibited a 3-fold higher activity when produced in eukaryotic cells. Precise quantification of the R2 concentration at each step of the purification indicated that the activity was not altered during the purification procedure. Moreover, we have observed that the level of R2 expression, in eukaryotic cells as well as in prokaryotic cells, did not influence R2 activity.

Extensive characterization of the recombinant R2 purified from eukaryotic and prokaryotic expression systems has shown that both types of pure R2 preparations were similar in their 76 kDa dimer contents (more than 95 %) and in their ability to bind the R1 subunit. However, we have found that the higher activity of R2 produced in eukaryotic cells is more probably related to a higher capability of binding the iron cofactor as well as a 3-fold greater ability to generate the tyrosyl free radical.

INTRODUCTION

Ribonucleotide reductase (EC 1.17.4.1.) plays a key role in the synthesis of DNA precursors in all living cells. This enzyme catalyses the reduction of the four abundant ribonucleotides into their corresponding limiting deoxyribonucleotides [1]. Some prokaryotes, including *Escherichia coli*, most eukaryotes and some DNA viruses such as herpes simplex virus (HSV), possess iron-dependent ribonucleotide reductases. The most extensively studied *E. coli* enzyme is the prototype of the eukaryotic and viral ribonucleotide reductases. All of them are formed by the association of two non-identical subunits called R1 and R2, each consisting of two identical polypeptide chains. The R1 subunit binds the substrates and the allosteric modulators, provides thiol groups involved in the reduction reaction and interacts with thioredoxin and/or glutaredoxin for the regeneration of active thiol groups. The R2 subunit contains the cofactor essential for ribose reduction, a tyrosyl radical adjacent to an oxo-bridged dinuclear iron centre (reviewed in [2,3]).

Crystallographic studies as well as direct measurement of the iron content have unambiguously demonstrated that the *E. coli* R2 contains two stable diferric centres (one per polypeptide chain) [4,5]. These centres are essential for the generation of the tyrosyl radical, a process that *in vivo* could require a complex enzymic system [6]. Theoretically, each centre should be able to generate one tyrosyl radical giving two radicals per R2 subunit. However, the tyrosyl radical content measured by EPR spectroscopy on pure preparations has been found to be between 1.2 and 1.4 per R2 and has never attained the maximal value of 2 (reviewed in [5]). The reason for this discrepancy is unknown but it has been postulated to be due to protein instability during purification [7].

The R2 subunits coded by eukaryotic genes have been purified from natural or recombinant sources for calf thymus, mouse, HSV and vaccinia virus reductases. All these R2 subunits have, after purification, much lower radical contents (0–0.6 radical per R2 molecule) than *E. coli* R2 [8–11]. This difference seems to be related to a low stability of the diferric centres in the eukaryotic and viral subunits ([12,13]; G. Matton, B. Massie and Y. Lange-lier, unpublished work). In contrast, obtaining *E. coli* R2 devoid of iron (named apo-R2) necessitates a drastic chelating treatment that produces a transient loss of the dimeric structure [15,16]; most of the studied eukaryotic and viral R2 subunits were obtained at the end of the purification with only 0.2–0.4 iron ions per R2 molecule, which correlated well with their low radical content [10,11,17]. Ferrous treatment of recombinant mouse R2 expressed in *E. coli* produced good reinsertion of iron and, correspondingly, a good radical-to-R2 ratio of 1.6. However, similar treatments for the R2 subunits coded by the human viruses, HSV and vaccinia gave at best 0.4 and 0.6 radicals per R2 molecule respectively, when expressed at high level in *E. coli* [8–10].

HSV ribonucleotide reductase is essential for efficient viral DNA replication in non-dividing cells where the host ribonucleotide reductase is absent [18,19]. Strong interest in this enzyme as a therapeutic target arose from the discovery that a nonapeptide corresponding to the HSV R2 C-terminus specifically inhibits enzyme activity by impairing subunit interaction [20–22]. Even though empirical modifications of this nonapeptide have led to the synthesis of shorter compounds exhibiting very high inhibitory potency *in vitro* [23–25] and antiviral activity *in vivo* [26], the knowledge of the tertiary structure of the HSV holoenzyme would facilitate the design of smaller inhibitory compounds able to reach their R1 intracellular target more

Abbreviations used: BHK, baby hamster kidney; HSV, herpes simplex virus.

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efficiently. As a first step towards this goal, *E. coli* expression vectors and purification methods were developed for HSV-1 R1 and R2 [9,10,17,27].

We have previously reported that overproduction of HSV-2 R2 in eukaryotic cells with a recombinant adenovirus yielded a protein exhibiting an activity similar to that produced in HSV-2-infected cells [28]. With an improved HSV-2 R2 recombinant adenovirus, which raised the R2 production to a level of 15% of total soluble protein [29], the purification of HSV-2 R2 from eukaryotic cells has become as easy as the reported HSV-1 R2 purification from *E. coli* [10,17]. Moreover we have purified the HSV-2 R2 from *E. coli* by using two types of expression vector, one being the same that others have used to obtain pure HSV-1 R2.

Here we show that the R2 produced in eukaryotic cells has a 3-fold higher activity than the R2 expressed in prokaryotic cells. This difference is related to a higher capacity of the R2 produced in eukaryotic cells to generate the tyrosyl free radical. Moreover, using a recently developed ^{59}Fe -binding assay (G. Matton, B. Massie and Y. Langelier, unpublished work), we have found that the higher radical content is well correlated with a higher capacity to bind iron.

MATERIALS AND METHODS

Cells and viruses

293 and baby hamster kidney (BHK)-21/C13 cells were grown in monolayer at 37 °C in (respectively) Dulbecco's modified Eagle's medium or α -modified medium supplemented with 10% (v/v) foetal bovine serum, glutamine and antibiotics. 293S cells, adapted to suspension culture (a gift from Michael Matthews), were grown in Joklik's modified Eagle's medium supplemented with 5% (v/v) calf serum and antibiotics. Monolayer or suspension cultures of *Spodoptera frugiperda* 9 (Sf9) insect cells were grown at 27 °C in TNM-FH medium [30] supplemented with 10% (v/v) foetal bovine serum and antibiotics.

HSV-2 (strain HG-52) stocks were produced in BHK-21/C13 cells by using a low multiplicity of infection as described previously [31]. Ad5/ $\Delta\text{E1}\Delta\text{E3}$, an adenovirus type 5 from which E1 and E3 transcription units have been deleted [32], and the Ad5-recombinant viruses were propagated, as detailed previously [28,29] by infecting monolayer or suspension cultures of 293 cells, a human kidney embryo cell line expressing Ad5 E1A and E1B genes [33]. *Autographa californica* nuclear polyhedrosis virus, a baculovirus used to generate the BAC-recombinant viruses, was propagated by infecting monolayer cultures of Sf9 cells as described [30].

Construction of plasmids and recombinant viruses

pAdBM5-R2 was constructed from the previously described shuttle plasmid pAdBM1-RR₂^{HSV} [28] and used to generate the recombinant viruses Ad5BM5-R2 as reported recently [29]. To construct pGEMTAC-R2 the fragment containing the HSV-2 R2 coding sequence was excised by *Bgl*II-*Bcl*II double digestion from pSVRR₂^{HSV}-16, the plasmid that served to construct pAdBM1-RR₂^{HSV}, and inserted into the prokaryotic vector pGEM-1 (Pharmacia) under the control of the TAC promoter. For the cloning of the HSV-2 R2 gene into the plasmid pET-11C (Novagen), the fragment containing the HSV-2 R2 coding sequence released from pSVRR₂^{HSV}-16 by *Bam*HI-*Bcl*II double digestion was mixed with a synthetic double-stranded 43-mer oligonucleotide (5'-CTAGAAATAATTTGTTAACTTT-AAGAAGGAGATATACCATG-3') with compatible *Xba*I and *Bam*HI overhangs and ligated to *Xba*I-*Bam*HI-digested pET-

11C. The resulting plasmid, pET-R2, contains the restored ATG in-frame in the context of an optimal ribosome-binding site for expression with the T7 RNA polymerase promoter. The *E. coli* expression vector pMD2-R2 containing the HSV-2 R2 gene fused to the first six amino acids of the β -galactosidase gene in the plasmid pUC8 [34] was kindly provided by M. Suh.

Expression of recombinant HSV-2 R2 subunit and preparation of crude extracts

E. coli

Recombinant HSV-2 R2 subunit was produced in *E. coli* strain BL21/DE3 containing the plasmid pET-R2 or in strain JA221 containing either the plasmid pMD2-R2 or the plasmid pGEMTAC-R2. For unknown reasons, high levels of R2 expression (approx. 20% of total soluble proteins) were obtained with pMD2-R2 in strain JA221 of *E. coli* without the need of induction by isopropyl β -D-thiogalactoside, which facilitated the large-scale production of R2. Overnight JA221 or BL21/DE3 bacterial cultures were diluted 100-fold into 1 litre of Luria-Bertani medium containing ampicillin (100 $\mu\text{g}/\text{ml}$). JA221 cells were grown to a D_{590} of 1.8 at 37 °C, whereas BL21/DE3 cells were supplemented with 500 μM isopropyl β -D-thiogalactoside at a D_{590} of 0.6 and allowed to grow for an additional 4 h at 37 °C. After being harvested, the pelleted cells were washed in 25 mM Hepes buffer, pH 7.6, resuspended in the same buffer, frozen in liquid nitrogen and thawed on ice. KCl, PMSF and egg-white lysozyme were added to obtain final concentrations of 80 mM, 1 mM and 300 $\mu\text{g}/\text{ml}$ respectively, and the mixture was incubated on ice for 20 min. After another cycle of freezing and thawing, cell debris was removed by centrifugation at 44000 *g* for 60 min at 4 °C. The major part of the supernatant (crude extract) was kept frozen until further purification. A minor fraction was passed on to a small Sephadex G-25 column to remove ribonucleotide reductase inhibitory molecules before measuring R2 reductase activity.

Adenovirus, baculovirus and HSV-2

The production and extraction of R1 and R2 subunits, either from 293S cells infected by Ad5BM1-R2, Ad5BM5-R2 and Ad5BM5-R1 or from Sf9 cells grown in suspension and infected by the recombinant baculovirus BAC-R2, were as recently described [29]. Quiescent BHK-21/C13 cells (2×10^8) in 850 cm^2 roller bottles were infected for 8 h with HSV-2 at a multiplicity of 10 plaque-forming units per cell.

Purification of HSV-2 R2 recombinant subunit

The purification of the recombinant R2 subunit was accomplished in two steps. The first step consisted of two successive salt precipitations as described previously [35]. Briefly, 5% streptomycin sulphate in buffer A [50 mM Hepes buffer (pH 7.6)/2 mM dithiothreitol] was added to the crude extract to a final concentration of 1%. After having been stirred for 1 h at 4 °C, the pellet containing the precipitated nucleic acids was removed by centrifugation at 12000 *g* for 20 min at 4 °C. Then ammonium sulphate was added to the supernatant to obtain 25% saturation. After 30 min of stirring at 4 °C and centrifugation as above, the pellet was dissolved in buffer A and dialysed overnight against the same buffer.

For the second step, anion-exchange chromatography was performed as described by Lankinen et al. [17]. The ammonium sulphate precipitates were dialysed against two changes of 20 mM Bistris/HCl (pH 5.8)/10% (v/v) glycerol (buffer B) in collodion bags (Schleicher and Schuell). For eukaryotic extracts, a white

precipitate appearing during dialysis was removed by centrifugation at 24000 *g* for 40 min at 4 °C. The supernatant (30–50 mg) was loaded on an 8 ml anion-exchange FPLC Mono Q HR 10/10 column (Pharmacia) previously equilibrated with 10 column volumes of buffer B. After washing with 5 column volumes of the same buffer, a linear increase of 2.4 % per min of buffer C (buffer B containing 1 M KCl) was applied for 5 min with a flow rate of 2 ml/min. Then buffer C was maintained at 12.5 % for 20 min, followed by a linear increase of 0.9 % per min for 15 min. Fractions containing recombinant R2 eluting at 125 mM KCl were pooled, concentrated by ultrafiltration with Centriprep-10 (Amicon), washed twice with buffer A containing 10 % (v/v) glycerol to remove KCl and immediately frozen at –80 °C. Protein concentrations were determined by the Coomassie Blue method of Bradford [36]. Concentrations of the highly purified R2 preparations were also measured by light absorbance with a molar absorption coefficient ($\epsilon_{280-310}$) of 104000 M⁻¹·cm⁻¹ [10].

Quantification of HSV-2 R2 protein

At the different steps of purification, the relative amount of R2 protein was quantified. Proteins were separated by SDS/PAGE [37], stained with Coomassie Blue and the percentage of R2 protein in extracts was evaluated by laser densitometric scanning (LKB Pharmacia) of stained gels, with pure pMD2-R2 or pET-R2 as standard. As this method was inaccurate for the extracts with low R2 concentration, a sensitive radioimmuno-competition assay was developed with P9 serum, a polyclonal rabbit serum raised against a synthetic nonapeptide corresponding to the C-terminus of HSV-2 R2 [38]. Typically, pure R2 in 5 mM glycine (pH 9.2; 2 µg/ml) was adsorbed overnight at 4 °C on polystyrene wells (50 µl per well). The wells were washed 10 times with PBS/0.25 % Tween-20 containing 0.02 % sodium azide, and twice with PBS. Then 250 µl of 5 % (v/v) milk was added per well for 1 h at room temperature. Previously, serial dilutions of protein extracts containing an unknown amount of R2 proteins had been mixed with an equal volume of 5 % milk containing P9 serum diluted 1:4000. After incubation overnight at 4 °C, 100 µl portions of the mixtures were added to the saturated wells and incubated for 5 h at room temperature. After 10 washings with PBS/0.25 % Tween-20 and two washings with PBS, 100 µl of ¹²⁵I-labelled antibody (Amersham) raised against rabbit IgG (50000 c.p.m.) in 5 % milk were added for an overnight incubation at room temperature. Wells were then washed 10 times with PBS and radioactivity was counted in a γ -radiation counter. In each competition assay, serial dilutions of pure pMD2-R2 or pET-R2 were used as standard.

Measurement of reductase activity

The specific activity of the R2 subunit was determined by adding to limiting amounts of R2 subunit excess amounts of HSV-2 R1 subunit produced by 293S cells infected with the recombinant adenovirus Ad5BM5-R1 and purified as described recently (specific activity 55 units/mg) [29]. Ribonucleotide reductase activity was assayed by monitoring the reduction of [³H]CDP as previously reported [35]. The standard reaction mixture contained 50 mM Hepes buffer, pH 7.8, 4 mM NaF, 50 mM dithiothreitol, 50 µM CDP, 0.25 µCi of [³H]CDP and 0.31 µM contaminating iron (present mainly in Hepes and dithiothreitol as measured by atomic spectrophotometric absorption with a Perkin-Elmer instrument equipped with a heated graphite furnace) in a final volume of 60 µl. One unit of ribonucleotide reductase was defined as the amount of enzyme generating 1 nmol of dCDP/min.

Sedimentation analysis of purified R2

R2 subunits (50 µg), purified from *E. coli* (JA221) containing pMD2-R2 expression vector or Ad5BM5-R2-infected 293S cells, were applied on the top of 4.2 ml 20–50 % glycerol gradients containing 50 mM Hepes buffer, pH 7.8, 2 mM dithiothreitol and 100 mM NaCl. Catalase ($s_{20,w}$ 11.6) was added as a marker. Sedimentation of proteins was performed at 310000 *g* for 24 h at 4 °C. Fractions of 200 µl were collected and a 50 µl portion of each fraction was analysed by SDS/PAGE. After staining of the gel with silver nitrate [39], the amount of R2 and catalase in each fraction was evaluated by laser densitometric scanning of the gel. The R2 sedimentation coefficients and related molecular masses were evaluated as detailed [40] by using for the catalase standard a molecular mass of 250 kDa.

Measurements of the binding potential of R2 to R1

Crude extracts of the recombinant Ad5BM5-R1 subunit were prepared as described above for the R2 subunit and passed on to a Sephadex G-25 column. Fixed amounts of this HSV-2 R1 extract (25 µg) were mixed with 50 mM Hepes (pH 7.6)/1 mM bacitracin and increasing concentrations (1 nM to 1 µM) of purified R2 in a final volume of 60 µl. Then the specific binding of the ¹²⁵I-labelled photoreactive peptide (4'-azido-Phe³²⁸,3',5'-[¹²⁵I]Tyr³²⁹)HSV R2(328–337) was measured as reported [41]. In some experiments Chelex-100 (Bio-Rad), a chelating ion-exchange resin with a high selectivity for heavy metals, was used to remove iron from reductase assay reagents and from the Ad5BM5-R1 protein extract in accordance with the procedure described by the manufacturer. The EBDA/LIGAND program [42] was used to analyse the competition studies.

EPR spectroscopy

The tyrosyl free-radical content of pure R2 preparations (1 mg/ml) were quantified at 77 K with a Varian Model E-102 X-band spectrometer at 9.1 GHz and 20 mW. The EPR spectra of pET-R2 and Ad5BM5-R2, which exhibited identical line shapes, were similar to that previously published for HSV-1 R2 produced in *E. coli* [10]. The spectra were quantified by signal-surface (double integral) comparisons with a standard sample of *E. coli* R2 of known tyrosyl free radical content. This sample was calibrated with, as standard, a solution of 1 mM Cu²⁺ in 1.2 M NaClO₄, pH 1.8.

Treatment of purified R2 with Iron

A freshly prepared argon-purged solution of 1 mM ferrous ammonium sulphate was added to pure R2 preparation (1 mg/ml) in buffer A (molar ratio of R2 to iron, 1:6). The resulting solution was incubated for 10 min at room temperature and immediately frozen in liquid nitrogen for EPR measurements. Occasionally, ferrous ammonium sulphate was directly added to the reductase assay with a molar ratio of R2 to iron of 1:6.

⁵⁹Fe-binding assay

The assay was developed from the assay described by Ormö and Sjöberg [43] and will be detailed elsewhere (G. Matton, B. Massie and Y. Langelier, unpublished work). Briefly, Ad5BM5-R2 (0.35 µM) or pET-R2 (0.87 µM) was added to increasing concentrations (0.15–0.5 µM) of ⁵⁹Fe (ferrous sulphate 0.05 M, 30.6 mCi/mg; NEN–Du Pont) in a buffer containing 50 mM Hepes, pH 8.1, and 50 mM dithiothreitol. For concentrations above 0.5 µM, ferrous ammonium sulphate was added to ⁵⁹Fe. The final sample volume was 150 µl. After incubation for 15 min

at 37 °C, two aliquots of 15 µl were withdrawn for scintillation counting, and the remaining 120 µl were transferred to the upper sample reservoirs of Ultrafree-MC filter units with polysulphone PTTK membranes (cutoff 30 kDa, Millipore). After a centrifugation at 3280 *g* for 30 s in a Canlab biofuge 15 centrifuge (rotor HFA 17.1), two aliquots of 15 µl were withdrawn from the filtrate volume for scintillation counting and calculation of the free iron concentration. The specific binding of the iron to the protein was calculated by subtracting the non-specific binding measured in the absence of R2. A concentration of 0.31 µM of contaminating iron was included in the total iron concentration.

RESULTS

Activity of the recombinant HSV-2 R2 subunit

To produce and purify the large amounts of the HSV-2 R2 necessary for crystallographic studies, the efficiencies of eukaryotic and prokaryotic expression systems were compared not only in terms of yield but also in terms of protein quality. Unexpectedly, it was consistently observed that the protein produced by eukaryotic cells infected with either a recombinant baculovirus (BAC-R2) or the two recombinant adenoviruses Ad5BM1-R2 and Ad5BM5-R2 [28,29] exhibited a higher activity than that produced in prokaryotic cells with three different expression vectors, pMD2-R2, pGEMTAC-R2 and pET-R2. Such a conclusion was made possible by precise quantification of the R2 concentration either on crude extracts or at the different steps of the purification procedure. Estimates of the specific activities of R2 determined on crude extracts or on purified preparations (for the vectors giving the highest yields) are summarized in Table 1. For those measurements, iron and pure R1 were always present in excess sufficient to maximize the activity. For the eukaryotic expression vectors, a mean value of 171 units/mg was calculated. This value, similar to the value of 160 units/mg obtained for the authentic R2 subunit produced in HSV-2-infected BHK cells, is 2.6-fold higher than the mean value of 67 units/mg calculated for the prokaryotic expression vectors. For both types of expression system we have also observed that the level of R2 expression (0.5–15% and 0.3–45% for eukaryotic and prokaryotic ex-

pression vectors respectively) did not influence significantly the specific activity of R2. As the activity of R2 was not altered during the purification procedure, the R2 purified from eukaryotic cells was on average 2.8-fold more active than that purified from prokaryotic cells (Table 1, and results not shown). Together these results indicate that HSV-2 R2 overproduction in eukaryotic cells yields a subunit with a specific activity identical with that of the subunit synthesized during HSV-2 infection, whereas overproduction in *E. coli* yields a subunit that appears to be defective.

Biochemical characterization of the purified recombinant HSV-2 R2 subunits

The recombinant HSV-2 R2 subunits purified either from *E. coli* bearing pMD2-R2 or pET-R2, or from 293S cells infected with Ad5BM5-R2 were extensively characterized to determine the basis for the difference in their activity. Figure 1 shows a Coomassie Blue-stained gel of the different steps of purification for pMD2-R2 and Ad5BM5-R2. The slightly lower mobility of pMD2-R2 is most probably due to the presence of the six additional amino acid residues of β -galactosidase because pET-R2, the other bacterially produced protein, co-migrates with Ad5BM5-R2. The two-step purification protocol yields preparations containing respectively more than 98 and more than 95% of the R2 protein as estimated by densitometric scanning of gel lanes containing 10 µg of protein.

Analysis of primary structure

Sequencing of the N-terminus of the *E. coli*-expressed pET-R2 revealed a perfect match with the amino acid sequence MDPAV-SPASTDPLDTHAS expected from the nucleotide gene sequence. The N-terminus of the *E. coli*-expressed pMD2-R2 gave the amino acid sequence MTMITNSDPAVSPASTDP with the expected six additional amino acid residues of β -galactosidase. We also observed that half of the molecules lacked the first two residues, indicating that the second ATG codon was used for protein synthesis. Attempts to determine the N-terminal sequence

Table 1 Expression level and activity of R2 subunit with different expression systems

Abbreviation: n.d., not determined.

Expression system	Crude extracts			Purified R2	
	% R2	Specific activity		% R2	Activity (Units/mg R2)
		(Units/mg total)	(Units/mg R2)		
Eukaryotic					
Ad5BM1-R2-infected 293 cells	4.0	6.6	165	n.d.	n.d.
Ad5BM5-R2-infected cells	15.0*	27.0	179	> 95	196
Bac-R2-infected SF9 cells	2.0	3.4	170	n.d	n.d.
			Average = 171		
HSV-2-infected BHK-21 cells	0.5	0.8	160	n.d.	n.d.
Prokaryotic					
<i>E. coli</i> (JA221)-pMD2-R2	21.0*	14.0	66	> 98‡	57
<i>E. coli</i> (BL21/DE3)-pET-R2	45.0*	33.0	73	> 98‡	70
<i>E. coli</i> (JA221)-pGEMTAC-R2	0.3†	0.2	63	n.d.	n.d.
			Average = 67		

* Determined by comparison with known amounts of pure R2 on Coomassie Blue stained gel.

† Determined by the radioimmunoassay with P9 serum.

‡ Determined by laser densitometric scanning of a Coomassie Blue-stained lane containing 5 µg of protein.

Figure 1 Analysis by SDS/PAGE of recombinant R2 protein purification

Protein extracts of the HSV-2 R2 protein produced either in 293 cells infected with the recombinant adenovirus Ad5BM5-R2 (lanes 2–4) or in growing bacteria containing the plasmids pMD2-R2 (lanes 5–7) or pET-R2 (lane 8) were analysed at different steps of purification by SDS/PAGE and staining with Coomassie Blue. Lane 1, high-molecular-mass markers (BRL; molecular masses in kDa are shown at the left); lanes 2, 5 and 8, crude extracts (25, 20 and 15 μ g respectively); lanes 3 and 6, ammonium sulphate precipitate (8 and 4 μ g respectively); lanes 4 and 7, 125 mM KCl Mono Q eluate (4 μ g).

Figure 2 Velocity sedimentation analysis of recombinant R2 subunit

Purified R2 preparations (50 μ g) were loaded on 20–50% glycerol gradients and sedimentation was performed at 310 000 g for 24 h at 4 $^{\circ}$ C. Collected fractions were analysed by silver staining of SDS/polyacrylamide gels and the amount of R2 was evaluated by laser densitometric scanning. Values are expressed in arbitrary units. Symbols: $\diamond$, Ad5BM5-R2; $\square$, pMD2-R2; the vertical arrow shows the position of catalase.

of the Ad5BM5-R2 protein revealed that a post-translational modification had probably added a chemical group on the N $^{\alpha}$ -moiety because Edman degradation could not proceed even with a sample of 100 pmol. This was confirmed by the removal of the N-terminal methionine residue by CNBr treatment followed by 18 cycles of sequencing on the 20 kDa fragment corresponding to residues 2–180, which gave the expected sequence. The N-terminal modification, although not experimentally identified, is most probably an N $^{\alpha}$ -acetylation because an aspartic residue at the penultimate position is known to both prevent the removal of the N-terminal methionine and promote the addition of an N $^{\alpha}$ -acetyl group in eukaryotic cells [44].

Figure 3 Competition of the R2 protein for the binding of a photoreactive peptide to the R1 subunit

Increasing concentrations of purified Ad5BM5-R2 ($\square$) or pMD2-R2 ($\circ$) were mixed with the recombinant Ad5BM5-R1 protein extract (25 μ g) to measure their potential to compete with the binding of the 125 I-labelled photoreactive peptide (4'-azido-Phe 328 ,3',5'-[125 I]Tyr 329)HSV R2(328–337) to R1. R1-bound peptide was quantified as described in the Materials and methods section. The 100% value corresponds to the amount of photoreactive probe covalently linked to R1 in assays without R2, and each point represents the mean of four experiments performed in duplicate.

R2 dimers

Lankinen et al. [17] reported that pure preparations of HSV-1 R2 overexpressed in *E. coli* could contain a high proportion (approx. 50%) of molecular species migrating on non-denaturing polyacrylamide gels at a position consistent with the monomer. To determine whether the presence of a higher proportion of these presumably inactive monomers in the pMD2-R2 or pET-R2 (results not shown) preparations compared with the Ad5BM5-R2 preparation might explain the difference in their activities, we analysed the native state of both proteins by sedimentation velocity on a glycerol gradient. As shown in Figure 2, peaks of R2 were detected at positions expected for dimers (5.2 and 5.3 S) for Ad5BM5-R2 and pMD2-R2 respectively. The small amounts of R2 (approx. 5% of the total) found in the fractions corresponding to monomers were similar for both recombinant proteins and would probably represent trailing of the dimers during gradient collection.

Affinity of R2 for R1

The potential of R2 to interact with R1 was evaluated by two strategies. First, by adding excess amounts of pure R1 to glycerol gradients containing a limiting amount of Ad5BM5-R2 or pMD2-R2, we observed that the sedimentation of most of the R2 molecules (more than 90%) was shifted from the dimer position to 14.4 S, the position expected for the 360 kDa holoenzyme (results not shown). This result demonstrated that most of the R2 molecules were able to bind R1.

Secondly, the R2–R1 affinity was evaluated with a competition assay [41] by using the photoaffinity peptide (4'-azido-Phe 328 ,3',5'-[125 I]Tyr 329)HSV R2(328–337), which was initially developed to demonstrate the involvement of the R2 C-terminus in the interaction between the two subunits [22]. As shown in Figure 3, both Ad5BM5-R2 and pMD2-R2 competed similarly for the photoaffinity peptide. From these results, IC_{50} values of 0.10 μ M for Ad5BM5-R2 and 0.09 μ M for pMD2-R2 were obtained. A similar value was measured for pET-R2. Therefore

Figure 4 Scatchard plots for binding of iron to R2 at 37 °C

Ad5BM5-R2 (Δ) or pET-R2 (○) were mixed with increasing concentrations of iron and the amount of bound iron was determined by ultrafiltration after an incubation of 10 min at 37 °C. A concentration of 0.31 μM of contaminating iron was included in the total iron concentration. The specific binding of iron to the R2 was obtained after correction for the non-specific binding measured in the absence of R2. The curves were calculated by linear regression analysis. [Fe]b represents the bound Fe; [Fe]f represents the free Fe. [R2] represents the total R2 concentration.

we concluded that the lower activity of the R2 produced in *E. coli* was not due to a lower affinity for the R1.

Our observations, described below, that the R2 purified from Ad5BM5-R2-infected cells had, at the end of purification, low iron and radical contents led us to determine whether variation in the iron content could influence the R2–R1 affinity. Our standard competition binding assay contained enough contaminating iron to saturate the R2 at most of the R2 concentrations used in the assay. The addition of excess iron (6-fold over R2) or performing the assay under conditions of contaminating iron depletion (Chelex treatment) with Ad5BM5-R2 subunit did not influence the IC_{50} value (results not shown). These results, showing that the apo-form of Ad5BM5-R2 has the same ability to compete with the binding of our affinity probe as the fully active form, strongly suggest that the R2–R1 affinity is not affected by the R2 iron content.

Tyrosyl radical and iron content

The maximum values (100%) are considered here to be two tyrosyl free radicals and four iron ions per dimer. Measurements by spectroscopy of the tyrosyl radical content on several preparations of purified R2 diluted to between 10 and 20 μM in 50 mM Hepes buffer (pH 7.8) revealed low radical contents of 5–10% for Ad5BM5-R2 and 5–15% for pMD2-R2 or pET-R2. After aerobic treatment with a 6-fold molar excess of ferrous ammonium sulphate, the radical content of Ad5BM5-R2 increased to 55–60% whereas that of pET-R2 or pMD2-R2 did not increase above 20%. The presence of dithiothreitol during the treatment generally gave approx. 10% higher values. Anaerobic treatment in the presence of a reductant (dithiothreitol or ascorbate) followed by oxygenation, which has been described as improving radical formation in mouse R2 [10], did not perform better than the aerobic procedure.

Attempts to measure iron content in trichloroacetic acid-precipitated protein extract by the usual colorimetric method [15]

gave highly variable results. More reproducible results were obtained with an ultrafiltration assay for ^{59}Fe binding that we had originally developed to evaluate the iron-binding stability in the HSV-R2 protein (G. Matton, B. Massie and Y. Langelier, unpublished work). As can be seen from the Scatchard plot presented in Figure 4 for iron binding to Ad5BM5-R2 and pET-R2 at 37 °C, the R2 produced in bacteria has a lower iron-binding potential (K_d 0.42 μM; 0.72 binding sites) than that produced in eukaryotic cells (K_d 0.18 μM; 2.3 binding sites). Thus it seems that 60% of the Ad5BM5-R2 molecules and only 20% of the pET-R2 molecules are able to form 2 diferric centres. Pretreatments of highly concentrated R2 solutions (10 mg/ml in 20 mM Hepes, pH 8.0) with 20 mM EDTA followed by the removal of EDTA by passage through a fast desalting column did not affect significantly the binding parameters of either protein. Thus it seems unlikely that the smaller iron binding in pET-R2 could be due to a higher contamination by a competing metal unless tightly bound.

Together these results strongly suggest that HSV-2 R2 protein preparations obtained from bacterial expression are less active because they contain a high proportion of molecules unable to bind the iron and consequently to generate the tyrosyl free radical.

DISCUSSION

The R2 subunit of HSV type 2 ribonucleotide reductase was overproduced with prokaryotic and eukaryotic expression systems. The recombinant R2 purified by a two-step procedure exhibited a 3-fold higher activity when produced in eukaryotic cells. Precise quantification of the protein by radioimmunoassay demonstrated that the purification procedure did not significantly alter its enzymic activity. Moreover the sensitivity and precision of the radioimmunoassay, allowing the determination of the specific activity of R2 in crude or partly purified extracts, led us to conclude that regardless of the expression system used, the R2 produced in eukaryotic cells was on average 2.5-fold more active than that produced in prokaryotic cells. This has been observed for the expression of R2 by using a recombinant baculovirus and two recombinant adenoviruses in eukaryotic cells and by using three different prokaryotic expression vectors in *E. coli*. We have also observed that the level of R2 expression, in eukaryotic cells as well as in prokaryotic cells, did not influence R2 activity.

Most of the biochemical analyses performed on Ad5BM5-R2 and *E. coli*-produced R2 (either pMD2-R2 or pET-R2) did not reveal any differences that could explain the lower activity of the latter. Acetylation of the N-terminal methionine, the most likely modification that blocked the Edman degradation when the subunit was expressed in eukaryotic cells, should not affect its enzymic activity. Sequence comparisons of R2 proteins from different species clearly showed that this part of the molecule is not conserved and varies considerably in length. The N-terminus of *E. coli* R2 has a disordered structure and even though it possesses seven amino acid residues involved in dimer interactions, the position of the N-terminal methionine residue did not reveal any obvious interaction important for protein activity that could be affected by acetylation [4,45]. Therefore the acetylation of this residue, which in many eukaryotic proteins has been associated with resistance to proteolysis [44], might only contribute to the high stability of the HSV-2 R2 that we previously noted in HSV and recombinant-adenovirus-infected cells [28]. Also, regardless of whether eukaryotic or prokaryotic cells were used for the R2 production, the purified proteins were in the dimeric form (approx. 95% of the molecules) and exhibited a similar affinity for the R1 subunit.

Measurements of radical content and iron-binding parameters revealed that the HSV-2 R2 expressed in *E. coli* exhibited a lower radical content well correlated with a lower capacity to bind iron. Moreover, as it is almost exclusively the number of binding sites that is affected, the most obvious conclusion is that a very high proportion (80 %) of HSV-2 R2 molecules purified from *E. coli* are unable to bind iron. Interestingly, the two other groups, which have expressed HSV-1 R2 in the same strain of *E. coli* and with the same vector that we used, have also obtained an R2 with a low radical content (approx. 25 %) associated with a low capacity for binding iron [9,10,17].

Our conclusion that the R2–R1 affinity is not affected by the presence of iron in the R2, which had not previously been supported by direct experimental results for either HSV R2 or other R2, however, could be inferred from the following observations. Studies on *E. coli* and HSV R2 have, indeed, shown that only the flexible C-terminal part of this subunit is involved in the interaction with R1 [46,47]. In addition, a report on the X-ray structure of *E. coli* apo-R2 suggests that the removal of iron does not alter the surface structure of the subunit. The only detectable minor movements arise in the amino acid residues involved in the formation of the diferric centres [48].

From the study presented here, we can only speculate on the origin of the iron-binding defect of the R2 produced in *E. coli*. On this matter, two hypotheses can be proposed. First, a post-translational modification taking place only in one of the two types of cells could affect (1) the protein structure in a way that the atoms that interact with iron would or would not be positioned optimally, or (2) accessibility of iron to the centre of the protein. Phosphorylation, which occurs on the protein produced in eukaryotic cells, is a likely candidate. Secondly, the translation of the HSV-2 R2 mRNA could be more error-prone in bacteria than in eukaryotic cells owing to differences in the codon usages. Little is known about the effects of codon usage on the rate of mis-sense errors but it seems likely that a high level of mis-sense errors could result in a fraction of R2 molecules having an altered capacity to bind iron or to form the radical. Pertinent to this hypothesis is the observation that amino acid misincorporation into recombinant proteins expressed at high level in *E. coli* could be at least 10 times higher than the error rates estimated for the synthesis of normal bacterial proteins [49].

In summary, the adenovirus expression system of HSV R2 will be a better choice for X-ray crystallographic studies leading to the determination of the tertiary structure of HSV R2 subunit and will be of great help in initiating studies on radical intermediates.

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