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# Purification and Characterization of Recombinant Mouse and Herpes Simplex Virus Ribonucleotide Reductase R2 Subunit†

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ABSTRACT: Overexpression of recombinant mouse and herpes simplex virus ribonucleotide reductase small subunit (protein R2) has been obtained by using the T7 RNA polymerase expression system. Both proteins, which constitute about 30% of the soluble *Escherichia coli* proteins, have been purified to homogeneity by a rapid and simple procedure. At this stage, few of the molecules contain the iron–tyrosyl free-radical center necessary for activity; however, addition of ferrous iron and oxygen under controlled conditions resulted in a mouse R2 protein containing 0.8 radical and 2 irons per polypeptide chain. In this reaction, one oxygen molecule was needed to generate each tyrosyl radical. Both proteins had full enzymatic activity. EPR spectroscopy showed that iron-center/radical interactions are considerably stronger in both mouse and viral proteins than in *E. coli* protein R2. CD spectra showed that the bacterial protein contains 70% $\alpha$-helical structure compared to only about 50% in the mouse and viral proteins. Light absorption spectra between 310 and 600 nm indicate close similarity of the $\mu$-oxo-bridged binuclear iron centers in all three R2 proteins. Furthermore, the paramagnetically shifted iron ligand proton NMR resonances show that the antiferromagnetic coupling and ligand arrangement in the iron center are nearly identical in all three species.

Ribonucleotide reductase (EC 1.17.4.1) catalyzes the reduction of ribonucleotides to deoxyribonucleotides, the first unique step in the biochemical pathway leading to DNA synthesis (Thelander & Reichard, 1979; Lammers & Follmann, 1983; Reichard, 1988; Stubbe, 1990). Mammalian cells, DNA viruses of the herpes virus group, and some prokaryotes, notably *Escherichia coli*, have a heterodimeric, iron-containing enzyme of $\alpha_2\beta_2$ type. The larger (R1)[1] subunit binds substrates and allosteric effectors and provides redox-active sulfhydryl groups. The smaller (R2)[1] subunit contains binuclear ferric iron centers and a stable tyrosyl free radical essential for activity.

Extensive studies of the *E. coli* R2 protein and its iron/free-radical center, culminating in the elucidation of its three-dimensional structure by X-ray crystallography (Nordlund et al., 1990), have been made possible by development of systems for its overexpression. The crystallographic studies together with data from Lynch et al. (1989) clearly demonstrate that each R2 polypeptide chain contains one binuclear iron center. The tyrosyl radical content as determined by EPR spectroscopy seems to vary and has usually been found to be around 0.5 per polypeptide chain (Sahlin et al., 1989; Lynch et al., 1989).

Mammalian R2 has been purified to homogeneity from hydroxyurea-resistant, R2-overproducing mouse cells (The-

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[1] The terms R1 and R2 for the large and small subunits, respectively, replace the earlier species-based abbreviations, viz., B1 and B2 for the *E. coli* and M1 and M2 for the mammalian ribonucleotide reductase proteins.

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lander et al., 1985), and herpes simplex virus R2 was only recently obtained pure in recombinant form (Ingemarson et al., 1989); however, neither protein has hitherto been available in quantities sufficient for detailed characterization. R2 amino acid sequences, as deduced from genomic or cDNA sequences, are known for several species such as *E. coli*, bacteriophage T4, clam, mouse, *Saccharomyces cerevisiae*, vaccinia, herpes simplex virus types 1 and 2, varicella, and Epstein–Barr virus. While overall identity scores are low, basic structural features and amino acids involved in the iron center/free-radical complex are highly conserved (Nordlund et al., 1990).

We here report the first overexpression of recombinant mouse R2 and a high-yield system for overexpression of herpes simplex virus R2, both based on transcription by T7 RNA polymerase. Both proteins were purified to homogeneity following a rapid and simple purification procedure. Suitable conditions for regeneration of active protein with an iron–tyrosyl radical center from apoprotein are presented, and the stoichiometry of oxygen consumption in this reaction has been investigated.

The active proteins were characterized by optical absorption, circular dichroism, EPR, and NMR spectroscopy. Detailed information on the properties of the iron centers was obtained from observation of the paramagnetically shifted iron ligand NMR resonances.

## Materials and Methods

*Plasmid Constructs.* All proteins were expressed from T7 RNA polymerase responsive plasmids, which were constructed by using standard molecular cloning techniques. Plasmid DNA was extracted from bacteria lysed with lysozyme and Triton X-100, and was purified by two successive ultracentrifugations in cesium chloride. Plasmids were propagated in *E. coli* strains K-12 MC1061 (Casadaban & Cohen, 1980) or JM109 (Yannish-Perron et al., 1985) grown in LB medium at 37 °C, in the presence of carbenicillin, 50 μg/mL. Transfections were performed by using either the Hanahan (1983) or poly(ethylene glycol) (Chung et al., 1989) techniques.

Mouse R2 cDNA was removed as two fragments from the original Okayama–Berg construct, clone 10 (Thelander & Berg, 1986), by digestion with *Pst*I and *Bam*HI. The larger (1.3 kb) *Pst*I–*Bam*HI fragment was ligated into pUC18, opened with the same enzymes. The initial 50 bp of the open reading frame was isolated by *Nla*III digestion of the smaller (130 bp) *Pst*I–*Pst*I fragment, purified by polyacrylamide gel electrophoresis, and ligated as a *Pst*I–*Nla*III–*Pst*I dimer into the 1.3-kb fragment-containing pUC18, opened with *Pst*I. An *Eco*RI site was introduced immediately 5′ to the start codon by opening the construct with *Hin*dIII and *Sph*I (which cleaved at the re-formed *Nla*III site) and inserting the complementary oligonucleotides 5′-AGCTTGGCGAATTC*ATG*-3′ and 3′-ACCGCTTAA-5′ (Symbicom, Umeå, Sweden). All steps to this point were verified by dideoxyribonucleotide sequencing in M13.

The mouse R2 cDNA was then isolated as a 1.3-kb fragment by partial *Eco*RI and complete *Bam*HI digestion of the pUC18 construct and ligated into the T7 RNA polymerase responsive vector pET3a (Rosenberg et al., 1987; Studier et al., 1990) cleaved with *Nde*I and *Bam*HI, theoretically resulting in an AGGA-to-ATG spacing of 15 bp. This construct, called pETM2, was transfected into *E. coli* strain BL21(DE3) (Rosenberg et al., 1987) which contains a *lac*(IPTG)-inducible, chromosomal copy of the T7 RNA polymerase gene.

The gene encoding the herpes simplex virus (type 1) R2 protein was previously isolated and ligated into a *tac* promoter based expression vector, as described (Ingemarson et al., 1989). A 1.7-kb *Nco*I–*Bam*HI fragment, commencing one base pair upstream from the start codon, was transferred from this construct into the T7 expression vector pET8c (Rosenberg et al., 1987; Studier et al., 1990), opened with the same enzymes. The resulting construct, pETH2, was transfected into BL21-(DE3) bacteria as described above. The plasmids pET3a and pET8c, and the bacterial strain BL21(DE3), were kindly provided by F. Studier, Department of Biology, Brookhaven National Laboratory.

*Expression of Recombinant R2 Protein.* Mouse and herpes simplex virus R2 proteins were produced by induction of logarithmically growing BL21(DE3) bacteria, containing either plasmid pETM2 or plasmid pETH2, respectively. Typically, 3 L of LB medium containing carbenicillin, 50 μg/mL, was infected with 5 mL of overnight cultures, shaken (275/min) at 37 °C, and supplemented with IPTG (0.5 mM) at $A_{590}$ = 0.4–0.6. After a further 4 h, the cultures were chilled, centrifuged at 2500*g* for 15 min at 2 °C, gently resuspended in 100–200 mL of 50 mM Tris, pH 7.6, and 1 mM EDTA, and centrifuged at 1000*g* for 10 min, and the pellets were frozen in liquid nitrogen and stored at −70 °C.

The *E. coli* R2 protein was overexpressed in C600 bacteria, containing the *nrdB* gene in a heat-sensitive runaway plasmid pBS1, as previously described (Sjöberg et al., 1986; Larsson & Sjöberg, 1986).

*Purification of Recombinant R2 Proteins.* Each protocol involved disintegration of frozen bacteria, extraction into buffer, precipitation of nucleic acids with streptomycin sulfate, ammonium sulfate precipitation, and anion-exchange chromatography. All operations were performed in a cold room at +4 °C. Mouse and viral R2 preparation, which is a modification of the previously published procedure of recombinant *E. coli* R2 (Sjöberg et al., 1986), will be described in detail, and the bacterial R2 protocol only where it differs. Protein concentrations and purity were assessed by Coomassie Brilliant Blue dye binding, with reference to a bovine serum albumin standard (Bradford, 1976), combined with laser densitometric (LKB Pharmacia) scanning of TCA-precipitated samples separated on 10% SDS–polyacrylamide gels (Engström et al., 1979). Concentrations of highly purified proteins were measured by light absorbance and calculated from extinction coefficients, as discussed below.

Frozen bacterial pellets (W g) were finely ground in a mortar with 2×W g of cold aluminum oxide (Sigma), using liquid nitrogen as necessary to prevent thawing. The powder was either stored (−70 °C) or directly mixed and thawed rapidly on ice in 4×W mL of extraction buffer (50 mM Tris-HCl, pH 7.6, 1 mM PMSF, and 1 mM EDTA) and centrifuged for 40 min at 44000*g*, 2 °C. Streptomycin sulfate, 10% (w/v), pH 7.0, was added to the supernatant, to a final concentration of 2.5% while stirring on ice, followed by centrifugation for 20 min, 27000*g*, 2 °C. Solid ammonium sulfate (0.243 g/mL, nominally 40%) was then added to the supernatant and the precipitate recovered by centrifugation for 30 min, 27000*g* 2 °C. After being dissolved in extraction buffer, the extract was iron-reactivated (see below) or directly equilibrated (apo-R2 preparations) in 50 mM Tris-HCl, pH 7.6, 1 mM PMSF, and 1 mM EDTA on a column containing Sephadex G-25 medium. Then about 30 mg of partially purified R2 was loaded onto a 3-mL (3.8 × 1 cm) DEAE-cellulose column (DE 52, Whatman), previously equilibrated with 10 mM potassium phosphate, pH 7.0, 30 mM KCl, and 1 mM EDTA. The column was washed with 10 mL of the same buffer, and then R2 was eluted in 3–4 column volumes of 10

mM potassium phosphate, pH 7.0, 70 mM KCl, and 1 mM EDTA. The protein eluate was frozen directly at −70 °C or recovered by overnight dialysis at 4 °C against saturated ammonium sulfate, centrifuged for 30 min, 25000*g*, 2 °C, dissolved in 50 mM Tris-HCl, pH 7.6, and stored in aliquots at −70 °C.

*E. coli* R2 was prepared essentially as described previously (Sjöberg et al., 1986; Larsson et al., 1988). However, bacteria were disintegrated in a French press, ammonium sulfate precipitation was to 60% (0.39 g/mL), a 150–300 mM potassium phosphate salt gradient was used (peak at 220 mM), and eluted R2 was concentrated by ultradialysis and further purified on a Mono Q HR 5/5 (Pharmacia LKB Biotechnology Inc.) anion-exchange column as previously described (Sjöberg et al., 1987).

*Iron Center and Radical Regeneration in Mouse and Viral R2.* Initially, iron and tyrosyl radial content was increased ("reactivated") after the first ammonium sulfate precipitation by adding a 4-fold molar excess of ferrous iron (as a freshly prepared, argon-purged solution of ferrous ammonium sulfate, typically 4 mM in 50 mM sodium ascorbate/50 mM Tris-HCl, pH 7.6) to an argon-equilibrated protein R2 solution, at 25 °C. After approximately 30 s, the solution was aerated, and after a total of 5-min incubation, the protein solution was transferred to ice and immediately desalted.

Later, reactivation was made on pure protein preparations after anion-exchange chromatography by either of the following two methods. In the anaerobic procedure, which is a minor modification of the method described above, R2 protein solutions in 50 mM Tris-HCl, pH 7.0, and 0.1 M KCl were equilibrated with argon in an EPR tube fitted with a stopcock for 60 min on ice. Then ferrous iron/ascorbate (molar ratio 1:2) was added anaerobically using a 50-μL Hamilton syringe with a long needle as an argon-equilibrated solution in the same buffer. The resulting solution was incubated for 10 min at 25 °C, and then 200 μL of air was bubbled through the solution using the same syringe. After a further incubation for 10 min at 25 °C, the sample was frozen in liquid nitrogen for later EPR measurements (Ochiai et al., 1990).

In the aerobic procedure used earlier (Thelander et al., 1985), a solution containing 1.5 mM ferrous iron and 200 mM dithiothreitol was added to R2 protein solutions in the same buffer as above to give a final concentration of 10 mM dithiothreitol, air was bubbled through the solution, and the mixture was incubated for 15 min at 25 °C and finally frozen in liquid nitrogen.

*Enzyme Activity and R1 Preparations.* Ribonucleotide reductase R2 activity was determined from the rate of reduction of [$^3$H]CDP; 1 unit is defined as the amount of protein which, in the presence of excess R1 subunit, catalyzes the formation of 1 nmol of dCDP/min at 37 °C (Engström et al., 1979; Ingemarson et al., 1989). Mouse R2 was assayed in the presence of 15 μg of pure calf thymus R1 protein, which was prepared in the following way. Preparation of crude extract, precipitation with streptomycin and ammonium sulfate, and DEAE-cellulose chromatography were performed as described (Engström et al., 1979). R1, as 2000 mL of DEAE eluate, containing about 850 $A_{280}$ units of protein, was then bound to a 20-mL AC1 monoclonal antibody (Engström, 1982; Engström et al., 1984) Sepharose 4B immunoaffinity column (5 mg of antibody bound/mL of gel after CNBr activation) equilibrated with 50 mM Tris, pH 7.6, and washed with 50 mM Tris containing successively 1 M KCl (until the absorbance of the eluate was close to zero) and 100 mM KCl, and finally with argon-equilibrated Tris containing 10% glycerol. R1 was eluted with 25 mL of 0.1 M glycine, pH 11.5, and 10% glycerol, followed by 25 mL of Tris/glycerol, both carefully equilibrated with argon. The first 8 mL was discarded and the next 42 mL collected into one column volume of gently stirring 2 M Tris-HCl, pH 6.8, and 10 mM dithiothreitol. After concentration and precipitation by dialysis against saturated ammonium sulfate, the protein was collected by centrifugation, dissolved in 50 mM Tris-HCl, pH 7.6, desalted into the same buffer, containing 100 mM KCl, and stored at −70 °C. The procedure results in 2.5–5 mg of pure R1 protein.

Herpes simplex virus type 1 R1 subunit was provided in the form of an extract of BHK-21 cells infected with HSV-1 strain 17 ts1222, which makes nonfunctional R2 subunit at the nonpermissive temperature (a generous gift from H. Marsden, MRC Virology Unit, Glasgow) (Preston et al., 1988; Ingemarson et al., 1989).

*Electronic Paramagnetic (EPR) Spectroscopy.* EPR first-derivative spectra were generally recorded at 20 K using a Bruker ER-200 spectrometer equipped with a 10-in. (25.4 cm) magnet and an Oxford cryostat. The tyrosyl free-radical content was also routinely determined at 77 K, using a Varian Model E-109 X-band spectrometer and E-238 cavity, at 9 GHz, 20 mW. The spectra were quantitated by peak height comparisons using a standard sample of mouse or herpes simplex virus R2 protein of known tyrosyl free-radical content. The original absolute quantitation was made by comparing double integrals of mouse and herpes virus protein R2 spectra at 77 and 30 K with that of a 1 mM Cu(II)/10 mM EDTA spectrum recorded under similar conditions. The uncertainties involved in free-radical quantitation are as usual fairly large, of the order of 20%. Contributing factors to the uncertainty are, e.g., curved base lines in the rather low-intensity EPR spectra and varying packing density after freezing of the different samples [cf. Peterson et al. (1980)].

*Light Absorption Spectra and Determination of Extinction Coefficients.* Light absorption spectra in the wavelength range 240–800 nm were recorded by using a Hitachi Perkin Elmer 320 spectrophotometer, at 10 °C. Immediately after the spectrum was recorded, aliquots (8 μL) containing 2–4 μg of protein were transferred to amino acid analysis tubes and stored frozen. These samples were subsequently hydrolyzed in 6 M constant-boiling hydrochloric acid, at 110 °C for 24 h and lyophilized, and amino acids were quantitated by using a Biotronic LC5000, with a Shimadzu C-R2AX data module. The mass of the original sample was calculated from the mean abundance of the following amino acids: Asp/Asn, Glu/Gln, Ala, Val, Ile, Leu, Tyr, Phe, Lys, and Arg.

*Iron Assay.* Iron concentrations in TCA-precipitated protein extracts were determined by the colorimetric method of Massey (1957) as modified by Atkin et al. (1973), using ferrous ammonium sulfate standards. About 90% of the iron was released from mouse R2 using either 5% TCA or 0.8 M HCl to precipitate the protein [cf. Lynch et al. (1989)]. This was evident from experiments in which the iron center of the R2 protein had been regenerated as described above using $^{55}$Fe/ascorbate followed by gel filtration (cf. Table II).

*Circular Dichroism.* Circular dichroism (CD) spectra were recorded on a Jasco J-600 spectropolarimeter using a cuvette with 2-mm light path and base-line correction. The samples were approximately 100 μg/mL in 50 mM potassium phosphate buffer, pH 7.5.

A quantitative evaluation of the CD spectra was made by a computer program [Protein Secondary Structure Estimation (SSE) program, supplied by Japan Spectroscopic Co., Ltd.]. In this program, the experimental spectrum is fitted by a

Table I: Summary of Purification of Recombinant Mouse R2 Protein from 6 L of Bacterial Culture

| step | total protein (mg) | R2 protein | | yield (%) |
|---|---|---|---|---|
| | | %[a] | mg | |
| extract | 118[b] | 35 | 41 | 100 |
| ammonium sulfate | 35[b] | 85 | 30 | 73 |
| DEAE-cellulose | 10.5[c] | >98 | 10.5 | 26 |

[a] Determined by laser densitometric scanning of Coomassie-stained SDS–polyacrylamide gel. [b] Protein concentration determined by the method of Bradford (1976). [c] Protein concentration determined by the $A_{280-310}$ absorbance using an $E^{1\%}_{1cm}$ of 13.8.

least-squares method to a linear combination of four reference spectra representing $\alpha$-helix, $\beta$-sheet, $\beta$-turn, and random coil secondary structures (Chang et al., 1978; Brahms & Brahms, 1980). The four reference spectra were extracted from a set of basis spectra of seven proteins included in the SSE program (myoglobin, hemoglobin, cytochrome *c*, lysozyme, chymotrypsin A, ribonuclease A, and papain) for which the secondary structure composition is known.

*Nuclear Magnetic Resonance (NMR) Spectroscopy.* $^1$H NMR spectra (500 MHz) were recorded at 10 °C on a Bruker AM 500 spectrometer, interfaced with an Aspect 3000 computer. A super-WEFT pulse sequence (Inubishi & Becker, 1983) was used to suppress the water signal. The spectra were obtained by using a bandwidth of 83 kHz and 16K data points, and 20 000–77 000 transients.

Protein solutions for NMR studies were concentrated by centrifugation on Centricon 30 (Amicon) size-exclusion membranes at 5000*g*, 2 °C. The samples (500 $\mu$L) were prepared as approximately 1 mM solutions of R2 in 50 mM potassium phosphate, pH 7.5. The presented spectra were base-line-corrected using a best-fitting polynominal base line. A line-broadening factor of 35 Hz was used in Fourier transformation. The ppm standard was 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), using the water resonance at 4.9 ppm (10 °C) as a secondary reference.

## Results

*Purification of Recombinant R2 Protein.* Purification and yields of the recombinant mouse R2 protein from 6 L of bacterial culture are summarized in Table I; an SDS–polyacrylamide gel electrophoresis analysis is shown in Figure 1. Some proteolysis of the full-length (45 kDa) mouse protein invariably occurred, especially during the anion-exchange chromatography. Protease inhibitors [PMSF, EDTA, pepstatin, leupeptin, 1,10-phenanthroline, benzamidine, *N*-[*N*-(L-3-*trans*-carboxyrane-2-carbonyl)-L-leucyl]agmatine, and 3,4-dichloroisocoumarin] added alone or in combination had very little protective effect. Therefore, we designed a rapid stepwise elution of the DEAE-cellulose column resulting in preparations containing up to 88% of the full-length 45-kDa polypeptide. The most abundant partially degraded form was 43.5 kDa in length (Figure 1). The preparations also contained variable minor amounts of more degraded forms, 41 and 39 kDa in length, seen most pronounced in fractions eluting late from the DEAE-cellulose column.

The 45-, 43.5-, 41-, and 39-kDa polypeptides all react with rat anti-mouse R2 monoclonal antibodies in immunoblots [cf. Engström and Rozell (1988)]. Furthermore, the relative proportions of the 45-kDa polypeptide and the 43.5-, 41-, and 39-kDa polypeptides in mouse R2 preparations changed with time on handling and incubation in the cold room. There was a parallell decrease in the 45-kDa polypeptide and increase in the more degraded forms. Finally, this pattern of polypeptides was specific for preparations using the mouse R2 expression vector and was never observed using the herpes simplex virus R2 expression vector (see below), and still both the bacterial host and the protein preparation procedure were the same. Therefore, we conclude that the 45-, 43.5-, 41-, and 39-kDa polypeptides all represent true mouse R2 polypeptides. Reactivity in immunoblots with the monoclonal antibody YL 1/2 which recognizes the carboxyl-terminal residues -Ala-Asp-Phe of the mouse R2 protein [cf. Thelander et al. (1985)] indicates that proteolysis in all cases was N-terminal (data not shown).

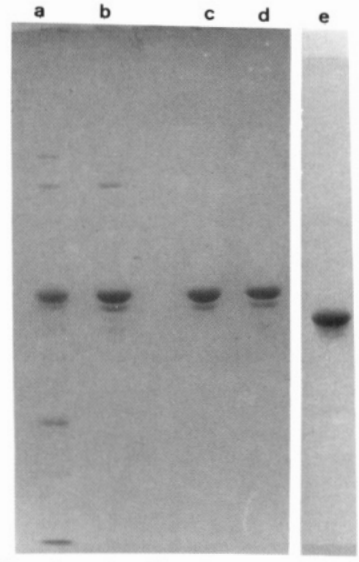


FIGURE 1: SDS–polyacrylamide gel electrophoresis of recombinant mouse and herpes simplex virus R2 protein preparations. All samples were analyzed on a 10% polyacrylamide gel. The sample wells are in the top of the figure and the position of the dye marker in the bottom. Lane a, 16 $\mu$g of a crude extract of mouse R2 protein. Lane b, 10 $\mu$g of a mouse R2 preparation after ammonium sulfate precipitation. Lane c, 6 $\mu$g of a mouse R2 preparation after DEAE-cellulose chromatography (early eluate). Lane d, the same as in (c) but a late eluate. Lane e, 6 $\mu$g of a herpes simplex virus R2 preparation after DEAE-cellulose chromatography (analyzed on a separate gel).

Specific activity of the recombinant mouse R2 protein was 280 units/mg measured on a preparation containing 85% 45-kDa polypeptide. However, at least the 43.5-kDa polypeptide also seemed to be enzymatically active since one especially degraded R2 preparation containing only 48% 45-kDa and about 20% each of the 43.5- and 41-kDa polypeptides had a specific activity of 240 units/mg. When assayed under normal assay conditions, i.e., at less than 1 $\mu$M R2 in the presence of 10 $\mu$M ferrous iron, reactivated R2 and the corresponding unreactivated "apoprotein" (iron content 10%) were equally active. The iron center/tyrosyl radical complex is thus rapidly formed under assay conditions. In contrast, unreactivated R2 was only 20% as active as reactivated R2 if iron was omitted from the assay.

Herpes virus R2 was produced in 100-fold higher yield than by the previously published method (Ingemarson et al., 1989). This allowed a purification procedure very similar to the one presented in Table I for the mouse R2 protein. Little if any proteolysis occurred in the presence of PMSF alone (Figure 1). The recombinant viral R2 had a specific activity of at least 51 units/mg, compared with the previously reported value of 15 units/mg for immunopurified R2 (Ingemarson et al., 1989) using the same crude extract as a source of herpes simplex virus protein R1 in both assays.

*Characterization of Protein R2 Tyrosyl Free Radical.* The EPR spectrum of pure recombinant mouse R2 is shown in Figure 2a and is essentially identical with that previously

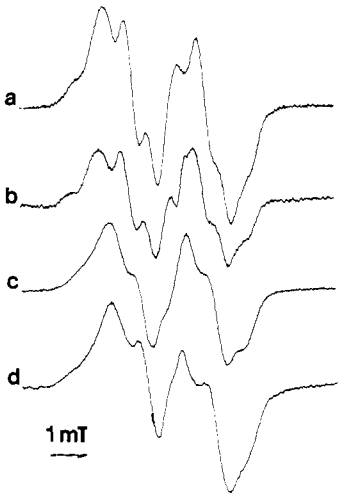


FIGURE 2: EPR spectra of tyrosyl radicals at nonsaturating microwave power conditions. (a) Recombinant mouse protein R2, 20 K. (b) Hydroxyurea-resistant mouse TA 3 cells, 30 K [from Sahlin et al. (1987)]. (c) Recombinant herpes simplex virus type 1 protein R2, 32 K [from Ingemarsson et al. (1989)]. (d) *E. coli* protein R2, 30 K [from Sahlin et al. (1987)].

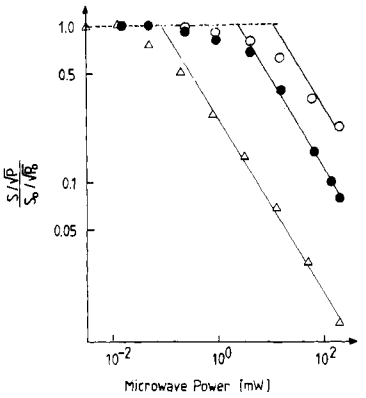


FIGURE 3: EPR microwave saturation curves at 30 K for proteins R2 from *E. coli* (△), recombinant mouse (●), and recombinant herpes simplex virus type 1 (○). The data for *E. coli* R2 were taken from Sahlin et al. (1987). $S$ is signal amplitude, $P$ is microwave power, and $S_0$ and $P_0$ are reference values at nonsaturating conditions.

published for R2-overexpressing mouse cells (Gräslund et al., 1982) (Figure 2b) and that obtained from pure native mouse R2 (Thelander, unpublished results). For comparison, the figure also includes the low-temperature EPR spectra from recombinant herpes virus protein R2 and *E. coli* protein R2.

The tyrosyl radicals of the R2 proteins from mouse, herpes simplex virus, and *E. coli* exhibit significant differences in their temperature-dependent microwave saturation behavior (Ingemarson et al., 1989). Figure 3 shows that, at 30 K, microwave saturation of the tyrosyl radicals in the mouse and viral R2 proteins occurs at much higher applied power than in *E. coli* R2 (Sahlin et al., 1987).

*Light Absorption Spectra and Extinction Coefficients.* Light absorption spectra for mouse and herpes simplex virus R2 proteins after iron/radical regeneration are shown in Figure 4 together with a spectrum for the *E. coli* protein. Above about 300 nm, the characteristic bands of the iron center and tyrosyl radical overlap with the tail of the aromatic amino acid contribution. The arrows indicate iron and tyrosyl radical light absorption bands previously reported for the *E. coli* protein R2 (Petersson et al., 1980). It is clear that these bands also appear in the recombinant proteins centered at about the same wavelengths. Note that, as in previous studies (Thelander et al., 1985), light absorption features were attributed to the

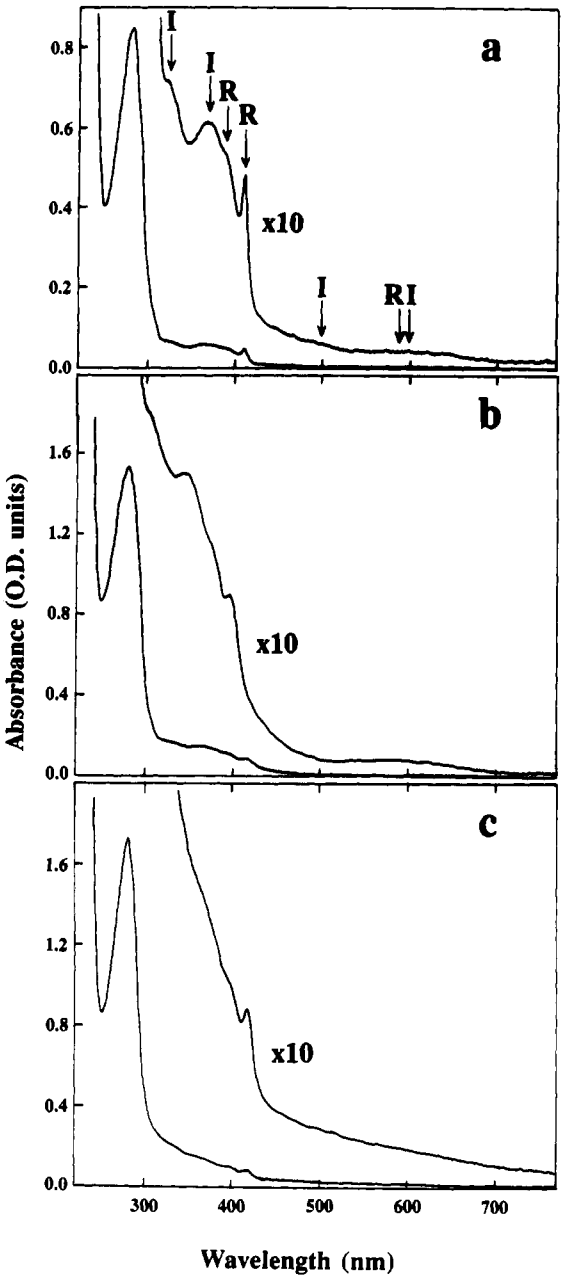


FIGURE 4: Optical absorption spectra of the small subunits of ribonucleotide reductase dissolved in 50 mM Tris-HCl, pH 7.0, and 0.1 M KCl. The sample conditions were (a) 42 μM reconstituted R2 polypeptide from *E. coli*, (b) 105 μM reconstituted R2 polypeptide from mouse, and (c) 95 μM reconstituted R2 polypeptide from herpes simplex virus type 1. The spectra were recorded in 2-mm cuvettes at 10 °C. The arrows denote the iron center (I) and tyrosyl radical (R) light absorption bands.

radical by their disappearance on hydroxyurea treatment. The herpes virus protein R2 spectrum (Figure 4c) has relatively weak and broad contributions from the iron/radical site and a more noticeable light-scattering tail than the other two spectra. The sharp band of the tyrosyl free radical at about 410 nm is visible in all three spectra, although shifted to 415 nm and somewhat broadened in the mouse and herpes simplex virus proteins.

The $\epsilon_{310}$ of *E. coli* protein R2 with full iron occupancy and about 50% free-radical content is typically 10–15% of $\epsilon_{280}$ (cf. Figure 4a). In many previous studies on *E. coli* protein R2, protein concentration has been estimated by measuring $\epsilon_{280}-\epsilon_{310}$, using the subtraction as a way to correct for a variable amount of iron and radical contribution in addition to possible light scattering. However, for accurate use of extinction

Table II: Generation of Iron–Tyrosyl Free-Radical Center in Recombinant Mouse or Herpes Simplex Virus R2 Protein

| R2 prepn | method | Fe added (mol/polypeptide) | tyrosyl radical content (%)[b] before | tyrosyl radical content (%)[b] after | iron content[a] (%)[b] before | iron content[a] (%)[b] after |
|---|---|---|---|---|---|---|
| mouse, 60% 45 kDa | Fe/ascorbate | 1.8 | 5 | 62 | 10 | 70 |
| mouse, 60% 45 kDa | Fe/ascorbate | 3.6 | | 59 | | 105 |
| mouse, 60% 45 kDa | Fe/dithiothreitol | 2.0 | | 20 | | |
| mouse, 60% 45 kDa | Fe/dithiothreitol | 4.0 | | 40 | | |
| mouse, 85% 45 kDa | Fe/ascorbate | 3.5 | 14 | 76 | | |
| mouse, 48% 45 kDa | Fe/ascorbate | 4.0 | | 58 | | |
| herpes simplex virus | Fe/ascorbate | 1.5 | 0 | 16 | 6 | 50 |
| herpes simplex virus | Fe/ascorbate | 2.9 | | 16 | | 55 |
| herpes simplex virus | Fe/ascorbate | 3.6 | | 23 | | 55 |
| herpes simplex virus | virus | Fe/ascorbate | 10.0 | 14 | | 115 |

[a] Iron content was determined after passage through a Sephadex G-25 column to remove excess iron. [b] 100% is one tyrosyl free radical and two irons per single polypeptide chain.

coefficients for protein concentration determination, measurements of $\epsilon_{280}$ should be made on apoprotein.

We have determined extinction coefficients at 280 and 310 nm for a number of samples from mouse and herpes simplex virus R2 proteins with low iron (≤20%) and low radical (≤10%) content, counting two iron ions and one radical per polypeptide as full occupancy. In both cases, $\epsilon_{310}$ was about 10% of $\epsilon_{280}$. The average value (±3000) of two determinations of $\epsilon_{280-310}$ was 62 000 $M^{-1}$ $cm^{-1}$ for mouse R2 and 52 000 $M^{-1}$ $cm^{-1}$ for R2 from herpes simplex virus, expressing concentration as molar 45-kDa and 38-kDa polypeptide, respectively. After treatment with $Fe^{2+}$ and $O_2$, the $\epsilon_{280-310}$ was found to increase 10–20%. A corresponding measurement of the extinction coefficient for reconstituted protein R2 from *E. coli* with high iron and radical content (152% and 60%, respectively) determined in parallel gave a value of $\epsilon_{280-310}$ of 88 000 $M^{-1}$ $cm^{-1}$.

*Iron and Tyrosyl Free-Radical Content.* Here the maximum achievable values (100%) are assumed to be one tyrosyl free radical and two iron ions per R2 polypeptide chain (i.e., monomer). Treatment of recombinant mouse R2 with ferrous iron and oxygen prior to the anion-exchange chromatography step, as described above, resulted in a large (at least 4-fold) increase in both iron (to 70–85%) and free-radical content (to 30%). This level of free radical and iron was obtained after the chromatography and subsequent steps despite the presence of EDTA at 1 mM in the column eluate.

A series of reactivation experiments using pure mouse or viral R2 preparations showed that as much as 0.8 tyrosyl radical per 45-kDa mouse polypeptide could be obtained (Table II). The anaerobic iron–ascorbate method was found to be more efficient in generating tyrosyl radical calculated per added iron than the aerobic iron–dithiothreitol method. After passage through a Sephadex G-25 column to remove unbound iron, iron analyses of a sample of mouse R2 reactivated with 3.6 Fe/45 kDa showed 2.0 irons per polypeptide chain.

In agreement with the results from the activity measurements, an R2 sample which contained only 48% 45-kDa polypeptide and around 40% 43.5-kDa plus 41-kDa polypeptide showed almost the same radical content after reactivation as the less degraded R2 preparations. Neither time nor oxygen was limiting in the reactivation reactions since more air or longer incubation times did not increase the radical yield.

Reactivation of pure herpes simplex virus R2 preparations never resulted in more than about 0.2 tyrosyl radical per polypeptide chain independent of the method used. Likewise, a greater excess of iron had to be added to viral R2 than to mouse R2 to reach full iron occupancy (Table II).

*Oxygen Consumption.* The following reaction can be postulated to generate the Fe–tyrosyl radical center in R2 proteins:

$$Fe^{II}_2 + \text{tyr–OH} + O_2 + X + H^+ \rightarrow Fe^{III}O^{2-}Fe^{III} + \text{tyr–O}^{\bullet} + X^{1+} + H_2O$$

In the reaction, $O_2$ is fully reduced to water, and there should be one $O_2$ consumed per tyrosyl radical formed. In our normal reactivation experiments, we always added a great and rather uncontrolled excess of oxygen in contrast to the precisely controlled amounts of iron and protein. We have recently shown that X in this reaction may be $Fe^{2+}$ (Ochiai et al., 1990).

To investigate the stoichiometry of the oxygen requirement, we now performed experiments where excess ferrous iron was added anaerobically to mouse R2 in EPR tubes under argon. After 10-min incubation at 24 °C, the sample was frozen and the tyrosyl radical content recorded by EPR. This gave the sum of radical originally present in the apoprotein preparation and radical generated by possible traces of oxygen in the added iron solution and represented the starting value in each experiment. Then the sample was thawed under argon, and substoichiometric amounts of oxygen were added strictly anaerobically in the form of water equilibrated with air at room temperature and therefore containing known amounts of dissolved oxygen. After incubation at 24 °C for 5 min under argon, the samples were frozen in liquid nitrogen, and the EPR spectrum was recorded. They were then thawed again, more oxygen was added, and the whole procedure was repeated.

The results from two experiments using different mouse R2 preparations are shown in Figure 5 where the formation of tyrosyl radical is plotted against nanomoles of oxygen added. Depending on how fast the oxygenated water was mixed in and on the condition of the protein, 0.3–1.0 tyrosyl radical was generated per added $O_2$. This indicates that the R2 iron center has a very high affinity for oxygen. The protein samples turned increasingly opalescent on repeated freezing and thawing, indicating denaturation which probably accounts for both the failure to reach full radical content and its reduction at later time points.

Table III: Secondary Structure Composition from Circular Dichroism Studies of R2 Proteins of Ribonucleotide Reductase from Mouse, Herpes Simple Virus Type 1, and *Escherichia coli*

| protein R2 from | α-helix | β-sheet | β-turn | random coil |
|---|---|---|---|---|
| mouse | 49 | 17 | 25 | 9 |
| herpes simplex virus type 1 | 52 | 21 | 13 | 14 |
| *E. coli* | 66 | 3 | 19 | 12 |

*Circular Dichroism.* Circular dichroism spectra recorded for the R2 proteins of mouse, herpes simplex virus type 1, and

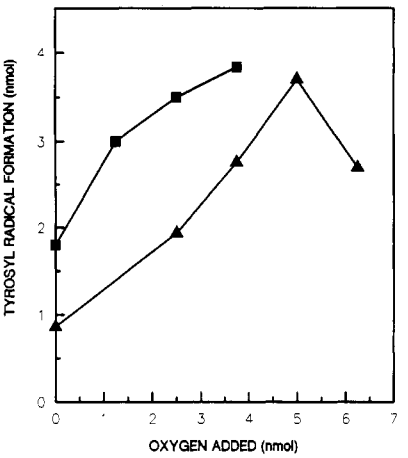


FIGURE 5: Oxygen consumption in tyrosyl free-radical generation. The upper curve (■) was generated by using 6.2 nmol (single polypeptide mole) of mouse R2 apoprotein (containing 85% 45-kDa polypeptide) and 22 nmol of Fe(II) in 200 μL of 50 mM Tris-HCl, pH 7.0, and 0.1 M KCl. The lower curve (▲) was obtained with a similar solution containing the same amounts of a mouse R2 preparation containing 60% intact 45-kDa polypeptide. Oxygen was added as 5- or 10-μL aliquots of water equilibrated with air at room temperature (24 °C).

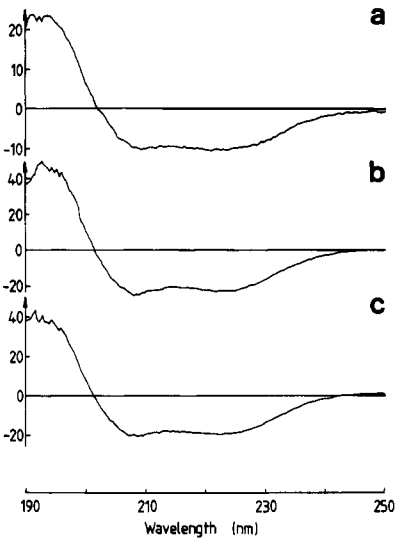


FIGURE 6: Circular dichroism spectra of the small subunits of ribonucleotide reductase dissolved in 25 mM potassium phosphate buffer, pH 7.5. The recording conditions were (a) 30 μg/mL protein R2 from *E. coli*, 2-mm cuvette, 5 °C; (b) 150 μg/mL protein R2 from mouse, 1-mm cuvette, 5 °C; (c) 75 μg/mL protein R2 from herpes simplex virus type 1, 2-mm cuvette, 10 °C. The vertical scale is ellipticity in millidegrees.

*E. coli* are shown in Figure 6. Evaluation of secondary structure components gave the results shown in Table III. Whereas the *E. coli* protein R2 contains almost 70% α-helix, the corresponding figures for the mouse and the herpes virus proteins are about 50%.

*NMR Spectroscopy.* $^1$H NMR spectra of R2 proteins in the normal protein region (0–10 ppm) are poorly resolved due to the high molecular weight of the proteins (data not shown). However, as was previously observed for the *E. coli* protein (Sahlin et al., 1986), the iron center of protein R2 gives rise to paramagnetically shifted proton resonances. Despite their low intensity, these resonances can be observed since they occur far beyond the normal protein resonance region.

Figure 7 shows the $^1$H resonances observed in the 16–32 ppm region of the R2 proteins from mouse and herpes simplex virus. The spectra were recorded in aqueous solution using a super-WEFT pulse sequence to suppress particularly the

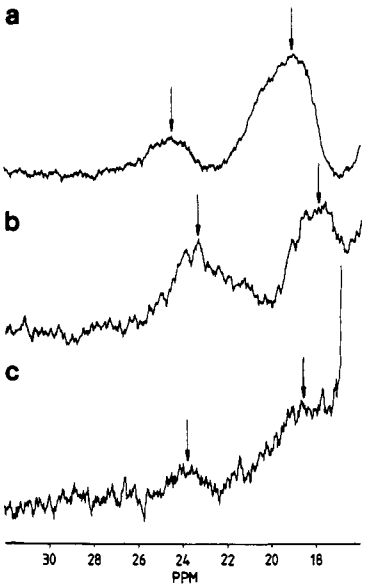


FIGURE 7: $^1$H NMR spectra (500 MHz) at 10 °C of the small subunits of ribonucleotide reductase dissolved in 50 mM potassium phosphate buffer, pH 7.5, containing 10% $D_2O$ for spectrometer lock. The experimental conditons were (a) 0.45 mM R2 polypeptide from *E. coli*, 152% iron, 20 000 transients; (b) 0.9 mM R2 polypeptide from mouse, 70% iron, 77 000 transients; and (c) 1.0 mM R2 polypeptide from herpes simplex virus type 1, 40% iron, 44 000 transients. The arrows indicate the paramagnetically shifted resonances.

Table IV: Paramagnetically Shifted $^1$H Resonances at 10 °C in R2 Proteins of Ribonucleotide Reductase from Mouse, Herpes Simple Virus Type 1, and *Escherichia coli*

| protein R2 from | concn (mM) | iron occupancy (%)[a] | resonance 1 (ppm) | resonance 2 (ppm) |
|---|---|---|---|---|
| mouse | 0.9 | 68 | 18 | 23 |
| herpes simplex virus type 1 | 1.0 | 43 | 18.5 | 23.5 |
| *Escherichia coli* | 0.45 | ~100 | 19 | 24 |

[a] Average number of iron pairs per polypeptide chain.

$H_2O$ resonance, and also to some extent the normal protein resonances (Inubishi & Becker, 1983). The corresponding spectrum recorded for the *E. coli* protein R2 is also included in the figure. In the previous study of the *E. coli* protein, the suggested assignments for the two proton resonances observed at 24 ppm (solvent exchangeable) and at 19 ppm (nonexchangeable) were to NH of a ligand imidazole ring and to methylene protons of ligand nonbridging carboxylic amino acid residues, respectively (Sahlin et al., 1986). These assignments are now supported by the recently reported three-dimensional crystal structure of *E. coli* protein R2, in which indeed both histidine and carboxylic amino residues are found to be iron ligands (Nordlund et al., 1990). Clearly, both mouse and herpes simplex virus R2 proteins exhibit resonances very similar to those of the *E. coli* protein (Table IV).

## Discussion

Recovery of 1 mg of native mouse R2 protein has previously (Thelander et al., 1985) involved harvesting more than $5 \times 10^9$ R2-overproducing TA 3 mammary tumor cells as ascites tumors in 20–50 mice; the same amount of recombinant mouse R2 protein was obtained from 500 mL of bacterial culture. The specific activity of the recombinant mouse R2 was 5-fold higher than the value obtained earlier for the material prepared from overproducing mouse cells when assayed under similar conditions. This probably reflects the much simpler and faster preparation procedure resulting in a more native protein. Like

many other enzymes, protein R2 rapidly loses activity on handling and repeated freezing and thawing.

Also the recombinant herpes simplex virus R2 protein showed a 3-fold higher specific activity than the value reported earlier (Ingemarson et al., 1989). However, the use of a crude cellular extract as a source of herpes simplex virus protein R1 compromises the assay, so no comparison with the specific activity of mouse R2 ought to be made. It is interesting that the T7 RNA polymerase expression system yielded 100-fold more viral R2 protein than the previously published method using a tac-promoter expression system (Ingemarson et al., 1989).

The observed N-terminal proteolysis of the recombinant mouse R2 protein, resulting in 43.5-, 41-, and 39-kDa polypeptides, may occur at protease-sensitive PEST sequences in its amino-terminal domain (Rogers et al., 1986; Rechsteiner, 1988). However, the 43.5-kDa polypeptide, which is the major degradation product, appears to be as capable as the intact protein of forming the iron/radical center and has similar specific activity. This suggests that the N-terminal amino acid residues are dispensable for activity in contrast to the C-terminal ones which are very important for subunit interaction (Cosentino et al., 1990). The mammalian PEST sequences have no homologues in herpes simplex virus or bacterial R2, which are much less susceptible to proteolysis during purification.

The recombinant protein R2 preparations ("apo R2") usually contained less than 15% iron and tyrosyl free radical, 100% representing two iron ions and one tyrosyl free radical per polypeptide chain. When the mouse R2 protein was used, the anaerobic reactivation method resulted in 0.8 radical and 2.0 irons per polypeptide chain. This is the highest radical content reported so far for any R2 protein [cf. Lynch et al. (1989)], and both values are in full agreement with the values predicted from the X-ray crystallographic structure of the *E. coli* R2 protein. In contrast, similar experiments using different preparations of recombinant viral R2 protein never resulted in more than about 0.2 radical per polypeptide chain. Furthermore, to get two irons per viral protein polypeptide chain, a greater excess of iron (5-fold instead of 2-fold) was required in the regeneration reaction. This indicates that the viral protein has lower affinity for iron than the mouse protein under these conditions.

The aerobic iron–dithiothreitol regeneration method was less efficient (per added iron) than the anaerobic iron–ascorbate method in forming tyrosyl free radical. However, under assay conditions in the presence of a large excess of Fe(II)—usually 20–40-fold—and dithiothreitol, the apoprotein rapidly formed the iron/radical center and showed the same specific activity as previously regenerated protein.

We have recently shown, by adding substoichiometric amounts of Fe(II) to recombinant mouse R2 apoprotein under anaerobic conditions followed by a brief exposure to air, that three Fe(II) were needed to produce one tyrosyl radical. Two of the irons formed the binuclear iron center, showing very high affinity for the protein, while the third supplied the fourth electron required to fully reduce one $O_2$ to water (Ochiai et al., 1990):

$$3Fe(II) + P + O_2 + tyr\text{–}OH + H^+ \rightarrow Fe^{III}O^{2-}Fe^{III}\text{–}P + H_2O + tyr\text{–}O^{\bullet} + Fe(III)$$

where P designates the iron binding site of the R2 protein.

Using controlled, substoichiometric amounts of oxygen in the presence of excess iron and protein, we now demonstrate that one $O_2$ indeed can produce one tyrosyl free radical. Essentially all added oxygen was used to generate the tyrosyl radical, indicating a very high affinity between oxygen and the R2 iron center.

The light absorption spectra of the recombinant mouse and herpes simplex virus proteins R2 show the characteristic iron center–tyrosyl radical bands above 300 nm as previously described for the *E. coli* and mouse proteins (Peterson et al., 1980; Thelander et al., 1985). In the *E. coli* protein, the iron bands at 325 and 370 nm (as well as weak bands at 500 and 600 nm) were attributed to the $\mu$-oxo-bridged dimeric iron center. A band at 380 nm and the sharp maximum at 410 nm were attributed to the tyrosyl radical. In the mouse and herpes virus proteins, the sharp maximum due to the tyrosyl radical appears around 415 nm. In a study of a series of model phenoxy radicals, light absorption spectra were reported to have maxima around 400–403 nm for radicals prepared by flash photolysis or chemical oxidation in various liquid systems, whereas maxima around 414–422 nm were reported for radicals in rigid solutions (e.g., polar glass) (Land et al., 1961). Obviously the optical spectral properties of protein R2 tyrosyl radicals in all three species compared in Figure 4 agree well with those of immobilized neutral phenoxy radicals.

Despite the uncertainties in the extinction coefficients, the values for the three proteins exhibit significant differences between species, which can be understood by considering their different amino acid compositions. The $\epsilon_{280}$ due to amino acids is determined mainly by the tryptophan and tyrosine residues, which contribute to the $\epsilon_{280}$ in the approximate ratio 3.6:1 (Wetlaufer, 1982). Protein R2 from mouse has 6 Trp and 9 Tyr, from herpes simplex virus 2 Trp and 14 Tyr, and from *E. coli* 7 Trp and 16 Tyr (Nordlund et al., 1990). For the apoproteins, we can therefore predict that their $\epsilon_{280}$ ratios should be 1.4:1:1.9, which compares favorably with the experimentally determined ratios (1.2:1:1.7 for mouse:herpes simplex virus:*E. coli*).

The circular dichroism results indicate a similar secondary structure composition for the mouse and herpes virus proteins, distinguishing them from the *E. coli* protein. The results for the *E. coli* protein are in good agreement with those previously reported (Sjöberg et al., 1982) and also with the three-dimensional structure determination (Nordlund et al., 1990). It is clear that protein R2 from *E. coli* is very rich in $\alpha$-helical structure (70%), with structural motifs mainly consisting of helix–turn–helix elements. The mouse and viral proteins are also dominated by $\alpha$-helix comprising 50% of their secondary structures. However, they appear to have a contribution also from $\beta$-sheet secondary structure. It is interesting to note these close similarities in secondary structures despite the fact that the amino acid homology is only 24% between *E. coli* and mouse and 27% between mouse and herpes simplex virus type 1 R2 proteins (Nordlund, 1990).

The iron sites of the different R2 proteins have been characterized by the paramagnetic shifts produced for proton resonances of its iron ligands. The spectra can be regarded as fingerprints of the iron site ligand composition and geometry. The almost perfect identity of the paramagnetically shifted $^1H$ resonances argues for almost identical iron centers in these three proteins, as regards types of iron ligands and their geometry as well as the antiferromagnetic coupling between the iron ions. The close similarity is also supported by essentially identical light absorption spectra attributed to the iron site in the three proteins. From amino acid sequence alignment, it is known that the likely iron ligands are absolutely conserved in the three proteins (Nordlund, 1990).

One striking difference observed between the proteins of different origin is that of the temperature-dependent relaxation

properties of the tyrosyl free radical. Previous studies (Sahlin et al., 1987) on the magnetic interaction between the iron site and free radical have indicated at least two possible explanations for these differences: the antiferromagnetic interaction between the iron ions might vary between species or the radical/iron site distance might vary. In view of the present findings, the most likely interpretation of the observed stronger iron site/radical interaction in the mouse and viral proteins is that the iron site/radical distances are smaller, since the iron sites themselves should be almost identical. Another observation in this context is that the relative intensities of the paramagnetically shifted NMR resonances are unexpectedly low for the mouse and herpes virus proteins compared to the *E. coli* protein R2, taking into account the protein concentration, iron loading, and number of transients collected. This might be an effect of signal broadening, caused by the stronger interaction between iron center and free radical.

The similarities and differences between the active sites of the *E. coli*, mouse, and herpes simplex virus R2 proteins form an interesting background for answering questions regarding the enzyme reaction and pathways of electron transfer as well as susceptibility to species-specific inhibitors of ribonucleotide reductase (Cosentino et al., 1990).

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**Registry No.** Fe, 7439-89-6; ribonucleoside diphosphate reductase, 9047-64-7; tyrosyl radical, 16978-66-8.

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