Radiation Inactivation of Ribonucleotide Reductase, an Enzyme with a Stable Free Radical

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ABSTRACT Herpes simplex virus ribonucleotide reductase (RR) is a tetrameric enzyme composed of two homodimers of large R1 and small R2 subunits with a tyrosyl free radical located on the small subunit. Irradiation of the holoenzyme yielded simple exponential decay curves and an estimated functional target size of 315 kDa. Western blot analysis of irradiated holoenzyme R1 and R2 yielded target sizes of 281 kDa and 57 kDa (approximately twice their expected size). Irradiation of free R1 and analysis by all methods yielded a single exponential decay with target sizes ranging from 128–153 kDa. For free R2, quantitation by enzyme activity and Western blot analyses yielded simple inactivation curves but considerably different target sizes of 223 kDa and 19 kDa, respectively; competition for radioligand binding in irradiated R2 subunits yielded two species, one with a target size of ~210 kDa and the other of ~20 kDa. These results are consistent with a model in which there is radiation energy transfer between the two monomers of both R1 and R2 only in the holoenzyme, a radiation-induced loss of free radical only in the isolated R2, and an alteration of the tertiary structure of R2.

INTRODUCTION

The ribonucleotide reductases (RR) are enzymes that catalyze the conversion of ribonucleoside 5′-diphosphate to the corresponding deoxyribonucleotide analog requisite for DNA replication. Herpes simplex virus (HSV) encodes for its own RR enzyme, which is an essential enzyme for the replication of HSV in resting cells (Goldstein and Weller, 1988; Jacobson et al., 1989). Like the mammalian and bacterial RRs, the HSV enzyme consists of two non-identical subunits (Lammers and Follman, 1983; Ingemarson and Lankinen, 1987). The small subunit (R2) is specified by a 1.2 kb mRNA encoding a 340-amino acid, 38,019 Da protein. The large subunit (R1) is specified by a 5.0 kb mRNA transcript that encodes a 1137-amino acid, 124,050 Da protein (McGeoch et al., 1988). On denatured polyacrylamide gel electrophoresis R2 migrates at the expected M_r of 38,000 (Mann et al., 1991; Lankinen et al., 1991; Lamarche et al., 1990). However, HSV R1 has been reported to migrate at an M_r of 140,000, possibly because of the presence of a hydrophobic N-terminal domain that retards the protein during electrophoresis (Furlong et al., 1991; Massie et al., 1995).

Structurally, the R2 contains a tyrosyl radical and a μ-oxo bridged binuclear ferric center that are crucial for the reduction process. R1 contains redox-active thiols that provide the hydrogen for nucleotide reduction (Stubbe, 1990). Association of the R1 and R2 is essential for catalytic activity. The C-terminus of R2 has been shown to be mobile in solution (Laplante et al., 1994) and is critical for this process (Filatov et al., 1992). These properties of HSV RR are common to other iron-containing RR and are crucial to their catalytic activity.

Knowledge of the molecular mechanism of enzymatic reduction of ribonucleotides is derived from elegant studies conducted with recombinant *Escherichia coli* ribonucleotide reductase (Stubbe et al., 1983; Erikson and Sjoberg, 1989). The R1 protein binds the substrate and provides the hydrogens for nucleotide reduction. R2 provides a tyrosyl free radical which subsequently, via radical transfer, provides a protein radical on R1 in close enough proximity to abstract the 3′-hydrogen atom of the substrate to yield the desired deoxyribonucleotide (Stubbe, 1990; Bollinger et al., 1991). The three-dimensional structure of the R1 and R2 proteins clearly supports this model (Uhlin and Eklund, 1994; Nordlund et al., 1990; Sjoberg, 1994) and shows that the radical-carrying tyrosine is inaccessible to solvent as it is buried in the interior of each protomer.

Although there exists a reasonable amount of information on the molecular structure of HSV RR subunits, there is less information on the nature of subunit interactions within the holoenzyme. However, it is known from direct binding studies that the affinity of recombinant R2 for R1 is in the 100 nM range (Krogsrud et al., 1993) and that R1 and R2 each form stable homodimers (Lankinen et al., 1991; Connor et al., 1993).

Radiation inactivation was chosen as the technique both to independently evaluate existing theories on the subunit interactions for RR and to further explore their nature. This technique is based on several general principles: gamma rays and high-energy electrons interact randomly with proteins, dependent only on their mass. In each interaction, large amounts of energy are deposited in protein molecules, resulting in breakage of many covalent bonds. Every polypeptide that is directly affected suffers severe and irre-

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versible structural damage and loses all biological activity. When irradiated in the frozen state, damage to individual molecules has no effect elsewhere. Only molecules that escape radiation damage retain both structure and complete function. Because of the random nature of the radiation interactions, the surviving biological functions decrease exponentially with radiation dose at a rate directly proportional to the mass of the active structure. In a complex mixture of polypeptides and other molecules, radiation damage to other molecules that are not involved in the measured activity has no effect on the measurement (Kempner, 1988). Several individual experimental tests have confirmed the basic principles of radiation target theory.

The method has been used successfully to study a wide variety of biologically active proteins (Kempner, 1988). These revealed the mass of all of the subunits required for the measured function—sometimes only one polypeptide of a complex. In addition to biological function, radiation target analysis can be applied to structure. The surviving monomers in irradiated proteins can be resolved by denaturing gel electrophoresis and quantitated by staining or antibody reactions. Analysis of these data yields the mass of those structures destroyed by a single radiation interaction independent of any biological function. Both of these approaches have been applied here to the irradiated holoenzyme RR and the individual subunits. The results give us new insight into the structure and function of this critical enzyme.

MATERIALS AND METHODS

Materials

Production of recombinant HSV-1 R2

Recombinant HSV-1 R2 protein was produced by induction of logarithmically growing BL21(DE3) pLysS *E. coli* that were transformed with the plasmid $pETH_2$. The protein was purified to apparent homogeneity and reactivated by the aerobic method essentially as described previously (Mann et al., 1991). Reactivated preparations of HSV-1 R2 had ~10–20% free radical content, as determined by electron paramagnetic resonance, courtesy of Dr. L. Thelander (University of Umea, Umea, Sweden). The iron content of the reactivated R2 proteins was typically 60–85% when determined as described previously (Krogsrud et al., 1993). These values for free radical and iron content are in agreement with previous reports (Mann et al., 1991; Filatov et al., 1992). Protein concentration for pure HSV-1 R2 was determined from the absorbance difference at 280 nm and 310 nm using a molar extinction coefficient ($\epsilon_{280-310}$) of 52,000 M^{-1} as described previously (Bradford, 1976; Bollinger et al., 1991). The concentration of HSV-1 R2 was calculated by assuming a 38 kDa R2 monomer. Under the standard assay conditions described below, the specific activity of the HSV-1 R2 preparations used in this study varied from 50 to 100 units/mg when tested in the presence of molar excess HSV R1. No activity was observed in the absence of HSV R1 subunit.

Production of recombinant HSV-2 R1

Recombinant HSV-2 R1 was obtained from confluent 293 cells that had been infected with the recombinant adenovirus vector Ad5-RR1 as described elsewhere (Massie et al., 1995; Krogsrud et al., 1993; Paradis et al., 1991). The HSV-2 R1 subunit was partially purified by using the following procedure. Infected cell pellets were resuspended in 1 volume of 50 mM HEPES, pH 8.0, 2 mM DTT containing the protease inhibitors aprotinin and leupeptin at a concentration of 50 μg/ml each, and the cell suspension was subjected to ultrasonic disruption using a Branson Sonifier Model 350. The protein homogenate was clarified by centrifugation for 10 min at 20,000 $\times$ *g* and the soluble proteins were precipitated by adding to the supernatant ammonium sulfate to a final concentration of 35% saturation. The precipitated proteins were collected by centrifugation, dissolved in a minimal volume of buffer A, and then dialyzed overnight against the same buffer. The dialysate was clarified by centrifugation and stored at −80°C. Protein concentration of the HSV-2 R1 preparation was measured by the method of Bradford (1976) using γ-globulin as a standard. The specific activity of the enzyme preparations used in this study ranged from 1 to 2 units/mg protein when measured in the presence of excess HSV-1 R2 as described below. All HSV R1 preparations had no activity in the absence of added HSV R2. The protein concentration of the R1 preparation was 15 mg/ml with a HSV-2 R1 content of ~10%, as determined by SDS-polyacrylamide gel electrophoresis and densitometric scanning of Coomassie Brilliant Blue-stained gels.

Production of ribonucleotide reductase holoenzyme

For the production of ribonucleotide reductase holoenzyme, baby hamster kidney cells were infected with HSV-1 strain F at a multiplicity of infection of 5 for 12 h as described elsewhere (Cohen et al., 1985). The HSV-1 ribonucleotide reductase holoenzyme was extracted from infected cell pellets and partially purified by following the protocol described for the preparation of the R1 subunit. The specific activity of the holoenzyme preparation was ~0.4 unit/mg protein when assayed as described below with a total protein concentration of 49.6 mg/ml.

Quantitation of RR subunits by SDS PAGE and Western blot

For immunoblot analysis, irradiated samples were run on 7.5% and 10% polyacrylamide gels for HSV R1 and R2, respectively. Twenty μg of partially purified R1 and 2 μg of purified R2 were loaded on the gel. In the case of the holoenzyme, 50 or 20 μg of partially purified enzyme were loaded for Western blots with the monoclonal antibodies 932 and 535, respectively (Ingemarson and Lankinen, 1987). For Western blotting, separated proteins were transferred to nitrocellulose membranes, washed three times with 1% dry milk powder in phosphate buffered saline, and probed with 10 μg/ml 932 antibody or 0.1 μg/ml 535 antibody for 2 h, followed by a 2 h incubation with 5 μCi sheep anti-mouse [^{125}I]IgG (Amersham, Québec, Canada). Dried membranes were exposed to phosphorscreens and quantitated on a Molecular Dynamics Phosphor Imager.

Radiation inactivation

Radiation inactivation was performed with 10 MeV electrons at −135°C as described (Harmon et al., 1985). The irradiated samples were stored for no longer than 1 month before assay.

Ribonucleotide reductase activity assay

The catalytic activity of ribonucleotide reductase was determined from the rate of conversion of [^{14}C]CDP to [^{14}C]dCDP as described elsewhere (Krogsrud et al., 1993). One unit of reductase activity is defined as that amount of enzyme which catalyzes the formation of 1 nmol dCDP per min at 37°C. Briefly, the standard reaction mixture, in a final volume of 60 μl, contained 50 mM HEPES, pH 8.0, 4 mM NaF, 30 mM dithiothreitol, 54

mM CDP, 0.1 μCi [^{14}C]CDP (524 Ci/mol; NEN Dupont), 1 mM bacitracin and enzyme. After 30 min incubation at 37°C, the reaction was stopped by the addition of 20 μl of 40 mM HEPES, pH 8.0, 4 mM $MgCl_2$ containing 2.5 units of calf alkaline phosphatase (Boehringer Mannheim), followed by incubation at 37°C for 15 min to dephosphorylate all nucleotides. Deoxycytidine was separated from cytidine using thin-layer chromatography and the radiolabel was quantitated using the radioanalytical imaging system AMBIS (Ambis Inc., San Diego, CA). The specific activity (unit/mg) of R2 samples was calculated from the linear portion of the activity versus R2 concentration (0.1–1 μg purified R2) curves in the presence of molar excess HSV R1 (40 μg of partially purified R1 per assay). Likewise, the specific activity of R1 samples was calculated from the linear portion of the activity versus R1 concentration (10–250 μg partially purified R1) curves in the presence of molar excess HSV R2 (5 μg purified R2 per assay). The biological activities of HSV-1 R2 and R1 were also determined using the solid-phase competition binding assay described below (Krogsrud et al., 1993).

Ribonucleotide reductase binding assay

Direct binding to R1 and competition for binding to R1 by R2 were evaluated using ^{125}I-labeled 3-iodo-desamino-Tyr-(N-Me)Val-Ile-Asn(δ-N, N-Et)Asp-Leu-OH (specific activity 74 ± 7 GBq/μmol), essentially according to the methods described earlier (Krogsrud et al., 1993).

Target size calculations

In the case of the enzyme assays and Western blot analysis where activity is represented by the amount of protein remaining in the gel band, the fraction of activity remaining following irradiation of samples was expressed as A_D/A_0, where A_D represents the enzyme activity following a dose of radiation D and A_0 represents the enzyme activity in the absence of irradiation. In the case of binding, activity was expressed as B/F, where B represents the amount of specific binding of radioligand and F represents the concentration of free radioligand. The fraction of binding activity remaining in irradiated samples was calculated by normalizing each value of B/F to that observed in non-irradiated controls, $(B/F)_0$. In either case least-squares linear regression analysis was used to calculate the radiation inactivation constant from:

$$\ln(\text{fraction remaining}) = k'D \tag{1}$$

where D is the dose of radiation in Mrad (Harmon et al., 1985). The rate of radiation inactivation constant, k', is corrected for its temperature dependence (Kempner and Haigler, 1982) by a factor S_t, which is equal to 2.8 for irradiations performed at −135°C.

For the analyses presented here the simplest form of target analysis was assumed, namely that the recognition site qualities of an enzyme or binding site are either completely abolished or remain unaffected. Thus, the loss of binding is due to a reduction in either V_{max} (maximal velocity of an enzyme reaction) or B_{max} (maximal binding site capacity) with no changes in K_m (enzyme affinity constant) or K_d (macromolecular dissociation constant), respectively. If this assumption is valid, the target size can be calculated from:

$$\text{Molecular mass (in Da)} = 6.4 \times 10^5 k' S_t. \tag{2}$$

For a complex radiation inactivation curve, the analysis for multiple independent targets was used (Kempner, 1995). Several different-sized molecules are present that either independently bind to the same ligand or (in the case of an enzyme) catalyze the same reaction. If only two such species were present, the activity remaining after exposure to a radiation dose D will be given by

$$A = Be^{-\beta D} + Ge^{-\gamma D} \tag{3}$$

where B and G represent the activity due to two species, respectively, in the unirradiated sample. The total activity initially is then $A_0 = B + G$; thus, substituting into the above equation the radiation inactivation curve is then described by

$$A_D/A_0 = (B/A_0)e^{-\beta D} + (G/A_0)e^{-\gamma D} \tag{4}$$

This curve initially drops off rapidly but then curves to the right and reaches a final single exponential at high radiation doses. This final slope is the activity due to the smaller mass species; extrapolation of the final slope back to the zero-dose axis permits determination of G. The individual values of β and γ were obtained by using nonlinear regression analysis.

Statistics

Differences between groups were analyzed statistically by Student's t-tests.

RESULTS

Irradiation of holoenzyme

Samples of HSV-1 RR holoenzyme dissolved in 50 mM HEPES buffer, pH 7.5 containing 2 mM DTT in glass ampoules were frozen on dry ice and sealed with an oxygen-gas torch before irradiation and thawed before analysis. This procedure did not affect the activity of the holoenzyme when compared to samples that had not been processed for irradiation studies. After exposure of the holoenzyme to different doses of radiation, the remaining enzyme activity decreased as a single exponential function of radiation dose (Fig. 1), leading to an average target size of 315 kDa (Table 1), which is similar to the predicted molecular size of 324 kDa. Analysis of the target sizes of R1 and R2 following irradiation of the holoenzyme and quantitation by Western blot yielded single exponential decay curves and average target sizes of 281 kDa for R1 and 57 kDa for R2 (Fig. 1, Table 1).

Irradiation of the free HSV R1 subunit

Partially purified recombinant R1 subunit was suspended in the same buffer as the holoenzyme. After exposure of the R1 subunit to different doses of radiation, the remaining functional R1 subunit was quantitated by enzyme assay following reconstitution with excess R2, Western blot, and tracer binding. All three techniques yielded single exponential decay curves and gave the following similar estimates: 153, 130, and 128 kDa, respectively (Fig. 2, Table 1).

Irradiation of the free recombinant HSV R2 subunit

Purified recombinant R2 subunit was suspended in 20 mM bis-TRIS, pH 6.5 containing 2 mM DTT and 0.15 M NaCl. After exposure of R2 subunit to different doses of radiation, the remaining functional R2 subunit was also quantitated by enzyme assay following reconstitution with excess R1,

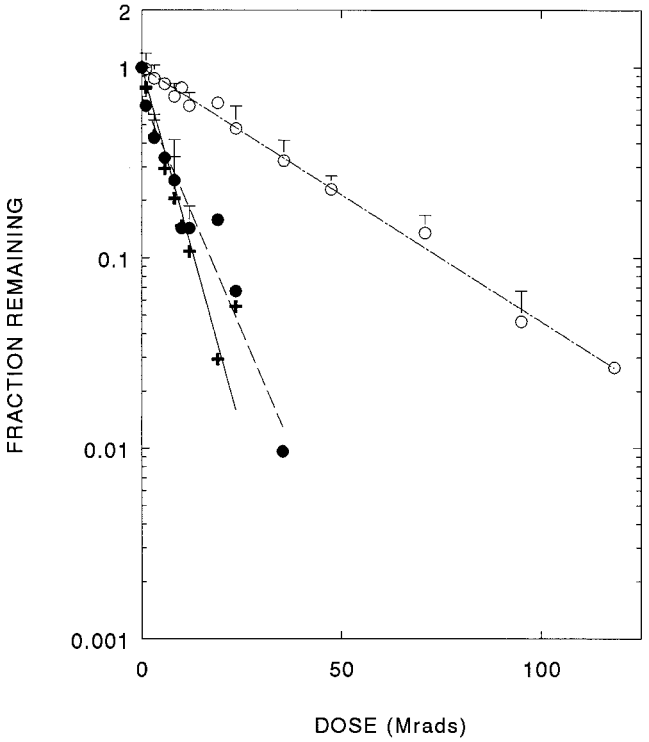

FIGURE 1 Effect of radiation on holoenzyme. Remaining enzymatic activity (+), surviving R1 subunits (●), and R2 subunits (○) detected by Western blot. Data are averages plus SD from three independent experiments.

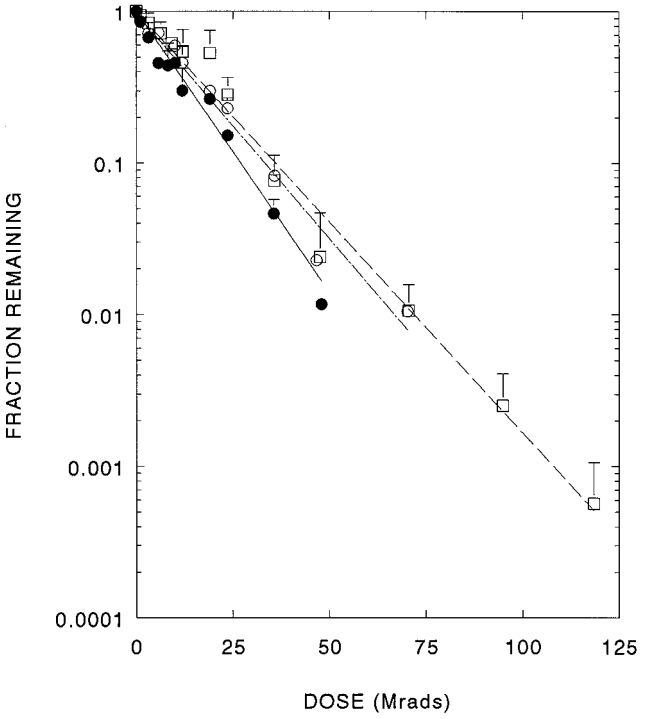

FIGURE 2 Effect of radiation on purified R1 subunits. Surviving R1 subunits determined by enzymatic activity (●) (after addition of excess R2 subunits), Western blots (○), and the binding of tracer (□). Data are shown as averages plus SD from three independent experiments.

Western blot, and competition for tracer binding. Surprisingly, by enzymatic assay the functional activity of R2 decreased as a single exponential with an estimated target size of 223 kDa (Fig. 3, Table 1) which is considerably larger than would be expected for either the monomer or the dimer. When Western blot analysis was used to quantitate R2, protein decreased as a single exponential with a target size of 19 kDa (Fig. 3, Table 1). Although this is smaller than would be expected for the R2 monomer, it indicates that only one monomer is damaged by each radiation event and there is no evidence for transfer of radiation energy to other monomers, as was seen in the holoenzyme.

TABLE 1 Target sizes from irradiation of RR and its subunits

	Property Measured				
Sample Irradiated	RR Activity	Western R1	Western R2	Tracer Binding (R1)	Inhibition of Binding (R2)*
Holoenzyme	315 ± 20	281 ± 48	57 ± 4	—	—
R1	153 ± 12	130 ± 15	—	128 ± 9	—
R2	223 ± 2	—	19 ± 2	—	20 (0.15) 210 (0.75)

The results are presented as the means ± SEM of three independent radiation runs.

*For R2 the target size estimates (determined from nonlinear regression) of the two molecular species and fraction of the total sites irradiated (in parentheses) are presented.

The amount of R2 capable of inhibiting tracer binding following radiation inactivation was quantitated from inhibition curves using R2 concentrations ranging from 1 to 10,000 nM. The destruction of R2 by irradiation resulted in a dose-dependent reduction of the inhibition of binding over all the concentrations of R2 used. In the absence of irradiation the IC_{50} for inhibition of tracer binding by R2 was 0.14 ± 0.03 μM. Two concentrations of R2 were chosen for measuring the amount of R2 remaining following irradiation, 0.1 μM and 1.0 μM, the amount of R2 remaining following each radiation dose being calculated from the inhibition curve by using the Hill equation. Upon radiation inactivation, both the high and low concentrations of R2 yielded biphasic exponential decay curves with increasing radiation dose (Fig. 3 shows only the results with the higher concentration of R2). The biphasic decay curve was resolved accounting for two target sizes. The target sizes and fraction of binding sites were 20 kDa, 0.15 and 210 kDa, 0.75, respectively (Table 1).

DISCUSSION

HSV RR is an $\alpha_2\beta_2$ heterodimer of M_r 324,000. Isolated R1 and R2 subunits each exist as stable protein dimers in

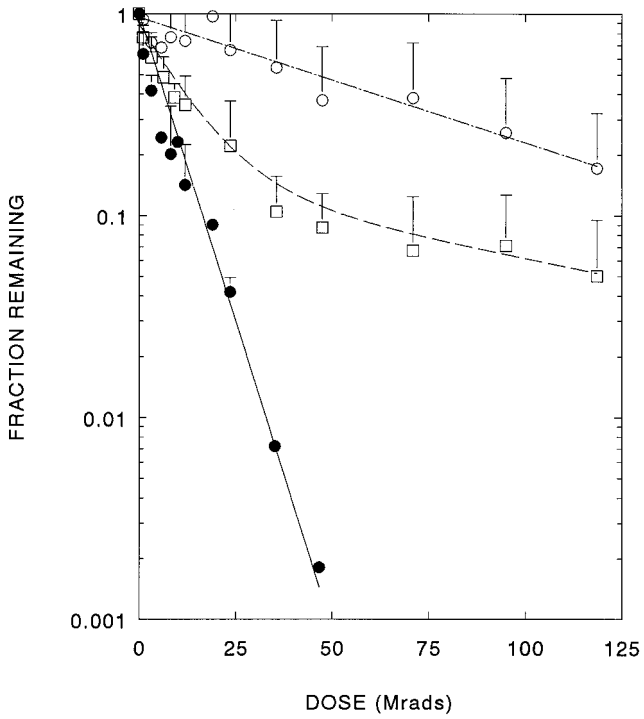

FIGURE 3 Effect of radiation on purified R2 subunits. The surviving R2 subunits were determined by enzymatic activity (●) (after addition of excess R1 subunits), Western blots (○), and competition (concentration of R2 in unirradiated sample, 1 μM) for tracer binding to the R1 subunit (□). Data are shown as averages plus SD from three independent experiments.

solution (Lankinen et al., 1991; Conner et al., 1993). The functional RR enzyme is believed to be a complex of the R1 and R2 because individual subunits do not have any detectable enzymatic activity (Mann et al., 1991; Lankinen et al., 1991; Lamarche et al., 1990; Furlong et al., 1991; Massie et al., 1995). Furthermore, truncated R2 derivatives lacking the essential C-terminal subunit interaction domain do not display enzyme activity with R1 due to an inability to associate (Filatov et al., 1992; Krogsrud et al., 1993).

The interactions between R1 and R2 and monomers for each of these subunits in the HSV RR holoenzyme were probed using radiation inactivation. Estimates of the functional target size (i.e., based on enzymatic activity) of the holoenzyme suggested that a tetramer of two R1 monomers and two R2 monomers was required for function. The target size for the structure (as assessed by Western blot) of both the R1 and R2 monomers in the irradiated holoenzyme was considerably larger than the published values of 124 kDa and 38 kDa, respectively (McGeoch et al., 1988) and corresponded to approximately twice their known molecular weights. These results indicate that in the holoenzyme there is transfer of radiation-deposited energy between monomers of R1 and also between the monomers of R2. Energy transfer between subunit monomers has been demonstrated previously (Kempner, 1995; Davis et al., 1996, 1997), possibly occurring as a consequence of very close juxtapositioning of monomers. The finding that the target size for each monomer in the holoenzyme fit closely with the mass of a homodimer implies, however, that energy transfer does not occur between R1 and R2. Because of the transfer of radiation-deposited energy between identical monomers in the holoenzyme, interpretation of the tetrameric functional target size is obscured. If only one monomer of each species were required for enzymatic activity, the expected target size would be 38 + 124 kDa; but because radiation damage appears in two monomers after only a single primary ionization, only the tetrameric target size can be observed. Thus, from this experiment alone it cannot be determined whether an $\alpha\beta$ dimer or an $\alpha_2\beta_2$ structure is needed.

Irradiation of the free R1 gave target sizes of the monomer that were independent of the method used for quantitation: enzyme activity, binding of ^{125}I-labeled peptide, or Western blot analysis. A target size by Western blot analysis equivalent to the monomer for irradiated free R1 indicates that energy transfer does not occur between purified monomers, even though this subunit exists in solution as a dimer. Thus, the minimum subunit for enzymatic and binding activity in R1 is the monomer.

The similar finding of a target size by Western blot analysis equivalent to the monomer for irradiated free R2 shows that radiation-deposited energy is not transferred between isolated R2 monomers either, even though it too exists as dimer. Because energy transfer did occur between monomers of R1 and between monomers of R2 during irradiation of the holoenzyme, this suggests that formation of the tetramer increases the interaction between two R1 monomers and also between two R2 monomers. The association of the subunits is probably accompanied by a change in conformation that results in a tighter interaction between the monomers of each subunit facilitating energy transfer. The tighter interaction between the monomers of each subunit may be important for stabilizing the holoenzyme complex, for optimal electron transfer from one subunit to the other, and for greater substrate binding at the active site.

The target size for enzyme activity in free R2 was consistently larger than the expected molecular weight for either the monomer or the dimer. RR is one of the few enzymes that are dependent on a native free radical (Pedersen and Finazzi-Agro, 1993; Sun et al., 1993). Therefore, the overestimation of the functional target size could be explained by a radiation-induced destruction of the critical tyrosyl free radical in R2, which was reported by Davydov et al. (1996). We analyzed their data for the disappearance of tyrosyl radicals in R2 as a function of radiation exposure at 77 K. Assuming no temperature correction (Kempner et al., 1986) is required, their data yield a target size of 191 kDa. This value is remarkably close to the activity target size for R2 (223 kDa) reported here. Confirmation of the disappearance of tyrosyl radicals in our samples was obtained by electron paramagnetic resonance measurements

on irradiated and control samples of R2. These demonstrated a loss of tyrosyl free radical at lower doses of radiation (data not shown; personal communication Dr. Lars Thelander, University of Umea, Umea, Sweden). This interpretation of the large target size for enzyme activity upon irradiation of R2 is consistent with the known structure and function of R2 in RR, but is in contrast with the observed effects of radiation on other proteins.

Previously published data indicated that a primary ionization caused by radiation in a protein molecule resulted in major structural damage (covalent bond breaks) and consequent loss of biochemical activity. Molecules not suffering a direct hit were undamaged and fully functional. No indirect effects of radiation (damage to one molecule resulting from reaction with a radiation product formed elsewhere) were observed in frozen samples irradiated at very low temperature. In the present case, most tyrosyl free radicals disappeared from purified R2 molecules that had not suffered substantial structural damage (estimated by scission of the polymer backbone). In the holoenzyme this did not happen; the tyrosyl free radical on R2 may be shielded by R1.

Irradiation of R2 and quantitation by competition for tracer binding yielded a complex inactivation curve. This is indicative of destruction of independent molecular species with different masses. The data yielded target sizes of 20 kDa and 210 kDa, species that contributed 15% and 75% of the original binding, respectively. The smaller value is comparable to the mass of R2; the larger target size is close to the value for enzyme activity (and for the loss of the tyrosyl free radical) in irradiated R2. The radiation destruction of the tyrosyl free radical could result in an altered R2 with reduced affinity for inhibition of tracer binding to R1. This is supported by our observation that depletion of the tyrosyl free radical of the R2 by the radical scavenger hydroxyurea resulted in an approximately 10-fold loss of affinity for R1 (unpublished observation). Thus the tyrosyl free radical plays a role in the formation of an optimal conformation for binding of R2 to R1.

This is the first radiation inactivation study of an enzyme containing a stable free radical. It appears that in this unusual molecule, the loss of a crucial free radical occurs by an indirect action of radiation, perhaps even in the frozen state. A role for free radicals in low-temperature irradiations has been suggested previously (Symons and Taiwo, 1987; Kempner and Miller, 1994; Kempner et al., 1986), but in molecules that did not contain a stable free radical. The well-defined protein structure of RR offers a new opportunity to examine this phenomenon.

In summary, radiation inactivation of HSV RR has confirmed the tetrameric structure of the holoenzyme in solution. It was revealed that only one R1 monomer, when combined with R2, was necessary for enzymatic activity, and that the R1 and R2 dimers undergo a conformation change upon formation of the holoenzyme. The use of this unique technique has enabled us to independently confirm several earlier findings on the function of RR and to create a more consistent model relating the structure and function of RR. Results of irradiation of the various forms of this protein indicate that indirect radiation effects can abolish the stable free radical if it is accessible to the solvent.

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