Effect of the Tyrosyl Radical on the Reduction and Structure of the *Escherichia coli* Ribonucleotide Reductase Protein R2 Diferric Site As Probed by EPR on the Mixed-Valent State†

Roman Davydov,‡,§ Margareta Sahlin,|| Sergei Kuprin,‡,⊥ Astrid Gräslund,‡ and Anders Ehrenberg*,‡

Departments of Biophysics and Molecular Biology, Stockholm University, S-106 91 Stockholm, Sweden

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ABSTRACT: It was recently shown by EPR that high yields of a sterically constrained mixed-valent species may be formed in radical free protein metR2 of *Escherichia coli* ribonucleotide reductase by γ-irradiation at 77 K [Davydov, R., Kuprin, S., Gräslund, A., & Ehrenberg, A. (1994) *J. Am. Chem. Soc. 116*, 11120]. This species, with $S = {}^1/_2$, essentially retains the ligand geometry of the original diferric center and should be a sensitive probe for structural changes in the diferric centers. Here we apply this probe and demonstrate that there is a structural difference between the diferric iron center of the complete site of protein R2, with a tyrosyl radical, and that of metR2, without radical. The EPR spectrum of the mixed-valent species of metR2 shows pure axial symmetry, while complete sites show rhombic distortion and a shifted high-field turning point. Differences also remain in the EPR of the first $S = {}^9/_2$ species obtained by annealing at 165 K, but disappear after relaxation at 200 K. In addition, the diferric center of a complete site is not reduced radiolytically until the associated tyrosyl radical has been reduced, indicating that an electron first reaching the iron center may be transferred to the radical. This route of electron transfer and the influence of the radical on the structure of the iron center are likely to have functional roles for the formation of the proposed substrate radical and regulation of redox processes within the enzyme. The sensitivity of the structure of the iron site to the structure of the Tyr-122 site is also demonstrated by the strong influence the mutation Y122F has on the EPR spectra of the corresponding mixed-valent species.

Ribonucleotide reductases (RNRs)[1] play an essential role in DNA biosynthesis, catalyzing the conversion of ribonucleotides to the corresponding deoxyribonucleotides (Reichard, 1993; Sjöberg, 1994). Class I RNRs (Reichard, 1993), e.g., from *Escherichia coli*, mammalian cells, or herpes viruses, are composed of two homodimeric proteins, R1 and R2. Of these, the *E. coli* enzyme has been most thoroughly characterized. Protein R1 binds both the NDP substrates and the dNTP and ATP allosteric effectors and contains the cysteine residues, which are essential for substrate reduction. Protein R2 contains in each polypeptide chain sites for a μ-oxo-bridged diiron cluster and a free radical in tyrosine-122. The combined diiron/radical site is required for enzymatic activity (Reichard & Ehrenberg, 1983; Reichard, 1993). Protein R2 with the tyrosyl radical reduced to normal tyrosine, but still with a diferric iron center, is called metR2 and is enzymatically inactive. The crystal structure of *E. coli* metR2 has been determined (Nordlund & Eklund, 1993). So far no three-dimensional structure has been reported for the enzymatically active tyrosyl radical containing active R2.

In the crystal structure of *E. coli* metR2, the Y122 hydroxyl group may be hydrogen-bonded to the carboxylate group of D84 (Nordlund & Eklund, 1993). This carboxylate is in turn coordinated to one of the irons of the iron center. However, results of studies with resonance Raman (Backes et al., 1989) and ENDOR (Bender et al., 1989) do not show any indication of the presence of a similar hydrogen bond in the radical-containing active form of R2. In fact, available data show that the proton of the phenolic group is dissociated, leaving the tyrosyl radical in a neutral and not H-bonded state. This suggests that the presence of the radical on Y122 may have an effect on the diiron site structure either directly or via effects on the local protein conformation. In previous studies Atkin et al. (1973) have shown that *E. coli* metR2 and active R2 give qualitatively similar Mössbauer spectra. The low intensity of these spectra, however, does not allow detailed quantitative comparisons. Futhermore, as established by Backes et al. (1989), reduction of the tyrosyl radical at position 122 to normal tyrosine or even replacement of Y122 by phenylalanine does not affect the Fe−O−Fe symmetric stretch mode at 493 cm^{-1}. However, these authors also reported that the oxidation state of Y122 in protein R2 has a strong effect on a photoinduced rearrangement in the diferric cluster. Thus, the available data do not allow any firm conclusions on the possible effect of the state of Y122 on the structure of the diiron site in *E. coli* protein R2.

Most other proteins containing oxygen-bridged diiron centers may in solution be obtained in a mixed-valent form in fairly good yields, often simply by chemical reduction or oxidation of the fully oxidized or reduced states, respectively. This has, however, not been possible with *E. coli* protein R2. In previous reports (Davydov et al., 1994; Ehrenberg

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‡ Department of Biophysics.

§ On leave from the Institute of Chemical Physics, Russian Academy of Sciences, Moscow, Russia.

|| Department of Molecular Biology.

⊥ Present address: Medical Nobelinstitute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, S-171 77 Stockholm, Sweden.

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[1] Abbreviations: RNR, ribonucleotide reductase; R1 and R2, protein components R1 and R2 of RNR; metR2, R2 without tyrosyl radical.

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Scheme 1

$$(Fe^{3+}, Fe^{3+})_{S=0} \xrightarrow[77\ K]{+e^-} \mathbf{I}\ (Fe^{2+}, Fe^{3+})_{S=1/2} \xrightarrow{T_{an}\approx 140\ K} \mathbf{II}\ (Fe^{2+}, Fe^{3+})_{S=1/2} \rightarrow$$

$$\xrightarrow{T_{an}\approx 160\ K} \mathbf{III}\ (Fe^{2+}, Fe^{3+})_{S=9/2} \xrightarrow{T_{an}\approx 190\ K} \mathbf{IV}\ (Fe^{2+}, Fe^{3+})_{S=9/2} \rightarrow$$

$$\xrightarrow{T_{an}\geq 230\ K} \mathbf{V}\ (\text{EPR silent product(s)})$$

et al., 1991), we have shown that on reduction of the diferric site of *E. coli* protein metR2 in solid solution at 77 K by mobile electrons, produced by ionizing radiation, a structurally constrained mixed-valent antiferromagnetically coupled state with $S = {}^1/_2$ is formed in yields as high as 40%. In this state, the iron center exhibits an axial EPR signal with $g_\perp = 1.936$ and $g_\parallel = 1.818$, observable even at 110 K. Because of restricted dynamics at 77 K, the mixed-valent (Fe^{2+}, Fe^{3+}) cluster produced by the reduction retains the ligand coordination structure identical to or very close to that of the initial diferric state. We have suggested (Davydov et al., 1994) that the EPR properties of this trapped kinetic intermediate are due to the bridging O^{2-} ligand of the fully oxidized center being retained in the mixed-valent diiron site. The one-electron reduction at 77 K may be regarded as producing an EPR probe for structural studies of the original (Fe^{3+}, Fe^{3+}) dinuclear iron site, which itself is normally EPR-silent. When a sample of this trapped mixed-valent diiron center of the *E. coli* protein is warmed for a brief period to 200 K, the constrained nonequilibrium ligand environment relaxes, and a new ferromagnetically coupled $S = {}^9/_2$ state is formed (Davydov et al., 1994). At higher temperatures, $T > 230$ K, the EPR signal of the mixed-valent cluster disappears. The transition (Scheme 1) from the primary, sterically constrained (Fe^{2+}, Fe^{3+}) site with $S = {}^1/_2$, species **I,** to the final product(s), **V**, stable at room temperature, proceeds via at least three EPR-distinguishable transient forms, **II**, **III**, and **IV**, stabilized at different, successively higher annealing temperatures, T_{an}.

The first intermediate, **II**, is stabilized after annealing at ≈140 K and displays a slightly rhombic EPR signal with *g*-values of 1.95, 1.92, and 1.80 (Davydov et al., 1994), vide infra Figure 4. At higher temperatures (≈160 K), this species transforms gradually to the ferromagnetically coupled $S = {}^9/_2$ mixed-valent state, species **III**, which gives an EPR spectrum with characteristic resonance features at $g = 14.8$, 6.7, and 5.4, vide infra Figure 5. The temperature dependences of these features suggest that the $g = 14.8$ resonance is from the $<\pm{}^1/_2|$ ground doublet and the ones at $g = 6.7$ and 5.4 are from the $<\pm{}^3/_2|$ excited Kramer's doublets (Davydov et al., 1994; Ehrenberg et al., 1991; Hendrich et al., 1991). The elevation of temperature to 200 K is accompanied by a prominent modification of the $S = {}^9/_2$ signal line shape (Davydov et al., 1994) Under anaerobic conditions at temperatures higher than 230 K, this mixed-valent form, species **IV,** transforms quite quickly to unidentified EPR-silent state(s).

In the present study, we show for the first time, by means of EPR, that the presence of the tyrosyl radical in RNR protein R2 affects the structure of the diiron center. Results also give evidence that electrons taken up by the diferric iron center are transferred to the radical, which becomes reduced. Conceivably, both of these properties of the diiron−radical center are of functional importance. In addition, we show that the replacement of Y122 by phenylalanine results in prominent changes in the structures of both primary and relaxed mixed-valent forms of the diiron site in protein R2.

EXPERIMENTAL SECTION

An *E. coli* C600/pBS1 strain overproducing protein R2 was used (Sjöberg et al., 1986). Protein R2 was isolated as previously described (Davydov et al., 1994). MetR2 was prepared by reduction of R2 with hydroxyurea as already described (Davydov et al., 1994). The mutant protein R2-Y122F was produced as described (Larsson & Sjöberg, 1986).

All protein solutions were prepared in 1:1 glycerol/50 mM Tris-HCl buffer of pH 7.3−7.6. Frozen glass-like protein solutions in EPR quartz tubes immersed in liquid N_2 were exposed to γ-rays from a ^{137}Cs source, at a dose rate of 65 krad/h. The irradiation time varied from 4 to 160 h. Most of the EPR measurements were made on samples irradiated by a dose of 1.5−3.0 Mrad. Annealings were made for suitable lengths of time at 142 K, 165 K, and 200 K in precooled *n*-pentane followed by reimmersion into liquid N_2. EPR measurements were performed on a Bruker Model ESP300 X-band spectrometer with an Oxford Instruments ESR9 liquid helium cryostat. For spin quantitation, CuEDTA (1 mM $CuSO_4$, 10 mM EDTA, pH 7.0) was used as a standard, and double integration of spectra was performed as described (Davydov et al., 1994) using standard Bruker software. Electronic spectra were recorded at room temperature on a Cary 119 spectrophotometer and at 77 K on an Aminco DW2 spectrophotometer.

The amount of tyrosyl radical in R2 samples irradiated at 77 K was determined at the same temperature both spectrophotometrically (up to 10 Mrad) and by EPR (for doses <4 Mrad). Before spectrophotometry, the trapped electrons in the γ-irradiated samples were photobleached by light from a 400 W tungsten lamp. The sharp absorption band at 410 nm was used for evaluation of tyrosyl radical content (Petersson et al., 1980). Alternatively, difference EPR spectra between γ-irradiated samples of R2 and metR2 at equal protein concentrations were used for the determination. It was assumed that the γ-ray-induced radicals were equal in the two samples and that any influence of the different yields of mixed-valent iron centers could be neglected. To correct for small differences, the metR2 spectrum was multiplied by an adjustable factor in order to obtain the best possible base line for the remaining tyrosyl radical EPR spectrum. At low doses, the results of both methods were in good agreement. However, because of the strong background, the EPR quantitation became more uncertain at high doses.

The relative yields (cross sections), *k*, of the reactions between mobile electrons and the diiron site or the tyrosyl radical in the unperturbed system were determined from the initial slopes of the yield vs. dose curves using the equation $k = C^{-1}\Delta P/\Delta D$, where $\Delta P/\Delta D$ defines the slope at zero dose in terms of incremental changes in the concentration of the product *P* and in the dose *D* and *C* is the original concentration of diiron sites or tyrosyl radicals. For our samples, the average values of 1.5 diiron sites (an average for this type of preparation) and 1 tyrosyl radical (determined before irradiation) per molecule of active R2 were used.

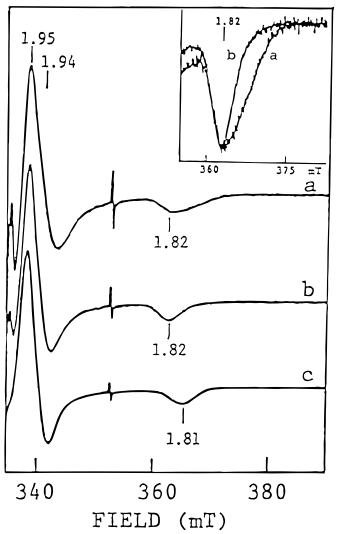

FIGURE 1: EPR spectra at 20 K of mixed-valent primary species of type **I** (cf. Scheme 1) produced radiolytically by γ-irradiation at 77 K in proteins R2 (a), metR2 (b), and R2-Y122F (c). Protein concentrations: 1 mM R2, 1 mM metR2, and 0.65 mM R2-Y122F. Solvent: 1:1 mixture of glycerol and 50 mM Tris-HCl, pH 7.6, 0.05 M KCl. γ-Irradiation dose 3.65 Mrad. Spectrometer settings: modulation frequency 100 kHz, modulation amplitude 5 G, microwave frequency 9.236 GHz, microwave power 1 mW, receiver gain 5×10^3. The inset shows a detailed comparison of high-field minima of (a) and (b) with magnifying amplitude scales.

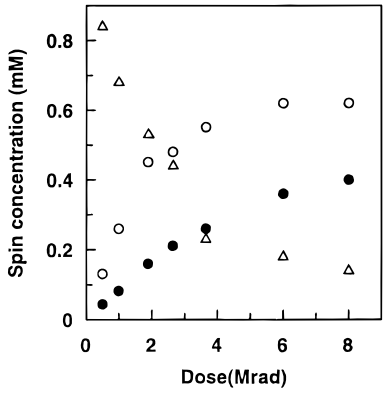

FIGURE 2: Yields of mixed-valent primary species of type **I** (cf. Scheme 1) of proteins R2 (●) and metR2 (○) and the remaining amount of tyrosyl radical in protein R2 (△) after increasing γ-ray doses at 77 K. The concentration of protein R2 was 1.0 mM with initially 1.5 mM (Fe^{3+}, Fe^{3+}) centers and 1.0 mM tyrosyl radicals. The sample of protein metR2 had the same protein and iron center concentrations.

RESULTS AND DISCUSSION

Influence of the Tyrosyl Radical on the Efficiency of the Reduction of the Protein R2 Diferric Site at 77 K. EPR spectra of the half-reduced diiron centers of active R2 and metR2 produced at 77 K by γ-irradiation are presented in Figure 1a and Figure 1b, respectively. The two primary kinetic intermediates of type **I** (cf. Scheme 1) exhibit similar but not identical axial EPR signals with $g_\perp$ around 1.935 and $g_{||}$ around 1.816. Figure 2 shows the reduction of the tyrosyl radical in active R2 and the formation of mixed-valent iron centers in active R2 and metR2 as functions of the γ-irradiation dose. From the initial slopes of the curves, the relative yields, *k*, were evaluated. For the appearance of mixed-valent-state EPR, these yields are 0.19 ± 0.03 and 0.062 ± 0.010 $Mrad^{-1}$ for metR2 and active R2, respectively. The corresponding relative yield of reduced radical in active R2, i.e., the disappearance of the radical EPR and light absorption, is found to be 0.33 ± 0.05 $Mrad^{-1}$. At high doses, the radical concentration decreases toward complete extinction. The total yield of mixed-valent centers formed from metR2 apparently levels off slightly above 0.6 mM, corresponding to 40% of the diiron sites. Also in the case of active R2 the total yield levels off at high doses, but more gradually than for metR2. Experiments were also made at the high dose of 14 Mrad, but high amounts of radiation induced radicals and possible protein damage made the interpretation difficult. Such problems could be neglected below 4 Mrad.

It is striking that the yield of mixed-valent diiron centers at low doses is about 3 times larger in the case of metR2 as compared with active R2. In general, a preparation of active R2 is a mixture of two kinds of sites: those containing the diferric center/tyrosyl radical combination, which we will call complete sites, and those consisting of only diferric centers, i.e., metR2 sites. There are also empty sites containing no iron. In the type of preparation of active protein R2 used in the present work, the ratio between the numbers of complete sites and metR2 sites has been found to be close to 2:1. Since the metR2 samples were obtained from active R2 by elimination of the tyrosyl radicals by reduction with hydroxyurea, i.e., conversion of the complete sites into metR2 sites, the ratio between the numbers of metR2 sites in the metR2 and active R2 samples is approximately 3:1. This concentration ratio is close to the ratio of the experimental initial yields for growth of mixed-valent site EPR in these two samples, 0.19 and 0.062 $Mrad^{-1}$, respectively. This suggests that in active R2 at low doses we only see the EPR from mixed-valent states formed from the metR2 sites.

Based on statistical considerations, it is reasonable to assume that the initial yields for low-temperature reduction of diferric centers of complete sites and of metR2 sites are approximately equal. But at low dose no mixed-valent EPR is seen from the complete sites despite the fact that they are twice as abundant as the metR2 sites in the active R2 preparation. At the same time, the tyrosyl radical EPR signal (and light absorption) disappears with a yield of 0.33 $Mrad^{-1}$, which is considerably higher than the assumed initial yield of diferric site reduction, ca. 0.19 $Mrad^{-1}$. These observations suggest that the tyrosyl radical features, EPR and light absorption, disappear for each electron captured by the complete site, diferric iron center + tyrosyl radical, and no mixed-valent EPR spectrum becomes observable from this site. The simplest mechanism to explain such a situation is that any electron first captured by the diiron center of a complete site only forms a transient mixed-valent state because an electron transfer immediately takes place from the mixed-valent species to the nearby tyrosyl radical. Another possibility would be an efficient exchange coupling between the two paramagnetic centers, the mixed-valent species and the tyrosyl radical, making them undetectable under the conditions applied. Recent relaxation studies on the radicals of active R2 have established weak couplings, both dipolar and exchange type, between the tyrosyl radical and excited paramagnetic states of the diferric center (Galli et al., 1995). Such an exchange coupling demands orbital overlap between the partners involved. This orbital overlap would then also exist between the mixed-valent diiron cluster and the tyrosyl radical, thus forming a route for electron transfer. Since the tyrosyl radical because of its high redox potential should be the better electron trap, we favor the

Table 1: Parameters Characterizing the EPR Spectra of the $S = {}^1/_2$ (Fe^{2+}, Fe^{3+}) Centers of R2, MetR2, and R2-Y122F Produced Radiolytically at 77 K

protein	g_1[a]	g_2[b]	g_3[c]	ΔH_1[d] (G)	ΔH_2[e] (G)	$\Delta H_{1/2}$[f] (G)
Directly after Irradiation (Species of Type **I** in Scheme 1)						
R2	1.950	1.934	1.816	28	53	81
metR2	1.949	1.936	1.818	24	42	49
R2 − 0.18metR2[g]	1.950	1.928	1.800			50
R2-Y122F	1.950	1.937	1.809	23	41	52
After Annealing at 165 K for 6 min (Species of Type **II** in Scheme 1)						
R2	1.950	1.930	1.805	38	60	63
metR2	1.950	1.929	1.805	39	59	54
R2-Y122F	1.950	1.937	1.806	23	41	52

[a] Measured at low-field maximum. [b] Measured at zero-crossing. [c] Measured at high-field minimum ($\approx g_{||}$ for an axial system). [d] Distance between low-field maximum and zero-crossing. [e] Distance between low-field maximum and nearest minimum at high field. [f] Full width at half-height of high-field minimum. [g] The fraction 0.18 of the spectrum for metR2 was subtracted from that of R2.

electron transfer mechanism as the most likely explanation of the observations.

The yield at low dose for reduction of the iron center of the mutant R2-Y122F (data not shown) was found to be 0.24 $\pm$ 0.03 $Mrad^{-1}$, which is somewhat larger than that of metR2, 0.19 $\pm$ 0.03 $Mrad^{-1}$. This shows that the nature of the side chain of residue 122 has some influence on the yield for electron capture of the nearby diiron cluster. Nevertheless, the limited degree of this influence also justifies the assumption made above that the intrinsic initial yields of reduction of the diferric clusters of complete and metR2 sites are approximately equal.

Why the total yield of mixed-valent centers at high γ-ray doses levels off considerably below 100% of the total concentration of diiron centers, as indicated in Figure 2, is difficult to settle. The initial yield and the apparent level at high doses were previously shown (Davydov et al., 1994) to depend on the amount of glycerol in the medium. It is known that ionizing γ-rays upon interaction with the medium generate strongly oxidizing positive holes in addition to the strongly reducing mobile electrons. Most of these holes are trapped and inactivated by the glycerol, but some holes are always left, particularly at the surface of and inside the protein, where glycerol availability is restricted, and are effective recombinating traps for the electrons. The building up of a barrier of such holes could hinder the electrons to reach an iron center shielded in this way. The possibility that already formed mixed-valent centers could be reoxidized to the diferric state by the holes, thus establishing an equilibrium, should also be considered. However, we know that with chemically preformed diferrous centers no oxidation to the mixed-valent state occurs under the same conditions of irradiation (Davydov et al., 1994).

Effect of the Tyrosyl Radicals of Complete Sites of Active Protein R2 on the EPR Spectrum of the Mixed-Valent Form of Their Diiron Sites. Figure 1 shows the EPR spectra of the primary mixed-valent forms of active R2, metR2, and the mutant protein R2-Y122F, produced by radiolytic reduction at 77 K at a dose of 3.65 Mrad. Parameters characterizing the spectra of Figure 1 are listed in Table 1. The (Fe^{2+}, Fe^{3+}) centers of these three proteins exhibit similar axial EPR spectra with $g_\perp$ and $g_{||}$ within the ranges 1.934−1.937 and 1.809−1.818, respectively. These spectra can be recorded

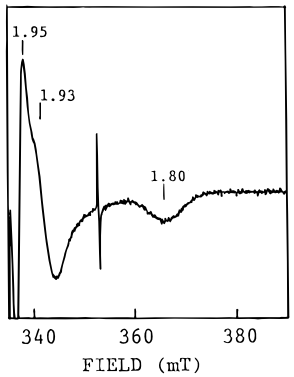

FIGURE 3: Difference EPR spectrum obtained by subtracting spectrum b of Figure 1 multiplied by 0.18 from spectrum a of the same figure. The spectrum is ascribed to the mixed-valent species of type **I** (cf. Scheme 1) formed in complete sites of protein R2, originally containing both the diferric iron center and the tyrosyl radical.

up to a temperature of 110 K without noticeable broadening. It is clear from Table 1 and Figure 1 that there are distinct differences between the EPR spectroscopic properties of the mixed-valent centers produced in tyrosyl radical-containing protein R2, radical-free metR2, and the mutant protein R2-Y122F. The EPR spectrum of the mixed-valent form of active R2 is broadened as compared with those of metR2 and R2-Y122F. For the mixed-valent form produced in active R2, the $g_{||}$ feature has its maximum at $g = 1.816$, is asymmetric, and is characterized by a half-width of 81 G. The line shapes of the corresponding features in the EPR spectra of mixed-valent metR2 and R2-Y122F are essentially symmetric and have half-widths of 48.5 and 52 G, respectively. Also, the $g_\perp$ feature obtained with active R2 is somewhat broadened in comparison with those for metR2 and R2-Y122F, as seen in Table 1.

Comparison of the $g_{||}$ signatures of the mixed-valent diiron centers induced in active R2 and metR2, shown in the inset of Figure 1, suggests that the EPR spectrum in the former case is a composite of two features: one originating from the metR2-type centers in active R2 and another one, with a lower $g_{||}$ value, originating from the complete sites originally containing a tyrosyl radical. Spectrum b of Figure 1 multiplied by a scaling factor was hence subtracted from spectrum a. The scaling factor was varied until a symmetrical $g_{||}$ feature was obtained. Figure 3 shows the resulting spectrum obtained with the scaling factor of 0.18. The parameters characterizing this difference spectrum are included in Table 1. The high-field peak at $g_{||} = 1.800$ is quite symmetric and has a half-width of 50 G, which is comparable to those obtained with metR2 and R2-Y122F. It should also be noted that the spectrum of Figure 3 in the $g_\perp$ region shows some rhombicity, which is not observed for radical free species in spectra b and c of Figure 1.

Within the model discussed above, it was assumed that the prepared active R2 contained approximately 33% of the diferric centers as metR2 centers, a figure in agreement with the slopes at low dose of the two yield curves of Figure 2. The scaling factor of 0.18 used for subtraction of spectra, in order to obtain the difference spectrum of Figure 3, corresponds to a metR2 content of 20%. Both of these values are within the range of metR2 contents obtained for this type of preparation. In any case, the rest of the discussion and the conclusions drawn, which are qualitative in nature, are not dependent on the exactness of this value.

Only the spectra obtained at the dose of 3.65 Mrad were subjected to the detailed analysis described above. However,

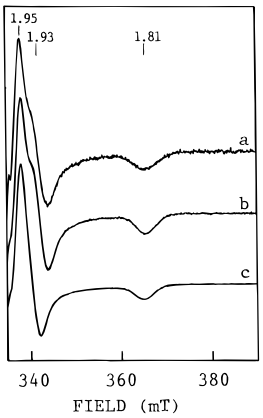

FIGURE 4: EPR spectra of mixed-valent species of type **II** (cf. Scheme 1) of proteins R2 (a), metR2 (b), and R2-Y122F (c) obtained by annealing samples of Figure 1 at 165 K for 6 min. Experimental conditions and spectrometer settings are as in Figure 1 except for temperature, 27 K; microwave frequency, 9.237 GHz; and microwave power, 2 mW.

Table 2: Parameters Characterizing the EPR Spectra of the Ferromagnetically Coupled $S = {}^9/_2$ (Fe^{2+}, Fe^{3+}) Centers of R2, MetR2, and R2-Y122F Arising When the Mixed-Valent Forms of These Proteins, Produced Radiolytically at 77 K, Are Annealed First at 165 K (6 min) (Type **III** of Scheme 1) and Later at 200 K (6 min) (Type **IV** of Scheme 1)

protein	T_{an}[a] (K)	g_1[b]	$\Delta H_{1/2}(g_1)$[c] (G)	g_2[d]	$\Delta H_{1/2}(g_2)$[e] (G)	g_3[f]	g_4[g]	$I_{g14}/I_{g6.7}$[h]
R2	165	14.6	182	6.7	96	5.425	5.262	2.37
metR2	165	14.9	146	6.7	82	5.426	5.263	1.66
R2-Y122F	165	16.77	55	6.61 8.8		5.46	5.242	
R2	200	13.84	204	6.67	82	5.424	5.263	6.2
metR2	200	13.89	204	6.68	80	5.42	5.259	6.2
R2-Y122F	200	16.77		6.61 8.8		5.46	5.242	

[a] Temperature of annealing. [b] Measured at most low-field maximum. [c] Full width at half-height of most low-field maximum. [d] Measured at second (for R2-Y122F second and third) maximum from low field. [e] Estimated width at half-height of second maximum from low field. [f] Measured at zero-crossing. [g] Measured at high-field minimum. [h] Ratio of intensities of maxima close to $g = 14$ and $g = 6.7$.

$g_{||}$ and the width at half-amplitude of the same peak were measured for several doses. For metR2, $g_{||}$ of the radiation-induced mixed-valent state remained at 1.817 independently of the dose, and the widths stayed close to 50 G. For the preparation of active R2, on the other hand, $g_{||}$ decreased continuously from 1.816 at 0.95 Mrad to 1.812 at 4.5 Mrad, while the width at the same doses increased from 63 to 80 G. At zero dose, the data extrapolated to the same $g_{||}$ and width as obtained with metR2. This behavior in the case of the active R2 sample is just what would be expected if, in addition to the peak at $g_{||} = 1.817$ obtained with metR2, the proportion of a contributing peak at $g_{||} = 1.800$ was increasing with increasing dose from a negligible level at very low doses.

The observations outlined above are in accord with the results already deduced from the yield vs dose curves. The metR2 sites and the complete sites of protein R2 respond differently to the reduction at 77 K. The additional important conclusion is now that the EPR spectrum of the mixed-valent diiron centers of the complete sites is distinctly different from the EPR spectrum of the mixed-valent form of the metR2-type centers. This means that the ligand geometry and/or electronic structure of the two types of mixed-valent diiron sites are somewhat different.

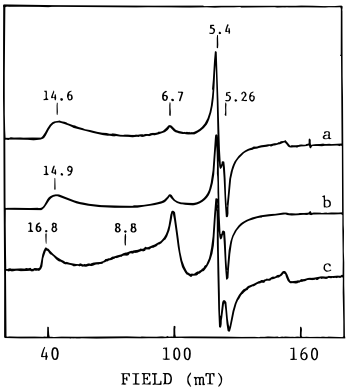

FIGURE 5: EPR spectra of mixed-valent species of type **III** (cf. Scheme 1) of the same samples as in Figure 4. Protein R2 (a), protein metR2 (b), and protein R2-Y122F (c). Experimental conditions and spectrometer settings were the same as in Figure 1 except for temperature, 3.6 K; microwave frequency, 9.235 GHz; and receiver gain, 2×10^4.

Next, the three samples giving the spectra of Figure 1 were annealed for 6 min at 165 K, which in the metR2 sample is known to form species **II** of Scheme 1 (Davydov et al., 1994). The new EPR spectra, which can be recorded up to 100 K without a noticeable change of shape, are shown in Figure 4, and the parameters read from the spectra are listed in Table 1. While the spectrum of the mixed-valent form of the mutant R2-Y122F remained unchanged after this annealing, the spectra of both R2 and metR2 are altered and now show very similar properties including a rhombic splitting of the low-field feature and a $g_{||} = 1.805$. The only marked difference between the two spectra is that the $g_{||}$ peak is slightly broader in the case of R2, 63 G, compared with metR2, 54 G. This broadening is increased to 73 G in the difference spectrum (not shown) taken between these two spectra using the same scaling factor, 0.18, as before.

It was shown previously (Davydov et al., 1994) that annealing at 165 K also causes the mixed-valent form of metR2 to change from the antiferromagnetically coupled $S = {}^1/_2$ state into a ferromagnetically coupled $S = {}^9/_2$ state (species **III** of Scheme 1). Similar transformations are found to take place in the mixed-valent samples prepared from active R2 and the mutant R2-Y122F as shown by the EPR spectra of Figure 5, recorded for the same samples as in Figure 4 but over a different field range. The corresponding characteristic spectral parameters are compiled in Table 2. The spectra obtained for R2 and metR2 have great similarities, but there are still some distinct differences: The most low-field maximum peaks are at $g = 14.6$ and 14.9, respectively; the widths of this feature and that at $g = 6.7$ are larger in the former case; and the ratio between the maximum amplitudes of these two low-field features is larger for R2 than for metR2 (cf. Figure 5 and Table 2). The spectrum obtained with the mutant protein is quite different with the most low field maximum at $g = 16.8$ and an additional broad feature at $g \approx 9$.

Previously it was found (Davydov et al., 1994) that annealing at 200 K caused the $S = {}^9/_2$ center of mixed-valent metR2 to relax a step further, as revealed by distinct changes of the EPR spectrum. Similar annealing was made with the present three samples, and the characteristic parameters of the new spectra (not shown) are compiled in Table 2. These parameters show that in this final state (state **IV** of Scheme 1) it is not possible to distinguish between the mixed-valent forms obtained from R2 and metR2. Both have relaxed to a state giving a broadened low-field peak with a maximum

at $g = 13.8-13.9$, and a much reduced intensity at $g = 6.7$. In contrast, the $S = {}^9/_2$ spectrum obtained with the mutant R2-Y122F is not affected at all by the annealing at 200 K.

Some samples of active R2 that had been exposed to doses of 3−6 Mrad, which abolished most of the tyrosyl radicals (cf. Figure 2), were thawed, refrozen at 77 K, and reirradiated. Before this second irradiation, these samples showed very weak EPR signals due to tyrosyl radicals. After irradiation, they gave EPR spectra due to mixed-valent diiron centers with shapes which were indistinguishable from those obtained from irradiated metR2. The intensities were only very slightly weaker. These results show that essentially all the diiron centers of R2, whether complete sites or metR2-type sites, which by the first irradiation were transformed into mixed-valent states, after thawing have a structure that cannot be distinguished from that of metR2 sites. It is also clear that radiation damage as far as the structure of the diiron sites is considered is negligible for doses below 6 Mrad.

Concluding Remarks. The results described above, based on observations made after radiolytic reduction of the samples at 77 K, demonstrate that the diferric iron cluster of complete sites in *E. coli* RNR protein R2 is affected by the adjacent tyrosyl radical in at least two important ways. First, the yield of mixed-valent iron clusters is greatly reduced in comparison with the metR2 case. In fact, data suggest that the diferric iron cluster of a complete site is not reduced to the mixed-valent state, stable at low temperatures, until the tyrosyl radical has been reduced. If an electron first reaches the diferric cluster, it is most likely transferred to the radical causing its reduction. This route of electron transfer is quite likely of importance for the catalytic reaction of the enzyme. This proposal is supported by the observation by Sahlin et al. (1989), recently confirmed by Silva et al. (1995), that during reductive titration of active protein R2 in solution the tyrosyl radicals first must be reduced completely before reduction of the iron centers starts, leading to the fully reduced state. Second, the ligand geometry and/or electronic structure of the mixed-valent diiron clusters originating from the complete sites are slightly but distinctly different from those of the metR2 sites, as deduced from their different EPR spectra. Based on the assumption that the configuration of a diferric site is retained upon reduction in solid solution at 77 K [cf. Davydov et al. (1994) and references cited therein], this also means that the detailed structure of a diferric cluster of a complete site is different from that of a differic cluster of metR2. There appears to be a small rhombic distortion in the former case, which is not present in metR2. Some of this structural difference between the mixed-valent forms remains even in the ferromagnetically coupled $S = {}^9/_2$ states formed by annealing at 165 K, and is abolished first by the relaxation of these states occurring upon annealing at 200 K. As to the possible cause and nature of this structural difference, it should be recalled that ENDOR studies of the tyrosyl radical have shown (Bender et al., 1989) that the radical is neutral and without any hydrogen bond to the tyrosyl oxygen, whereas the structure of metR2 obtained by X-ray crystallography shows (Nordlund & Eklund, 1993) that tyrosine in this case may form a hydrogen bond, probably to the bidentate iron ligand D84. Possibly such a difference in hydrogen bonding could cause different orientations and twistings of the tyrosine ring leading to small differences in the twisting and orientation of the peptide chain and the ligands surrounding the diiron center. This structural difference should remain in the corresponding mixed-valent states, formed at 77 K, and is relaxed completely first at 200 K. It is known from studies with X-ray diffraction and Mössbauer absorption that extensive motional freedoms become possible in a frozen protein when warmed to this temperature region (Frauenfelder et al., 1994).

Also in mouse and herpes R2 proteins, there is evidence for structural differences around the iron site depending on the presence or absence of the associated tyrosyl radical. Similar effects as presented here for *E. coli* protein R2 have also been observed for mouse R2 by Davydov et al. (unpublished experiments). For both mouse and herpes R2, Nyholm et al. (unpublished experiments) have found that the accessibility of the iron chelator deferrioxamine to the diiron sites was higher without than with a neighbouring tyrosyl radical. In addition, the sensitivity of the diiron site to the structure at the Tyr-122 site is demonstrated by the great influence the mutation Y122F has on the EPR spectra of the mixed-valent form in both $S = {}^1/_2$ and $S = {}^9/_2$ states, as seen in Figures 1 and 5, respectively.

Presently we can only speculate about the possible functional role of the discovered structural influence that the tyrosine/tyrosyl radical site has on the diferric site of protein R2. Conceivably, it could play a role in the regulation of redox properties of the diiron−radical site and/or routes of electron flow inside the protein. That this is possible is supported by the very recent redox results by Silva et al. (1995). (The report became available during the final printout editing of this paper.) They show that the mutation Y122F in metR2 indeed has a marked effect on the redox potential of the iron center.

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