Electron Paramagnetic Resonance Study of the Mixed-Valent Diiron Center in *Escherichia coli* Ribonucleotide Reductase Produced by Reduction of Radical-Free Protein R2 at 77 K

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Abstract: It is demonstrated that γ-irradiation at 77 K of glycerol-containing solutions of metR2, the radical-free form of protein R2 of ribonucleoside diphosphate reductase (EC 1.17.4.1) from *Escherichia coli*, or of methemerythrin from *Themiste zostericola*, gives high yields of mixed-valent (Fe^{2+}, Fe^{3+}) antiferromagnetically coupled states with $S = 1/2$. These sites are nonequilibrium forms with axial electron paramagnetic resonance (EPR) spectra, characterized by $g_{av} < 2$, which are fairly easy to saturate with microwave power. Upon annealing at temperature above 140 K, these primary species are transformed into new mixed-valent species, reflecting relaxation of the ligands of the iron sites toward equilibrium configuration. The mixed-valent nonequlibrium centers of hemerythrin relax at $T > 170$ K to a form with an EPR spectrum observable only below 35 K. This spectrum is identical to that obtained after chemical reduction of metHr. The antiferromagnetic coupling is maintained, but weakened, and the oxo bridge has been transformed into a hydroxo bridge. In the case of the mixed-valent centers of R2, the relaxation process is more complex. The ligands of the primary nonequilibrium form relax at 142 K into slightly different orientations, reflected in a change of the EPR spectrum from axial into rhombic symmetry. The change of the average **g** value is small, and the EPR resonance is still easy to saturate. Annealing at 165 K leads to a dramatic change of the EPR spectrum. The absorption at $g < 2$, corresponding to antiferromagnetically coupled centers, decreases, and new EPR features at low field appear, demonstrating formation of ferromagnetically coupled (Fe^{2+}, Fe^{3+}) centers with $S = 9/2$. A possible explanation is that a hydroxo or aquo bridge has been formed by protonation of the oxo bridge in the iron center. This EPR spectrum is difficult to saturate by microwaves, and may only be observed at temperatures below ca. 35 K. Annealing at about 200 K causes further changes of the spectral features. It is likely that adjustments of the iron-iron distance and positioning and orientation of ligands are taking place at these temperatures. Annealing at $T \geq 230$ K causes disappearance of the low-field EPR spectrum, more rapidly in the presence of O_2 than in its absence. The results show that one-electron reduction of μ-oxo-bridged (Fe^{3+}, Fe^{3+}) centers in proteins at 77 K generates nonequilibrium kinetically stabilized mixed-valent states. These primary products relax to new states upon annealing at $T > 77$ K. The route and final result of this structural relaxation are different for different proteins.

Iron-containing ribonucleotide reductases (RNR) catalyze reduction of the hydroxyl group at the C(2′) position of the ribose moiety in ribonucleoside diphosphates.[1-4] This is an essential reaction for the living cell. The products of the reaction are the four monomeric deoxyribonucleotide precursors needed for DNA synthesis. The best studied enzyme of this kind, RNR from *Escherichia coli*, consists of two nonidentical subunits denoted protein R1 (172 kDa) and protein R2 (86.8 kDa),[1a] each of which is a homodimer. Protein R1 which carries two binding sites for substrates contains redox-active dithiols which are oxidized in the course of the enzymatic reaction. Protein R2 contains a binding site for a pair of iron ions in each of its two identical polypeptide chains. In the active R2 the two ferric ions of an occupied iron site are antiferromagnetically coupled[5-7a] via a μ-oxo bridge.[8-12] In their vicinity there is an unusually stable tyrosyl free radical formed together with the iron site in a reaction among apoprotein, ferrous iron, and molecular oxygen.[7] Both the iron center and the tyrosyl free radical are needed for the enzymatic activity.[4-6] The tyrosyl free radical of active R2 may be reduced chemically, e.g., by hydroxyurea.[4] The product is inactive, radical-free protein R2 (metR2), in which the iron center, as inferred from Mössbauer data, is supposed to be identical to that of active R2.[5]

Similar oxo- or hydroxo-bridged binuclear iron centers have been found in other proteins such as hemerythrin, purple acid phosphatase, and methane monooxygenase.[13,14] Binuclear iron

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clusters normally exist in one of the three redox states, the fully oxidized (Fe^{3+}, Fe^{3+}), the fully reduced (Fe^{2+}, Fe^{2+}), or the intermediate, mixed-valent (Fe^{2+}, Fe^{3+}) state. Strong antiferromagnetic coupling between the iron ions of a diferric state makes it electron paramagnetic resonance (EPR) silent, as is the case of active protein R2 and metR2. Below 30 K the diferrous clusters of fully reduced protein R2 of RNR,[11] methane monooxygenase,[15] and the azide complex of deoxyhemerythrin[16] exhibit EPR spectra with one **g** value in the range 15−16, which arises from a ferromagnetically coupled (Fe^{2+}, Fe^{2+}) system with integer spin.[17] Below 30 K the mixed-valent forms of such dinuclear iron clusters have been found to show rhombic EPR spectra with $\mathbf{g}_{av} = 1.7-1.8$.[14]

A mixed-valent (Fe^{2+}, Fe^{3+}) form has been observed in several oxo-bridged binuclear iron centers of both proteins and model compounds,[13,14,18] but not until recently in RNR. A short-lived one-electron-reduced form of protein R2 was proposed as a possible intermediate in the redox reactions of the iron center in protein R2.[10] Recently, two studies were published presenting EPR data ascribed to mixed-valent forms of protein R2 from *E. coli*. Hendrich et al.[19] characterized an (Fe^{2+}, Fe^{3+}) form of *E. coli* protein R2 with ferromagnetically coupled iron ions from the observation of an $S = 9/2$ EPR spectrum. In their study, mixed-valent R2 was obtained by irradiation of a frozen aqueous solution of metR2 with X-rays at 77 K. Gerez et al.,[20,21] on the other hand, observed a weak transient rhombic EPR signal with $\mathbf{g}_{av} = 1.835$ ($\mathbf{g}_1 = 1.93$, $\mathbf{g}_2 = 1.835$, and $\mathbf{g}_3 = 1.64$) upon reduction of protein R2 by diimide or hydrazine. This signal was also assigned to an (Fe^{2+}, Fe^{3+}) form of R2, but with $S = 1/2$.

One of the approaches to study transient forms of redox centers is to stabilize them kinetically with the help of a so-called low-temperature or solid-state reduction method. Reduction of such centers of proteins in the rigid matrix of frozen solutions at 77 K is conveniently made by mobile electrons generated by ionizing radiation. This technique was successfully applied to the study of transient redox states of metalloproteins[19,22-39] as well as metal ion complexes,[40-46] and was also used by Hendrich et al.[19] to produce the $S = 9/2$ mixed-valent iron center of RNR, mentioned above. However, in the application of the method to metalloproteins it should be emphasized that the primary product arising from the reaction between a mobile electron and a protein metal ion in the rigid matrix of the frozen solution at 77 K as a rule will retain a constrained nonequilibrium conformation.[22-28,34] The results of previous experiments[22-28,34,40-46] provide evidence that, in a solid solution at 77 K, the geometry, i.e., bond lengths and bond angles of the coordination environment of the metal ion site which acquires an electron, remains unaltered or is only very slightly modified. This is because of the low mobility in the system at the low temperature. Therefore, we expect that after electron addition at 77 K the metal ions of protein R2 will retain the ligation geometry close to that of the fully oxidized initial state. In general the details of the equilibrium coordination sphere depend on the oxidation level of the metal ion. Thus, the conformation of the metal ion site, obtained upon single-electron reduction at 77 K, is in a constrained, nonequilibrium state. Only at some higher temperature structural relaxation to the corresponding equilibrium state will take place:

$$Me^{n+}L_n \xrightarrow[77\ K]{+e^-} \underset{\text{nonequilibrium constrained state}}{M^{(n-1)+}L_n} \xrightarrow{T > 77\ K} \underset{\text{equilibrium state}}{M^{(n-1)+}L_{n-1}}$$

In this scheme L_n and L_{n-1} denote the equilibrium ligand environments around Me^{n+} and $Me^{(n-1)+}$, respectively. This relaxation is often a complex process and may proceed in several steps.[22] The temperature where the relaxation is completed depends, inter alia, on the magnitude of the required structural changes and on the solvents used.[40-46]

In this study we present results from EPR studies of kinetically stabilized mixed-valent forms of radical-free protein R2 (metR2) from *E. coli*, and of methemerythrin produced by one-electron reduction of the oxidized protein at 77 K by mobile electrons generated by γ-irradiation. The primary, nonequilibrium, mixed-valent forms show axial, or nearly axial, EPR spectra, characterizing them as antiferromagnetically coupled $S = 1/2$ states. In the case of one-electron-reduced metR2 the ferromagnetically coupled state with $S = 9/2$ is found to be an intermediate in the relaxation process.

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Experimental Section

For production of *E. coli* protein R2, cells of the R2-overproducing wild-type C600/pBS1 strain[47] were used. Protein R2 of *E. coli* RNR was isolated and characterized according to the method described previously.[47,48] MetR2 was prepared by incubation of the active R2 with 10−20 mM hydroxyurea (Sigma) or (*N*-methylhydroxyl)amine hydrochloride (Aldrich) for 10 min at 25 °C and subsequent passage through Sephadex G25 in a centrifuge column.[48b] Apoprotein R2 was prepared according to Atkin et al.[5] Fully reduced protein R2 was made as described by Sahlin et al.[10] using $Na_2S_2O_4$ as a reductant and benzylviologen as a mediator. Anaerobic samples of metR2 were prepared by addition of 10 mM $Na_2S_2O_4$ prior to freezing.

Octameric methemerythrin from the worms *Themiste zostericola* was isolated according to Pearce et al.[49] The live animals were purchased from Pacific Baltimore Biomarine Laboratories Inc., Venice, CA.

The samples in the irradiation experiments contained about 1 mM protein, 50 mM Tris−HCl or NaP_i buffer (pH 7.3−7.6), 100 mM KCl, and 50% by volume glycerol, unless noted otherwise. Frozen protein solutions in quartz tubes were at 77 K exposed to γ-radiation from a ^{137}Cs source (dose rate of 65 krad/h) for 4−160 h. Most of the EPR measurements were performed on samples irradiated by a dose of 2.5−3.5 Mrad. The samples were annealed in isopentane ($T \geq 118$ K) or *n*-pentane ($T \geq 142$ K) baths for suitable times, followed by recooling to 77 K. EPR spectra were recorded on a Bruker ESP 300 X-band spectrometer using 100 kHz modulation with an Oxford Instruments ESR 9 liquid helium cryostat. Temperatures below 77 K were measured with a calibrated gold (0.03% Fe)/chromel thermocouple. Since the thermocouple is not situated at the sample position, temperature gradients in the sample region will result in a difference between the measured temperature and the actual sample temperature. As an independent control the sample temperature was also obtained from a Cu^{2+}−EDTA standard sample (1 mM $CuSO_4$, 10 mM Na_2EDTA, pH 7.8) of the same geometry as the protein samples.[50] A Curie dependence was assumed for its EPR signal amplitude, and 3.6 K was used as a reference point. This temperature was obtained with liquid helium pumped through the cryostat. When temperature differences were observed with the two methods (thermocouple and Cu^{2+}−EDTA standard), the latter method was considered to be more reliable. The frequency was monitored by a Hewlett-Packard 5350A microwave frequency meter.

The EPR signals for the $S = 1/2$ mixed-valent forms with $g_{av} < 2$ were quantitated under nonsaturating conditions by double integration of the first-derivative EPR spectra, using the frozen 1 mM solution of Cu^{2+}−EDTA as standard. In all samples the γ-irradiation also induced large amounts of free radicals, which gave a strong but fairly narrow EPR spectrum around $g = 2$; see Figures 1−3. These radicals disappeared, most likely by recombination processes, upon annealing at high temperatures; see Figure 3b. Hence, there was usually an overlap between the intense radical signal and the $g_{av} < 2$ signal, interfering with the quantitation procedure of the latter; see Figures 1−3. This interference was minimized by first noting the zero crossing between the two signals in the first-derivative spectrum, and then, when calculating the second integral, starting from the high-field side and stopping at the field of this zero crossing.

Lacking a simulation procedure, evaluation of relative intensities for specific parts of the spectra of the $S = 9/2$ mixed-valent form of protein R2 was attempted at different temperatures, by estimating the areas under the absorption-like features at turning points of the spectra, as explained under Results.[51]

Power saturation data were collected, in the power range from 100 nW to 200 mW at temperatures ranging from 3.6 to 25 K, by measuring the EPR absorption derivative peak signal intensity, I, as a function of incident microwave power, P. The data at each temperature were analyzed using the equation

$$\log(I/\sqrt{P}) = a - (b/2)\log(P_{1/2} + P) \qquad (1)$$

making it easy to determine the half-saturation power, $P_{1/2}$.[52]

Protein R2 and methemerythrin concentrations were determined spectrophotometrically on a Cary 219 spectrophotometer according to refs 48 and 49.

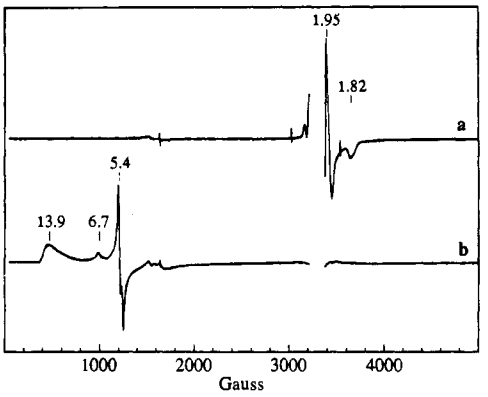

Figure 1. X-band EPR spectra of one-electron-reduced radical-free protein R2 (metR2) of *E. coli* ribonucleotide reductase produced at 77 K by γ-irradiation: (a) before annealing and (b) after 3 min of annealing at 200 K. Experimental conditions: 1 mM protein R2 in 50% glycerol, 50 mM Tris−HCl, pH 7.6, 100 mM KCl, γ-irradiation dose 3.6 Mrad. Instrument parameters: microwave power 1 mW, modulation amplitude 5 G, gain 5×10^3, sample temperature 16 K (a) and 3.6 K (b). The gaps in the spectra are where the free radical signal around $g = 2$ has been left out.

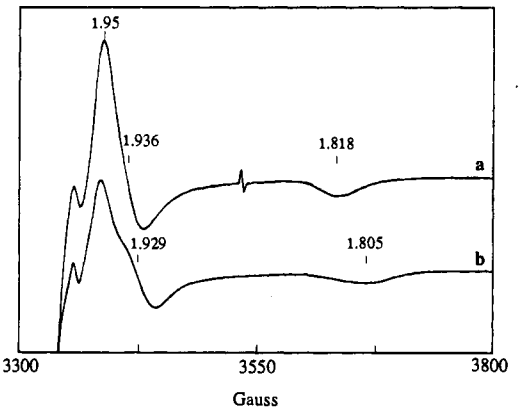

Figure 2. X-band EPR spectra of one-electron reduced met R2 of *E. coli* RNR produced at 77 K by γ-irradiation: (a) before annealing and (b) after annealing at 142 K for 20 min. Experimental conditions: as in legend to Figure 1. Instrument parameters: microwave power 1 mW, modulation amplitude 5G, gain 5×10^3, sample temperature 21 K.

Results

Formation of a Mixed-Valent $S = 1/2$ State in metR2 from *E. coli* by Reduction at 77 K. Before γ-irradiation the met R2 sample shows only a weak $g = 4.3$ signal due to adventitiously bound ferric ions. In Figures 1a and 2a EPR spectra are shown, obtained for metR2 at 77 K after γ-irradiation at the same temperature. Besides the intense signals centered at $g = 2.0$, due to various kinds of radiation induced free radicals, the weak $g = 4.3$ signal, and the H atom doublet with 505 G hyperfine splitting, an axial signal with two characteristic components at $g_\perp = 1.936$ and $g_\parallel = 1.818$ ($g_{av} = 1.90$) is clearly visible. This signal can be observed without noticeable broadening up to 100 K. A sample of active protein R2

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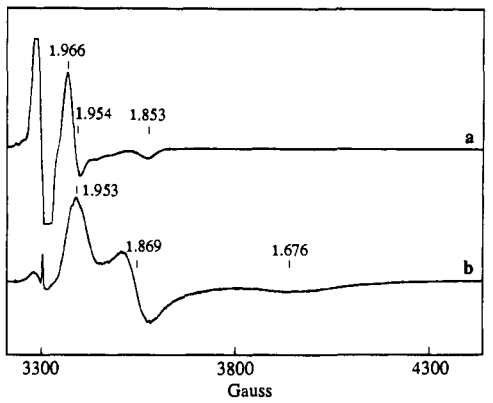

Figure 3. X-band EPR spectra of one-electron-reduced methemerythrin from *T. zostericola* produced at 77 K by γ-irradiation: (a) before annealing and (b) after annealing at 200 K for 3 min. Experimental conditions: 2 mM methemerythrin in 50% glycerol, 50 mM Tris–H_2SO_4, 0.1 M Na_2SO_4, pH 7.6, γ-irradiation dose 0.9 Mrad. Instrument parameters: microwave power 1 mW, modulation amplitude 5G, gain 5×10^3, sample temperature 77 K for (a) and 5 K for (b).

containing tyrosyl radical exposed to γ-rays at 77 K shows a similar EPR signal, but its intensity is 4–5-fold weaker than in the metR2 case (data not shown). This difference between active protein R2 and metR2 will be discussed elsewhere (manuscript in preparation). In the present study only mixed-valent states obtained by γ-irradiation of metR2 have been investigated. We have also found similar EPR signals in other diiron oxo-bridged proteins and model oxo-bridged diiron compounds irradiated at 77 K (manuscripts in preparation). As a comparison the EPR spectrum of methemerythrin, metHr, irradiated at 77 K is also presented in Figure 3a. This mixed-valent EPR signal is characterized by $\mathbf{g}_\perp = 1.954$ and $\mathbf{g}_\parallel = 1.853$ ($\mathbf{g}_{av} = 1.92$). It may be observed up to 130 K, but only below 100 K there was no visible broadening. No EPR signal of this type, with $\mathbf{g}_{av} \approx 1.92$, was observed in irradiated apoprotein R2, diferrous protein R2, deoxy- or semimetHr_R (chemically prepared mixed-valent hemerythrin[49]). Taken together these results show conclusively that the signals of Figures 1–3 originate from (Fe^{2+}, Fe^{3+}) sites produced in the proteins by the low-temperature irradiation. The mixed-valent species are formed when mobile electrons, generated by the ionizing radiation, migrate to and are trapped by the diferric sites, giving rise to one-electron-reduced iron clusters. The EPR signals are characteristic of $S = 1/2$ mixed-valent centers, with a pair of antiferromagnetically coupled high-spin ferric, $S = 5/2$, and high-spin ferrous, $S = 2$, ions.[53–56]

As shown in Figure 4, the yield of the mixed-valent R2 signal increases with increasing radiation dose, up to 5–6 Mrad, and then reaches a plateau. The initial slope of the curve and the level of the plateau both depend strongly on the glycerol content in the irradiated sample. The EPR signal intensity corresponding to the plateau level increases more than 50 times upon changing the glycerol content from 0 to 50% by volume.[57] Quantitation of the half-reduced protein R2 signal of Figure 2a versus a copper standard gave the concentration of unpaired spins in the sample as 0.6 ± 0.1 mM. The concentration of protein R2 in the sample was 1 mM. The plateau level of Figure 4a corresponds to at least 30% of the diiron centers being in a mixed-valent state. The line shapes of the EPR signals of irradiated metR2 or metHr are independent of the dose up to 5–6 Mrad, and show no differences between aerobically and anaerobically prepared protein samples.

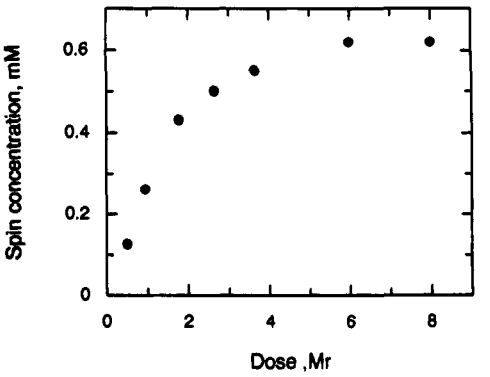

Figure 4. Dose dependence of the yield of mixed-valent metR2 of *E. coli* RNR produced at 77 K by γ-irradiation. The sample conditions were as in the legend of Figure 1.

At temperatures lower than 35 K the EPR signals of the primary mixed-valent forms of R2, Figure 2a, and Hr, Figure 3a, are saturated at relatively low levels of microwave power. For this mixed-valent form of protein R2 the half-saturation power, $P_{1/2}$, was found to be 0.01 ± 0.002 mW at 7.0 K, 0.12 ± 0.02 mW at 9.2 K, 0.5 ± 0.1 mW at 12 K, and 5.0 ± 0.6 mW at 20.5 K. Similar $P_{1/2}$ values were found for this mixed-valent form of Hr: 0.3 ± 0.1 mW at 11.3 K and 4 ± 1 mW at 20.5 K.

For microwave relaxation dominated by two-phonon-assisted coupling of the ground and first excited states, i.e., an Orbach process, the temperature dependence of $P_{1/2}$ is related to the energy separation between the ground and first excited states, Δ, by the equation[52a]

$$\ln P_{1/2} = \ln A - \Delta/kT \qquad (2)$$

where k is the Boltzmann constant and A is a parameter characteristic of the system under study. As a rule this equation has been well-fitted, except for the very low temperatures, in many systems studied.[52a,38,49,58] Using eq 2, we obtain $\Delta = 50 \pm 6$ cm^{-1} for the primary mixed-valent form of R2 and $\Delta = 40 \pm 5$ cm^{-1} for that of Hr.

Relaxation from the Primary Nonequilibrium States. It was expected that annealing at temperatures above 77 K would cause the primary nonequilibrium geometry of the mixed-valent centers and their immediate surroundings to relax toward, and finally reach, the equilibrium configuration. The following spectroscopic observations were made.

In Figure 3, trace a shows the spectrum of the primary mixed-valent nonequilibrium form obtained with metHr. Trace b was obtained after annealing the sample at 200 K for 3 min. The new EPR spectrum of trace b displays rhombic character, with the three **g** values 1.95, 1.87, and 1.68 ($\mathbf{g}_{av} = 1.83$). It can be observed only at $T < 35$ K. This spectrum is identical to that of the mixed-valent Hr, also called semimetHr_R,[59] which is produced by standard chemical reduction methods and is stable in solution at room temperature. The identity between the two

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spectra, which also extends to the temperature dependence and relaxation characteristics, shows that the primary mixed-valent species with an axial EPR spectrum, arising from low-temperature reduction of the diferric iron–oxo cluster of the protein, is not an artifact generated by γ-radiolysis but a real intermediate trapped by the slow kinetics at 77 K. Furthermore, the difference in the EPR properties, before and after annealing of mixed-valent Hr generated at 77 K, is evidence that the structures of the diiron cluster in metHr and the relaxed equilibrium form of mixed-valent Hr, i.e., semimetHr$_R$, are different. This conclusion is in good agreement with results of various other studies.[13,60]

The primary mixed-valent R2, obtained by low-temperature reduction of metR2, was first annealed at 118 K for 50 min. This did not produce any observable change of the EPR spectrum. However, after annealing at 142 K for 20 min, certain small but well-defined changes were observed, Figure 2, trace b. The maximum is shifted slightly to a lower field, and a shoulder has appeared on the high-field side, shifting the zero crossing from $\mathbf{g} = 1.936$ to $\mathbf{g} = 1.926$. The high field minimum is broadened, and its $\mathbf{g}$ value is shifted from 1.818 to 1.805. The appearance of this slightly rhombic spectrum with $\mathbf{g}_x = 1.95$, $\mathbf{g}_y = 1.92$, and $\mathbf{g}_z = 1.80$ ($\mathbf{g}_{av} = 1.89$) is independent of the length of the annealing within the range 10–50 min, and no line broadening was observed at 90 K. Experiments with progressive saturation gave $P_{1/2} = 0.4 \pm 0.1$ mW at 12.5 K and $P_{1/2} = 4 \pm 1$ mW at 20 K, which within experimental errors is identical with the results obtained before annealing. Annealing up to 142 K did not cause any EPR signals to appear elsewhere in the spectrum.

Drastic changes of the EPR spectrum resulted, however, from annealing at higher temperatures. Progressive annealing at 165 K caused a gradual loss of the $\mathbf{g} < 2$ spectrum and a simultaneous growth of new resonance features at $\mathbf{g} = 5.4$, 6.7, and 14.8, which can be observed only at $T < 35$ K. The new spectrum is shown in Figure 5, trace a. At 165 K the kinetics of the decay of the $\mathbf{g} = 1.95$ resonance and the appearance of the low-field features are characterized by $t_{1/2} = 4 \pm 1$ min. Under these conditions, there is direct proportionality between disappearance of the former spectrum and growth of the latter one. Annealing at higher temperatures resulted in changes of the low-field spectrum. Thus, incubation of the sample at 200 K for 3 min caused the low-field peak at $\mathbf{g} = 14.8$ to become broader and shift to $\mathbf{g} = 13.9$, as shown in Figure 1, trace b, and Figure 5, trace b. Simultaneously, the relative intensity of the $\mathbf{g} = 6.7$ peak decreased considerably, whereas no noticeable changes of the $\mathbf{g} = 5.4$ resonance line shape or intensity were observed. The ratios of the areas under the $\mathbf{g} \approx 14$ and $\mathbf{g} = 6.7$ features of Figure 5A, traces a and b, obtained after annealing at 5 and 200 K, are 1.25 ± 0.15 and 5 ± 0.5, respectively. The spectrum shown by trace b of Figure 5 is similar to that reported by Hendrich et al.[19] for X-irradiated metR2 after annealing at 200 K. It should be noted that the EPR spectrum of Figure 5, trace b, does not depend on whether the sample is prepared aerobically or anaerobically. Hendrich et al.[19] assigned the low-field resonances, observed in irradiated metR2, to mixed-valent diiron centers with $S = 9/2$. This spin state should originate from ferromagnetic exchange coupling between the high-spin ferric and high-spin ferrous ions of a mixed-valent cluster.

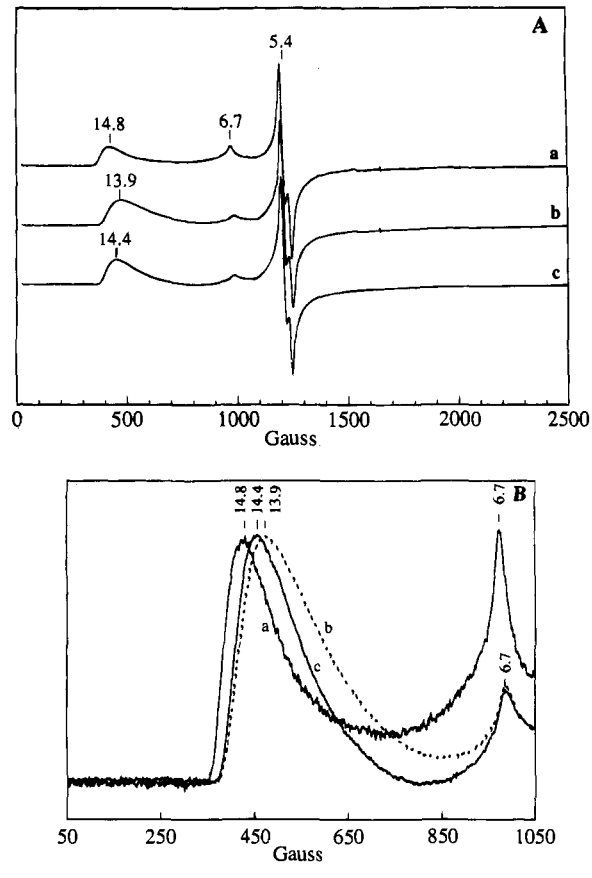

Figure 5. X-band EPR spectra of one-electron-reduced metR2 of *E. coli* RNR produced at 77 K by γ-irradiation. Panel A: After annealing at 165 K for 6 min (trace a) and at 200 K for 3 min (traces b and c). Experimental conditions: as in legend to Figure 1. Instrument parameters: microwave power 1 mW (traces a and b) and 100 mW (trace c), gain 5×10^3 for (traces a and b) and 1×10^3 for (trace c), modulation amplitude 5 G, sample temperature 3.6 K. Panel B: overlay of enlarged low-field regions of spectra a–c in panel A.

In anaerobic samples of metR2, frozen in the presence of sodium dithionite before irradiation, the low-field signals persist up to 230 K, at which temperature they decay with a half-life $t_{1/2} \approx 1$ min, whereas in samples prepared aerobically, the signals disappear quite rapidly, $t_{1/2} < 10$ s, already at 210 K. After annealing at these temperatures, the iron centers of irradiated metR2 become EPR silent. The higher rate of EPR signal loss in aerobic samples is most likely due to oxidation by O_2 of the $S = 9/2$ mixed-valent diiron centers. When an aerobic sample was recycled through the irradiation/annealing process, the EPR spectra of Figures 1 and 2 were obtained again, albeit slightly less intense (loss of intensity depending on strength of previous doses). In addition, when a sample at the stage corresponding to that of trace b of Figure 5, was exposed to γ-rays at 77 K, a low-intensity spectrum with $\mathbf{g}_{av} < 2$ was observed beside the $S = 9/2$ spectrum. This shows that the radiation damage of the protein is small, as far as structure and stability of the iron center is concerned.

Microwave saturation of the two types of $S = 9/2$ spectra could only be obtained at low temperatures, and was thus studied at 3.6 K. The following half-saturation powers, $P_{1/2}$, were found for the sample obtained after annealing at 165 K, 31 ± 4 mW ($\mathbf{g} = 14.8$), 27 ± 4 mW ($\mathbf{g} = 6.7$), and 23 ± 3 mW ($\mathbf{g} = 5.4$), and after annealing at 200 K, 19 ± 2 mW ($\mathbf{g} = 13.9$–14.4), 18

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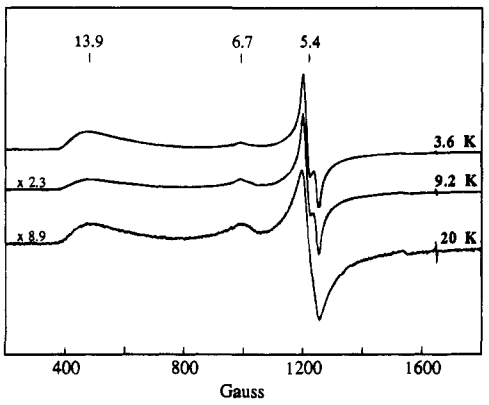

Figure 6. EPR signal of the sample corresponding to Figure 5A, trace b, at different temperatures. Instrument parameters: microwave power 1 mW, modulation amplitude 5 G, relative gains as shown in the figure, sample temperature 3.6, 9.2, and 20 K.

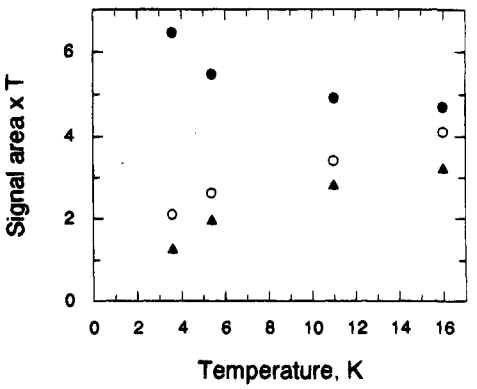

Figure 7. Temperature dependence of the relative population of EPR-active states giving rise to the $g = 13.9$ (●), $g = 6.7$(▲), and $g = 5.4$ (○) features. For $g = 13.9$ and $g = 6.7$ the data show the relative areas under the EPR features, multiplied by temperature. For $g = 5.4$ the data show the signal amplitude of the EPR feature multiplied by the square of the half-width of the feature, as described in the text, multiplied by temperature. The vertical scale is in arbitrary units.

$\pm$ 3 mW ($g = 6.7$), and 21 $\pm$ 2 mW ($g = 5.4$). After annealing at 165 K there are slight differences between the features in readiness to saturate. This causes the relative intensities to change with power. After annealing at 200 K the spectrum is slightly easier to saturate, but there are hardly any differences in saturation behavior of the peak amplitudes of the different features. However, a remarkable change in shape of the low-field peak of the EPR spectrum was observed at high powers in both cases, but particularly after annealing at 200 K. This is shown by traces b and c of Figure 5. A 100-fold increase in power, from 1 to 100 mW, sharpened the peak considerably and shifted the maximum from $g = 13.9$ to $g = 14.4$.

Figure 6 shows how the shapes and intensities of the features of the low-field spectrum, obtained after annealing at 200 K, depend on the observation temperature. Up to 16 K there is no noticeable broadening of the peaks at $g = 13.9$ and 6.7, but their relative amplitudes are changing. This is illustrated by spectra a and b of Figure 6, at 3.6 and 9.2 K, respectively. The features around $g = 5.4$ are significantly broadened already at 9.2 K. At temperatures above 16 K all spectral features are broadened, as illustrated by spectrum c of Figure 6, recorded at 20 K.

The features of the spectra of Figure 6 at $g = 13.9$ and $g = 6.7$ appear to represent absorption-like low-field turning points of separate powder spectra. The relative intensities of these peaks at the different temperatures were estimated by two methods. The first method is based on the observation that the area under a turning point absorption-like feature should be proportional to the concentration of the species giving this EPR signal, at least for a Kramers doublet.[51] The areas of the low-field peaks at $g = 13.9$ and 6.7 were determined by integration from ca. 300 G up to the first minimum at 800 G, and from this point to the next minimum at 1070 G, respectively. In the second method the products were taken of the peak amplitudes, measured from the low-field base line, and the peak widths, estimated at half-height, accounting for overlap interference between peaks. The results from the two methods were in reasonable agreement. The estimated values of spin concentration were corrected for a supposed general Curie-law dependence by multiplication with the absolute temperature of the recording. The results using the first method of evaluation are shown in Figure 7.

The temperature dependence of the $g = 5.4$ feature was more difficult to assess quantitatively. The low-field peak of this feature looks like a central derivative signature of a powder spectrum, whereas the high-field part appears as a high-field turning point. Because of the serious overlap between these two parts, the relative intensities were estimated in the following way. The amplitude of the low-field peak, which is part of the possible derivative signature, was multiplied by the square of the half-width at half-height, measured on the low-field side. On the high-field side, the amplitude of the negative peak, the possible turning point, was multiplied by the half-width at half-height, now measured on the high-field side. These two sets of relative intensities at different temperatures appeared equivalent. The former set of data, corrected for a Curie-law dependence, is also included in Figure 7.

It is obvious that the temperature dependences of the signals are not in agreement with the Curie law. With increasing temperature the amount of the paramagnetic species giving the $g = 13.9$ resonance decreases, while the amounts of those giving rise to the $g = 6.7$ and $g = 5.4$ signals increase. This non-Curie temperature dependence of EPR signals is characteristic of half-integer high-spin systems,[55] in the present case an $S = 9/2$ system. The temperature behavior of the spectrum of Figure 6 as well as simulation of this[19] indicates that the $g = 13.9$ resonance is from a ground-state doublet, and the $g = 5.4$ and $g = 6.7$ features are from Kramers doublets of excited states.

Discussion

Yield of the Mixed-Valent State of Protein R2. The results presented above show that mobile electrons arising from γ-radiolysis of the aqueous–glycerol glass at 77 K are capable of reducing the diferric sites of *E. coli* protein R2 in the radical-free met form and in methemerythrin. High yields of mixed-valent (Fe^{2+}, Fe^{3+}) states are formed. The maximal yields of the (Fe^{2+}, Fe^{3+}) states of metR2 and metHr amounted to approximately 40% and 50%, respectively, of the diiron center contents. In analogous experiments with the hydroxylase component of methane monooxygenase from *Methylococcus capsulata* Ericson et al.[37] and De Witt et al.[38] have observed above 70% conversion of the diferric clusters to the mixed-valent state. These high yields of mixed-valent species should allow application of various spectroscopic techniques (EPR, Mössbauer, MCD, ENDOR, Raman spectroscopy, etc.) for further studies. The low yield (~0.5%) of the mixed-valent form of metR2 in the analogous experiments by Hendrich et al.[19] could be explained by the absence of glycerol in the irradiated samples. Glycols are known to scavenge mobile electron holes, which arise during γ-irradiation, and are powerful oxidants.

***S* = 1/2 State in Mixed-Valent R2.** The EPR signals of the primary products of one-electron reduction of metR2 and metHr produced at 77 K (Figures 1–3) are characterized by

Table 1. EPR Parameters for Mixed-Valent Iron–Oxo Proteins and Model Compounds

protein or model compound	method of production	g value	g_{av}	T_{obs}(max) (K)	refs
E. coli protein R2	γ-irradiation, 77 K	$g_\perp = 1.936$ $g_\parallel = 1.82$	1.899	≤130	present paper
metHr from *T. zostericola*	γ-irradiation, 77 K	$g_\perp = 1.954$ $g_\parallel = 1.85$	1.924	≤130	present paper
E. coli protein R2	diimide, 293 K	$g_x = 1.93$ $g_y = 1.835$ $g_z = 1.64$	1.835		20
hemerythrin semimetHr$_R$	chemically, 293 K	$g_x = 1.950$ $g_y = 1.88$ $g_z = 1.66$	1.834	<35	13, 14
semimetHr$_O$	chemically, 293 K	$g_\perp = 1.95$ $g_\parallel = 1.72$	1.79	<35	13, 14
uteroferrin pink	chemically, 293 K	$g_x = 1.93$ $g_y = 1.73$ $g_z = 1.58$	1.746	<35	13, 14
acid phosphatase pink	chemically, 293 K	$g_x = 1.92$ $g_y = 1.77$ $g_z = 1.63$	1.773	<35	13, 14
methane monooxygenase	chemically, 293 K	$g_x = 1.92$ $g_y = 1.85$ $g_z = 1.72$	1.83	<35	37, 38
$Fe_2(O_2CH)_4(BIPhMe)_2$	chemically, 293 K	$g_\perp = 1.94$ $g_\parallel = 1.84$	1.87	<35	61
$Fe_2(bimp)(\mu\text{-}O_2CCH_2H_3)_2$	chemically, 293 K	$g_x = 1.97$ $g_y = 1.81$ $g_z = 1.72$	1.83	<35	62
$Fe_2O(O_2CCH_3)_2(Me_3TACN)_2$	electrochemically, 293 K	$g_\perp = 1.95$ $g_\parallel = 1.89$	1.93	<35	63

$\mathbf{g}_{av}$ = 1.90 and 1.92, respectively, and bear the characteristic features of EPR signals of mixed-valent binuclear iron clusters with antiferromagnetically coupled ferric ($S = 5/2$) and ferrous ($S = 2$) ions. This EPR signal in X-irradiated *E. coli* protein R2 was also observed in the study by Hendrich et al.,[19] but was misinterpreted as a free radical signal. We have now also observed EPR signals with similar **g** values and relaxation characteristics, i.e., slow enough relaxation to permit observation by EPR at 77 K, in other iron–oxo proteins (protein R2 from mammalian sources and herpes virus, met HrN_3, HrO_2, some mutant proteins R2) and μ-oxo-bridged diiron model complexes ($[Fe\ EDTA]_2O$, $[Fe(phen)_2]_2O$, etc.) irradiated at 77 K (to be published elsewhere). It should be noted that the line shapes, **g** values, and relaxation characteristics of the EPR signals of the mixed-valent species of metR2 and metHr produced at 77 K differ distinctly from those of the mixed-valent forms of R2 and metHr as well as other iron–oxo proteins and model compounds produced by standard chemical reduction at room temperature (Table 1). The mixed-valent species of metR2 and metHr generated at 77 K exhibit close to axial EPR spectra with $\mathbf{g}_{av}$ = 1.90–1.92, and may be observed even at temperatures as high as 130 K (Table 1). The EPR spectra of the mixed-valent states of iron–oxo proteins and binuclear iron model complexes produced at room temperature are as a rule rhombic and characterized by lower values of $\mathbf{g}_{av} \approx 1.75$–1.85. These species also have shorter relaxation times so that they may be observed only close to liquid helium temperatures (Table 1).

The dependence of the spectroscopic properties of mixed-valent derivatives of metR2 and metHr on the method of generation is due to the specific conditions for the reduction in rigid solutions at 77 K. The mixed-valent binuclear iron cluster trapped upon reduction in a rigid solution at 77 K has a ligation geometry close to that of the initial diferric cluster. If the cluster structure depends on the iron oxidation level, the mixed-valent state generated at 77 K will be constrained and not in equilibrium. It has to relax toward a new equilibrium state at higher temperatures. This behavior is clearly demonstrated with methemerythrin. The room temperature conversion of metHr to the mixed-valent semimetHr is known to be accompanied by protonation of the μ-oxo ligand and as a consequence lengthening of the Fe–O distance.[13,64] Therefore, the primary spectrum of Figure 3A originates from a constrained (Fe^{2+}, Fe^{3+}) cluster which is structurally close to the oxo-bridged diferric cluster of metHr (Scheme 1). After annealing at or above 170 K, this constrained species transforms into the equilibrium form of semimetHr$_R$ (Figure 3B) with a μ-hydroxy bridge instead of a μ-oxo bridge in the diiron center.[60,64]

The spectroscopic properties and relaxation behavior of the mixed-valent diiron derivatives of metR2 and metHr produced at 77 K are mainly determined by the presence of the μ-oxo bridge. In contrast, the hydroxylase component of methane monooxygenase from *M. capsulata* contains a μ-hydroxo bridge in its native diiron cluster. In that case reduction at 77 K results in the formation of a rapidly relaxing mixed-valent species, which gives a rhombic EPR spectrum close to that from its mixed-valent state produced at room temperature.[37,38]

EPR spectra of mixed-valent diiron centers were initially described using the vector coupling model.[54,55] This model assumes that the extra electron is mainly localized on one of the iron ions and relates an effective **g** tensor of the dimer primarily to the **g** tensor of the ferrous ion of the pair. Theoretical analyses performed by Bertrand et al.[54d] and by Bominaar et al.[65] give evidence that the **g** tensor rhombicity of the Fe^{2+} ion is extremely sensitive to small distortions of the geometry of the ligand-induced crystal field. The axial character of the primary spectra with $\mathbf{g}_{av} < 2$ indicates that the extra electron in metR2 reduced at 77 K is located on an iron ion which is characterized by a high degree of symmetry of the surrounding ligands. Already the first Mössbauer studies of *E. coli* protein R2[5] established a nonequivalence of the coordination geometries of the two iron ions, reflected by differences in

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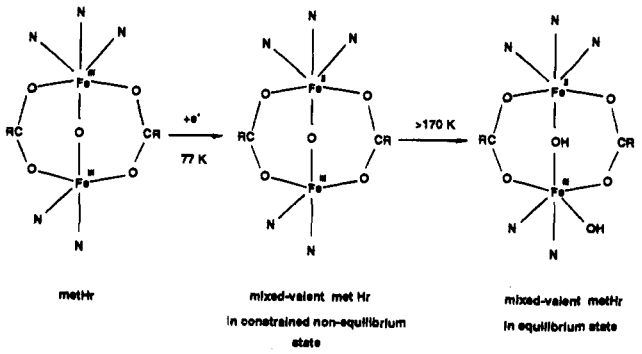
Scheme 1



quadrupole splittings and electric field gradient asymmetry parameters. This nonequivalence was confirmed in the X-ray crystallographic structure of metR2 from *E. coli*.[12] Each of the two iron ions was found to have only one histidine imidazole as a ligand. The other ligands are all oxygens from carboxylates, the O^{2-} bridge, and water molecules. One iron ion (Fe(2)) is octahedrally coordinated with small deviations from regular geometry, while the coordination of the other iron ion (Fe(1)) is more distorted, mainly due to the fact that Asp84 is a bidental ligand to this iron ion.[12] There is also spectral evidence that in the fully oxidized native state of *E. coli* protein R2 only one iron ion is six-coordinated while the other one may have only five ligands,[66] thus confirming the nonequivalence of the iron sites in protein R2. We therefore propose that it is the more symmetrical site Fe(2) that acquires the additional electron in mixed-valent R2 generated from metR2 at 77 K, and gives rise to the axial EPR spectrum of Figure 2a.

McCormick et al.[60] have shown that the **g** values of the iron dimer are sensitive to both the zero-field splitting, $D_{Fe^{2+}}$, and the coupling constant, J. For positive $D_{Fe^{2+}}$ the relation $\mathbf{g}_\perp > \mathbf{g}_\parallel$ always holds, but for negative $D_{Fe^{2+}}$ the reverse relation $\mathbf{g}_\parallel < \mathbf{g}_\perp$ is valid, except for numerically very small values of J ($-J \leq 5$ cm^{-1}). For mixed-valent R2 and metHr obtained at 77 K we have found that $\mathbf{g}_\parallel < \mathbf{g}_\perp$. This indicates that $D_{Fe^{2+}}$ is negative. The sign of $D_{Fe^{2+}}$ is known to be determined by the splitting of the T_2 ground state. $D_{Fe^{2+}}$ is negative when the $d_{xz,yz}$ orbitals are below the d_{xy} orbital in energy.[60]

The magnitude of the antiferromagnetic coupling constant, J, for the primary form of the mixed-valent diiron cluster generated in metR2 at 77 K, may be estimated from Δ, the energy separation between the ground and first excited state. From the temperature dependence of $P_{1/2}$, $\Delta = 50 \pm 6$ cm^{-1} was derived according to eq 2. For the antiferromagnetically coupled (Fe^{2+}, Fe^{3+}) system with a ground spin state of $S = 1/2$, Δ is a function of both the coupling constant, J, and the zero-field splitting energy, D. Provided that $|D| \ll |J|$, the approximation $|J| \approx (1/3)|\Delta|$ is applicable.[38,60] With J defined by the Hamiltonian $\mathcal{H} = -2J\hat{S}_1\hat{S}_2$ we obtain $J = -17 \pm 2$ cm^{-1} in the present case. We found a similar value of J (-13 cm^{-1}) also for the primary mixed-valent derivative of metHr, generated at 77 K. This value of J is close to that for (Fe^{2+}, Fe^{3+}) centers reported for the equilibrium forms of semimetHr[49] (-15 cm^{-1}) and semimetHrN_3[49] and indicates weak antiferromagnetic exchange. Interestingly, the J values for the (Fe^{2+}, Fe^{3+}) sites obtained from metR2 and metHr at 77 K are much smaller than those of the diferric forms of R2 (about -90 cm^{-1})[7,67a,b] and metHr (-130 cm^{-1})[13] despite structural similarity. The significant decrease of the J value should be due to a change of the dominating exchange pathway[68] in mixed-valent R2 compared to metR2. Lengthening of the Fe–O distance could possibly also contribute.[69]

$S = 9/2$ State in Mixed-Valent R2. The transformation from the primary $S = 1/2$ mixed-valent form of metR2 into the final $S = 9/2$ form at 200 K proceeds through at least two EPR-detectable transient states.

First, annealing at 142 K effects transformation from the original axial EPR spectrum with $\mathbf{g}_{av} = 1.90$ into a new one of slightly rhombic character with $\mathbf{g}_{av} = 1.89$. A reasonable explanation of the spectral changes is that at 142 K small adjustments of ligands around the iron ions are allowed. Small orthorhombic distortions of the ligand geometry around the ferrous ion could in principle cause noticeable shifts of the EPR signal components of the coupled center.[54d,56a]

Second, annealing at 160–170 K causes the EPR signal with $\mathbf{g}_{av} = 1.89$, due to an $S = 1/2$ state, to disappear, and the simultaneous growth of low-field features assigned to an $S = 9/2$ state of the exchange-coupled mixed-valent diiron center with its most low field peak at $\mathbf{g}_1 = 14.8$. Next, annealing to 200 K results in distinct changes of the line shapes and the relative intensities of the separate features, with the most low field peak moved to $\mathbf{g}_1 = 13.9$, and a decrease of $P_{1/2}$ for this peak and that at $\mathbf{g}_2 = 6.7$. No noticeable difference in the $\mathbf{g} = 5.4$ resonance line shape was observed after increasing the annealing temperature from 165 to 200 K.

The analysis of the EPR signals of Figure 5 in the framework of an $S = 9/2$ spin Hamiltonian formalism[70] performed by Hendrich et al.[19] have shown that the $\mathbf{g} = 13.9$ resonance originates from a ground-state $\pm 1/2$ Kramers doublet and the $\mathbf{g} = 5.4$ resonance from an excited $\pm 3/2$ doublet state. Pierik et al.[71,55] have come to a similar conclusion for a similar system from the analysis of EPR signals from (6Fe–6S) iron–sulfur proteins with total spin $S = 9/2$. From the temperature dependence analysis of the low field-signals, Figures 6 and 7, a similar interpretation is likely also for the $\mathbf{g} = 14$ and $\mathbf{g} = 5.4$ signals observed by us in mixed-valent R2. However, our results indicate that also the $\mathbf{g} = 6.7$ feature originates from an excited-state doublet, but this feature does not appear in the spectrum simulated by Hendrich et al.[19] Further simulations applying Hendrich's approach are in progress in our laboratory. Using an increased width of a Gaussian spread in rhombicity, it seems possible to obtain, at least qualitatively, the $\mathbf{g} = 6.7$ feature from the same spin system that gives rise to the $\mathbf{g} = 5.4$ feature. An alternative explanation would involve two $S = 9/2$ spin systems, similar to the model proposed by Pierik et al.[71b] to explain the heterogeneity of the EPR signal from a (6Fe–6S) iron–sulfur protein from *Desulfovibrio vulgaris*. Also in that case they observed a feature around $\mathbf{g} = 6.8$ alongside a $\mathbf{g} = 5.6$ resonance. Such heterogeneity is not an uncommon phenomenon in high-spin metalloproteins.[55,72]

Switch of Exchange-Coupling Mode: Possible Structural Implications. A few remarks should be made on the transformation in the (Fe^{2+}, Fe^{3+}) site of protein R2 from antiferromagnetic to ferromagnetic coupling upon annealing. A superexchange mechanism which is responsible for the antiferromagnetic interaction in any (Fe^{2+}, Fe^{3+}) state is known to be strongly dependent on both the geometry and the chemicalstructure of the bridging ligand.[69,69,71,73,74] Hotzelmann and Wieghardt[73] have reported that protonation of the oxo bridge

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results in switching the mechanism of spin exchange coupling from antiferromagnetic to ferromagnetic in a binuclear (μ-oxo)-bis(μ-acetato)chromium(III)vanadium(III) complex. Mixed-valent ferromagnetically coupled $S = 9/2$ states have been reported for two model systems: the bis(μ-phenoxo) (Fe^{2+}; Fe^{3+}) complex[74a,b] and the tris(μ-hydroxo) (Fe^{2+}, Fe^{3+}) complex.[74c] The oxidized forms of these complexes differ considerably both in bridging systems and in the number of coordinated nitrogen and oxygen ligands. Our results on the (Fe^{2+}, Fe^{3+}) $S = 9/2$ site in protein R2 indicate that a significant conformational transformation takes place at temperatures above 160 K, and reveals itself in changes of the magnetic properties of the site.

Protein R2 is the first iron−oxo protein in which the diiron center has been found to be ferromagnetically coupled in the mixed-valent state. A transformation of the exchange-coupling mode from antiferromagnetic to ferromagnetic has been described for the complex formation of deoxyhemerythrin with N_3^-. In that case it was suggested[16] that the changes in magnetic properties of the diiron center were due to protonation of a bridging hydroxo group to form an aquo-bridged ferrous ion pair. Similarly one may speculate that in the $S = 9/2$ mixed-valent center of protein R2 the μ-oxo bridge becomes protonated during annealing above 160 K. The origin of the spectral distinctions between the $S = 9/2$ states arising after annealing at 165 and 200 K is currently unclear. Judging from the results of our simulations, it seems likely that they are due to a slight decrease of rhombicity of the Fe^{2+} ligation geometry, and/or narrowing of the width of the distribution of the crystal field parameters.

Disappearance of the Mixed-Valent EPR Signal. Under anaerobic conditions the $S = 9/2$ centers transform to EPR-silent states with a prominant rate already at temperatures around 230 K ($t_{1/2} \approx 1$ min). In the presence of O_2 the $S = 9/2$ state decay rate increases significantly. In this case the disappearance of the mixed-valent intermediate should be due to oxidation by O_2 to a diferric state. The low stability of the $S = 9/2$ mixed-valent form of *E. coli* protein R2 at 230 K explains the failure of previous attempts to observe mixed-valent R2 at room temperature after exposure to standard chemical reducing agents ($Na_2S_2O_4$ + benzylviologen, dithiothreitol, etc.).

Disappearance of the $S = 9/2$ signal in protein R2 above 230 K may be due to disproportionation of two mixed-valent forms of protein R2 into fully reduced (Fe^{2+}, Fe^{2+}) and oxidized (Fe^{3+}, Fe^{3+}) sites. Another possibility to explain the loss of the EPR signal could involve intramolecular structural changes in the $S = 9/2$ center, resulting in a new mixed-valent state with the property to show only a very weak EPR signal even at very low temperatures. Mixed-valent diiron systems which are or may appear EPR silent are known: The (Fe^{2+}, Fe^{3+}) complexes with *N,N'*-(2-hydroxy-5-methyl-1,3-xylylene)bis[*N*-(carboxymethyl)glycine][75] and with a (μ-phenoxo)bis(μ-carboxylato)-dimetal core[76] have been reported to be EPR silent or to have a very weak EPR signal, respectively.

Comparison with Earlier Studies. As mentioned above, Gerez et al.[20,21] observed a transient rhombic EPR signal with $g_{av} = 1.835$ during anaerobic reduction of protein R2 by diimideor hydrazine. This mixed-valent species could, however, not be produced with other reducing agents under similar conditions. A possible explanation could be that, unlike in the $S = 9/2$ form of R2 described here, in the $S = 1/2$ mixed-valent state observed by Gerez et al. the iron site has incorporated as a ligand either the reductant itself or its oxidation product. This may have a stabilizing effect on the mixed-valent state.

Our present results resolve some problems in earlier studies of mixed-valent states in *E. coli* protein R2 of ribonucleotide reductase. Most previous efforts to produce a mixed-valent state at room temperature have failed simply because the EPR-active mixed-valent state is not stable under those conditions. It may become stabilized only if a suitable ligand is available.[20] The previously observed ferromagnetically coupled center produced in *E. coli* protein R2 by X-irradiation at 77 K[19] is not a primary product. It is an intermediate, in our case stabilized in the temperature range 165−220 K, in the chain of structural relaxations and reactions taking place with an increase of temperature.

Concluding Remarks

In the present paper only the radiolytic reduction of *E. coli* protein R2 in the form of radical-free metR2 has been discussed. It has been proposed that in the enzymatic reaction the oxidation equivalent stored in the form of a tyrosyl radical in active protein R2 is used to transiently oxidize the ribose moiety of the substrate to a free radical, which is needed for activation of the site for the subsequent reduction. In this activation process the iron site may play a role in the electron transfer between the substrate and the tyrosyl radical. This possibility will be discussed in more detail in a following paper, where the one-electron reductions of metR2 and active R2 are compared.

The temperature-dependent changes of EPR spectral properties of proteins R2 described here reflect a relaxation from a primary nonequilibrium mixed-valent state generated at low temperature toward a new conformational equilibrium via several transient states, stabilized at higher temperatures. In certain respects, this relaxational process may be regarded as the reversal of the process of the formation of the oxygen-bridged iron center from a diferrous center in the reactions which generate the diferric iron−tyrosyl radical site in active protein R2.

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