EPR Studies of Mixed-Valent [$Fe^{II}Fe^{III}$] Clusters Formed in the R2 Subunit of Ribonucleotide Reductase from Mouse or Herpes Simplex Virus: Mild Chemical Reduction of the Diferric Centers

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Ribonucleotide reductase (RNR) catalyzes the reduction of all four ribonucleotides to their corresponding deoxyribonucleotides, an essential step in DNA synthesis in living cells. In *Escherichia coli*, herpes simplex viruses (HSV), and mammals, the enzyme is composed of two nonidentical homodimer subunits designated R1 and R2.[1] The smaller R2 subunit contains a stable tyrosyl free radical and a binuclear iron center within each polypeptide chain.[1] The three-dimensional structure of *E. coli* protein R2 has been determined.[2] In agreement with spectroscopic studies the structure shows two iron clusters, each consisting of two O_2-derived μ-oxy-bridged antiferromagnetically coupled ferric ions.[3] A variety of spectroscopic results show that a similar iron/free radical site exists in protein R2 from mouse and HSV type 1 (HSV1).[4]

Dinuclear non-sulfur iron clusters exist in several enzymes with different functions.[1,5] This class of iron–oxygen proteins also includes hemerythrin with a diferric oxy bridge,[6] the hydroxylase component of methane monooxygenase (MMOH) with a diferric hydroxy bridge,[7] purple acid phosphatases (e.g., uteroferrin),[8] rubrerythrin,[9] and a desaturase.[10] In general, these binuclear iron sites exist in three oxidation states:[5-11] diferric resting [$Fe^{III}Fe^{III}$], mixed valent [$Fe^{II}Fe^{III}$], and fully reduced [$Fe^{II}Fe^{II}$]. The mixed-valent forms of diiron clusters have been characterized for hemerythrin,[12] the purple acid phosphatases,[8a-d] MMOH,[13] and iron model compounds.[11] The diferric and diferrous oxidation states have been extensively studied in the *E. coli* R2 protein.[3] However, the mixed-valent state of *E. coli* protein R2 was previously produced only in small (<5%) amounts[14] and only by using drastic conditions. In R2 protein from mouse or HSV1 only the oxidized form has previously been characterized.[4] In the present communication, we report the first direct spectroscopic evidence for a high yield of a mixed-valent state by mild chemical reduction of the diiron clusters in the R2 proteins of mouse and HSV1 RNR.

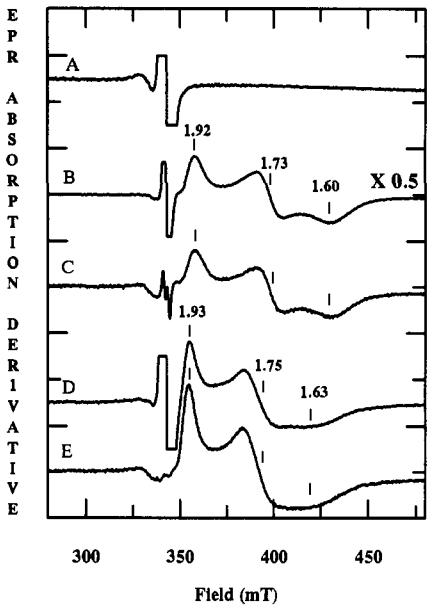

Figure 1. EPR spectra of RNR R2 proteins after incubation with dithionite/PMS or hydrazine. EPR spectra (Bruker ESP300 spectrometer with an Oxford ESR 900 helium flow cryostat at 9.62 GHz, 4 K, 15.8-mW microwave power, 100-kHz 0.1-mT modulation of amplitude) of the mixed-valent R2 protein obtained by reduction of R2:[15] (A) 0.2 mM *E. coli* R2 after 25-min incubation at 0 °C with 2 mM PMS and 4 reducing equiv from dithionite; (B) 0.2 mM mouse R2 as in spectrum A; (C) 0.2 mM mouse R2 after 90-min incubation at room temperature with 5 mM hydrazine; (D) 0.2 mM HSV1 R2 after 2-min incubation at 0 °C with 2 mM PMS and 4 equiv from dithionite; (E) HSV1 R2 as in spectrum C. The strong truncated $g = 2.00$ signal in the presence of PMS is from PMS.

In EPR-monitored chemical reduction experiments with R2 proteins[15] from *E. coli*, HSV1, and mouse, all the samples were incubated at 0 °C and pH 7.6 with 2 mM phenazine methosulfate (PMS) as mediator and dithionite as reductant (corresponding to four electrons per dimer). First the characteristic tyrosyl radical spectra at $g = 2.004$[1,4] disappeared in all three proteins. Subsequently in mouse and HSV1 R2 proteins, the EPR spectra exhibited new signals at $g < 2$ (Figure 1). These rhombic spectra are attributable to antiferromagnetically coupled mixed-valent states of the iron clusters (single $S = {}^1/_2$ species). The apparent g values are $g = 1.92$, 1.73, and 1.60 for the mouse protein (Figure 1B) and less resolved $g = 1.93$, 1.75, and 1.63 for the HSV1 protein R2 (Figure 1D). In the *E. coli* R2 protein (Figure 1A)

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Table 1. Effect of Incubation Time and Reducing Agents on the Formation of the Mixed-Valent State of RNR R2 Proteins[a]

	PMS/dithionite		hydrazine (5 mM)	
samples	2 min	25 min	2 min	90 min
E. coli	0%	0%	0%	0%
mouse	17%	39%	0%	9%
HSV1	11%	10%	0%	13%

[a] Amounts of spins ($S = {}^1/_2$) are estimated relative to the total protein concentration by double integration versus 1 mM Cu^{2+}. Experimental conditions[15] were as in Figure 1.

there is no detectable $g < 2$ signal under the same conditions. Identical $g < 2$ signals were detected when R2 proteins from mouse (Figure 1C) and HSV1 (Figure 1B) were instead reduced by incubation with 5 mM hydrazine at pH 7.5, while no signal was observed with *E. coli* R2 (data not shown). The observed g values from R2 resemble most the acid forms of mammalian purple acid phosphates[8a-d] with $g = 1.93$, 1.75, and 1.59.

In our search for optimal conditions to produce the mixed-valent state we found that the presence of glycerol (20%) and a low incubation temperature were generally favorable for a high yield. Table 1 shows the effect of incubation time and method of reduction on the intensity of the mixed-valent EPR signal of the R2 proteins. With 2 mM PMS as mediator and four electrons per protein R2 from dithionite as reductant at 0 °C, the signal reached maximum intensity after 20–25 min of incubation for R2 protein from mouse (39%) and 2 min from HSV1 (11%). When the incubation was performed at room temperature, in the presence or absence of glycerol, the same mixed-valent signals were detectable, but with lower intensity. With 0.5–2 mM ascorbate as reductant and/or 0.1 mM PMS as mediator, the mixed-valent states were formed, but again with lower yields. Introduction of air into the EPR tubes with mixed-valent protein R2 resulted in instantaneous oxidation of the PMS, no significant increase at $g = 4.3$, no formation of tyrosyl radical, and only small loss of the $g < 2$ signal.

The mixed-valent EPR signals are difficult to saturate with microwave power. At 3.6–3.7 K the $P_{1/2}$ of the $g = 1.73$ and $g = 1.75$ signals of the mixed-valent R2 proteins are 14 mW (mouse) and 25 mW (HSV1). For both proteins, $\ln P_{1/2}$ was found to be a linear function of the inverse absolute temperature (3.6–11 K, data not shown). The temperature of the EPR samples was obtained from the Curie dependence of a 1 mM copper perchlorate solution under nonsaturating microwave power conditions. From the slopes of the lines ($\ln P_{1/2}$ vs $1/T$) we could estimate[16a] the coupling constant J for the Heisenberg exchange ($H = JS_1S_2$) to be $J = -15 \pm 2$ cm^{-1} for mouse R2 and $J = -11 \pm 2$ cm^{-1} for HSV1 R2. Similar small J values have been determined for mixed-valent hemerythrin,[12b] MMOH,[16] and purple acid phosphates.[8a,b] Recent ENDOR[17] and ESEEM[17b] studies of both hemerythrin and MMOH mixed-valent clusters show a strongly hyperfine coupled proton from a hydroxy bridge. Thus, the small J values of the two R2 proteins fall in a range typical for demonstrated hydroxy bridges while an oxy bridge should show stronger magnetic coupling.[5,11] We conclude that, as in hemerythrin,[17] the oxy bridge in the diferric R2 proteins from mouse and HSV1 most likely converts into a hydroxy bridge upon reduction to the mixed-valent form.

In the previous studies of the mixed-valent forms in *E. coli* protein R2, reduction by hydrazine at basic pH resulted in an $S = {}^1/_2$ species.[14c,d] In agreement with the present study this state could not be formed in *E. coli* R2 by hydrazine at pH 7.6. Preliminary experiments show that, for the mixed-valent R2 produced by the methods presented here, the EPR spectrum is not affected by the presence of glycerol, azide (0.5 M), or hydrazine (10 mM). In contrast, azide binding is known to change the EPR spectrum of mixed-valent hemerythrin.[12] In MMOH relatively large hydrocarbons, which bind to[7c] or can approach[13c] the iron cluster, readily change the mixed-valent EPR signal.[13c,16b] By low-temperature X-irradiation of *E. coli* protein R2 a ferromagnetically coupled $S = {}^9/_2$ system was observed,[14a,b] which however was not stable in solution.[14a,b,18]

Our data show that the redox properties of protein R2 from mouse and HSV1 are significantly different from those of protein R2 from *E. coli*. In *E. coli* R2, the redox potential for a direct two-electron transfer resulting in the diferrous state has been determined at pH 7.6 and 4 °C to be −115 mV.[19] This agrees with the fact that we cannot observe any reduction with PMS (E_m of +141 mV[20a] at 4 °C and pH 7.0) of the *E. coli* R2. In fact, R2 protein from mouse[21] and HSV1 might be considered related to MMOH in terms of the redox potentials of the iron clusters.[20] The MMOH studied by Lipscomb *et al.*[20a] has $E_{m1} = +76$ mV and $E_{m2} = +21$ mV at 4 °C and pH 7.0, for the first and second electron transfers, respectively. Reduction with PMS alone of this MMOH yields about equal amounts of all three redox states,[16b] which might be the case also for mouse R2 protein.[21] Furthermore, it seems that the iron center is much less reactive and more tightly bound to the R2 protein in *E. coli* than in HSV1/2 and mouse.[22]

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(15) R2 protein[3b,4a] samples (0.2 mM in total volume of 200 μL in 0.1 M Tris-Cl pH 7.5 containing 20% of glycerol) were incubated in EPR tubes with sodium dithionite at 0 °C. The samples were made anaerobic by repeated cycles of evacuation and flushing with O_2-free argon gas. Phenazine methosulfate (2 mM) was added to the EPR tubes, and the samples were incubated for 2 min at 0 °C, then frozen in liquid nitrogen for EPR measurement, and thawed for further incubations. All solutions were prepared in anearobic buffered solutions. Sodium dithionite was quantified before use by titration with potassium ferricyanide solution. Anaerobic incubation with 5 mM hydrazine, made from neutralized hydrazine dihydrochloride, was made as above but at room temperature.

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