High Field EPR Studies of Mouse Ribonucleotide Reductase Indicate Hydrogen Bonding of the Tyrosyl Radical

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Ribonucleotide reductase catalyzes by free radical chemistry the reduction of ribonucleotides to deoxyribonucleotides. The R2 protein of a class I ribonucleotide reductase contains a stable tyrosyl radical of neutral phenoxy character, which is necessary for normal enzymatic activity. Here we present the EPR spectra from the tyrosyl free radical in the R2 protein from mouse at 9.62, 115, and 245 GHz. We show that the g-value anisotropy of the mouse R2 radical, when precisely determined from high field EPR spectra, is similar to that of the hydrogen bonded dark stable tyrosyl radical of photosystem II and different from that of the E. coli R2 radical. Because the g-value anisotropy is an important indicator of the hydrogen bonding status of the tyrosyl radical, this result suggests that the mouse R2 radical has its tyrosylate oxygen hydrogen bonded with a D2O exchangeable proton, whereas this hydrogen bond is absent in the E. coli enzyme. It is suggested that the observed proton may be derived from the tyrosine that will become a tyrosyl radical.

Free radicals on tyrosyl residues have been found in the enzyme ribonucleotide reductase (RNR) from several different sources, as well as in photosystem II (PSII) and prostaglandin H synthase (1). The highly regulated RNR catalyzes the reduction of ribonucleotides to deoxyribonucleotides, which are precursors in the synthesis of DNA in all living organisms (2–5).

Several classes of RNR have been described. Class I enzymes contain a stable tyrosyl radical of neutral phenoxy type and a diferric iron-oxygen center in the small subunit protein R2 (2–5). The catalytically active form of class I RNR consists of a 1:1 complex of the two subunits, proteins R1 and R2, each of which is a homodimer. The crystal structures of Escherichia coli proteins R1 and R2 have been determined (6–8). The tyrosyl radical, which is necessary for normal enzymatic activity, is found about 5.2 Å from one iron of the iron-oxygen cluster in the E. coli enzyme (6). A weak magnetic coupling exists between the tyrosyl radical and the iron-oxygen cluster (5, 9–12). Model building studies of the R1-R2 complex suggest that the radical/iron clusters in the E. coli RNR-R2 are about 35 Å from the substrate binding active site in protein R1 (8). Class I RNR can be further divided into categories a and b based on sequence homologies (13). Although eukaryotes belong to class Ia along with the E. coli RNR, a number of prokaryotes belong to class Ib. The X-band EPR spectra of the tyrosyl radical of class Ia proteins are all similar to that of the E. coli RNR-R2. In a class Ib enzyme on the other hand, the EPR spectrum of the RNR tyrosyl radical of the Salmonella typhimurium R2F protein is strikingly similar to that observed for the YD0 tyrosyl radical of PSII (12, 13). This difference between several class Ia enzymes and the class Ib enzyme is due to a different dihedral angle arrangement for the β-protons relative to the ring of the tyrosyl radical in the two groups, whereas the spin density distribution of the radical remains similar in both cases (14, 16).

Here we present the EPR spectra from the tyrosyl free radical in RNR from mouse (17) at 9.62, 115, and 245 GHz and compare it with the E. coli tyrosyl radical, which has been well characterized by electron nuclear double resonance (ENDOR) and electron spin echo envelope modulation (ESEEM) (14, 18). Here we show for the first time that the g-value anisotropy of the mouse RNR-R2 radical, when precisely determined from high field EPR spectra (19–21), is similar to that of the YD0 tyrosyl radical of PSII (21) and different from the E. coli RNR-R2 radical (22). Because the g-value anisotropy seems to be an important indicator of the hydrogen bonding status of the (tyrosyl) radicals (22–24), this indicates that the mouse RNR-R2 radical has its tyrosylate oxygen hydrogen bonded, whereas this hydrogen bond is absent in the E. coli enzyme.

The fate of the phenol hydrogen on the tyrosine, which is lost when the tyrosyl radical is formed in RNR-R2 (5, 25), may be of importance for understanding the long range electron (possibly accompanied by a proton) transfer (4–8, 25) that is postulated to occur in the enzymatic reaction.

EXPERIMENTAL PROCEDURES

Cloned mouse RNR-R2 (17, 26) was produced in E. coli and prepared essentially as described before (26). To ensure efficient removal of iron in the apoprotein before reconstitution, the protein was pretreated with 5 mM hydroxyurea and 5 mM EDTA and gel filtrated before reconstitution as described in (27). Active protein was produced from iron-free apoprotein by reconstitution in 50 mM HEPES, pH 7.5, 100 mM KCl with ferrous iron and oxygen as previously described, using 6 FeII per RNR-R2. Exchange of apoprotein and reconstitution in D2O was done using deuterated buffer corrected for deuterium effects and with centrifuge columns of Sephadex G-25, a procedure developed by Penefsky.

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and described before (12, 27). After reconstitution in H₂O or D₂O buffer, the samples were exchanged into 20% glycerol, 50 mM HEPES, pH 7.5, 100 mM KCl in H₂O or D₂O by the Sephadex G-25 centrifuge columns, thereby removing excess iron. The iron and radical content of the samples were as previously reported judged from light absorption spectra and EPR characteristics (26). The *Salmonella* RNR-R2F sample was as before (16). The radical content was at least one radical per reconstituted RNR-R2 protein. EPR spectroscopy at X-band, 9.62 GHz, and 245 GHz (using the 1223 67-mu laser line of methanol gas as the frequency source for the EPR instrument) was done as before (16, 20) using at maximum 3-G modulation amplitude. The 115-GHz spectrum was obtained with the same basic instrument set up (20) as at 245 GHz but using a Lithuanian (Elmika company) carcinotron as a source of frequencies for microwaves for the EPR. Simulations of the X-band EPR spectra were made as before (16) using iteration minimization routines to fit the spectra (28).

**RESULTS**

**Low Temperature RNR Radical Spectra at Three Different Microwave Frequencies**—The active mouse RNR-R2 tyrosyl free radical has been studied in the temperature range 4–43 K at EPR microwave frequencies from 245 to 9.6 GHz as was previously done with the tyrosyl radical in the *S. typhimurium* R2 protein (class Ib RNR) (16). In Fig. 1 is shown the EPR spectra of a mouse RNR-R2 sample reconstituted in D₂O (see below) at different frequencies 9.62 (at 15 K), 115 (at 43 K), and 245 GHz (at 4 K) and as a reference (Fig. 1, *dotted line*) the *S. typhimurium* protein R2F (a class 1b enzyme) spectrum at 245 GHz (at 4 K). In agreement with previous analyses and simulations of the mouse R2 spectrum (10) at X-band, the spectrum is dominated by the 20-G hyperfine splitting from one of the β-protons and with smaller contributions from the four protons on the aromatic ring and the second β-proton. These features are similar to the hyperfine parameters found for other tyrosyl radicals of class Ia RNR enzymes. The small differences in X-band EPR spectra for different class Ia RNR tyrosyl radicals are mainly attributed to small changes in the dihedral angles _θ_H defined by the locations of the β-methylene protons, β-methylene carbon, ring carbon C1, and its _p_₉ axis. As already pointed out, when comparing class Ia and Ib RNR radicals, only minor differences can be observed in spin densities of different tyrosyl radicals (14, 16),^2^ and this should also be true when comparing radicals within class Ia. We obtained a good simulation of the X-band spectrum (Fig. 1, *top panel*) using the following parameters: _g_ = 2.0076, 2.0043, and 2.0022 for the components of the _g_-tensor; and hyperfine coupling constants (in G) _A_β₁ = 21.4, 19.0, and 21.45; _A_β₂ = 9.5, 2.5, and 5.7; _A_₅H₁ implies _θ_H to be a few degrees smaller than for the _E. coli_ tyrosyl radical.

At 245 GHz the _g_-value anisotropy of the mouse RNR-R2 radical signal dominates the EPR spectrum (Fig. 1, *lowest panel, solid line*) as previously observed for the *S. typhimurium* RNR-R2F (16) and the _E. coli_ RNR-R2 (24) radicals as well as of the dark stable _Y_H in PSII (21, 24). The spectrum at 245 GHz shows a rhombic Zeeman powder pattern. In addition, we can observe partially resolved hyperfine couplings (around 20 G) at all three _g_-values from the same β-proton, which dominates the spectrum at 9.6 GHz. Possibly the report that the _E. coli_ RNR-R2 spectrum at 245 GHz and 4 K in water did not show a resolved hyperfine coupling from the β-proton (24) could be explained by the fact that a 15-G modulation amplitude was used in that study, whereas we used a maximum 3-G modulation amplitude here for the mouse RNR-R2. The isotropic _g_-value was determined at X-band with a carefully calibrated field and frequency (16). The result was used to calibrate the _g_-value scale at the high field measurements. From this calibration we could determine the mouse RNR-R2 _g_-values to be 2.0076, 2.0043, and 2.0022 with _g_-iso = 2.0047. The _g_-value is significant lower than the corresponding _E. coli_ and _S. typhimurium_ values of about 2.0090. The _g_-value close to that expected for a free radical, indicating good accuracy in the _g_-iso.
The spectrum at 115 GHz is dominated by the hyperfine splitting of the $\beta$-proton. The g-value anisotropy is not clearly resolved at this frequency. In comparison, the E. coli radical at 145 GHz had the g-value anisotropy as well as the hyperfine splitting somewhat better resolved (22, 31). Table I shows the determined $g_1$-values of the different tyrosyl radicals. In principle it is only this g-value component that varies in the different tyrosyl radicals studied to date (22, 24). Quite unexpectedly we observe that the mouse RNR-R2 radical has its $g_1$-value significantly lower than the E. coli or S. typhimurium radicals, and it is in fact similar to what is observed for the dark stable YD$^-$ in PSII (21, 24).

Reconstitution of Mouse RNR-R2 in D$_2$O Increases Resolution of Hyperfine Couplings of the Tyrosyl Radical—Previous studies have shown that performing the reconstitution reaction in D$_2$O instead of H$_2$O gave rise to a somewhat different line shape of the mixed valent iron EPR signal of RNR-R2 from mouse (27) and also affected the results in our saturation recovery EPR experiments on the tyrosyl radical of mouse R2 (12). The microwave power saturation $P_s$ value at 25 K of mouse RNR-R2 tyrosyl radical is a factor of two lower in a sample reconstituted in and kept in D$_2$O (12). We have now studied the direct effects of reconstitution in D$_2$O on the EPR spectrum of the native tyrosyl radical of mouse RNR-R2 at 245 GHz and 4 K (Fig. 2). At 245 GHz the hyperfine coupling of one of the $\beta$-protons of the radical is easily observed in a D$_2$O reconstituted sample (Fig. 2A), whereas it is not well resolved after reconstitution in H$_2$O (Fig. 2C). If a sample is first reconstituted in D$_2$O and the solvent is afterwards changed to H$_2$O, a spectrum nearly identical to that of the H$_2$O reconstituted sample is observed (data not shown). The corresponding results were obtained for a sample reconstituted in H$_2$O and then exchanged into D$_2$O, which gives a rather weakly resolved EPR spectrum (Fig. 2B). The sample in D$_2$O demonstrates that at least one proton is weakly magnetically coupled to the tyrosyl radical, and it can be exchanged with deuterons from D$_2$O. The smaller line width in the D$_2$O sample arises because a deuteron has a 6.5 times lower gyromagnetic ratio than a proton (and $I = 1$). Clearly both spectra in Fig. 2 (B and C) have less resolved hyperfine couplings compared with Fig. 2A. All three principal g-values are affected as well as the general line width. Similar deuterium-dependent solvent effects have been reported for semihydroquinone radicals at 95 GHz (23). In that case the effect was attributed to the presence of a hydrogen bond with a solvent exchangeable proton(s).

**DISCUSSION**

The tyrosylate radical oxygen is probably hydrogen bonded in mouse RNR-R2. In recent studies Un and co-workers suggest that measurements of the g-value anisotropy and especially the $g_1$-value could be used as a probe for the presence or the absence of a hydrogen bond to the phenol oxygen of tyrosyl radicals (22, 24). Table I summarizes the $g_1$-values reported from high field EPR studies of several tyrosine radicals. For the E. coli RNR-R2 radical ENDOR studies (14, 18) have shown the absence of a hydrogen bonded proton consistent with a large value of $g_1$ around 2.0090. For the dark stable YD$^-$ in the PSII radical, ENDOR and ESEEM have demonstrated the presence of an exchangeable hydrogen bonded proton consistent with a small value of $g_1$ around 2.0075 (29, 30). Together with the simulations by Un et al. (24), the present results (Table I) with a $g_1$ of 2.0076 for the neutral mouse RNR-R2 tyrosyl radical suggest that it has a hydrogen bond to the phenolate oxygen. The observation may be related to the results in Fig. 2, which indicates a D$_2$O exchangeable proton weakly coupled to the tyrosyl radical.$^3$ The chemical nature of the positive charge interacting with the tyrosyl oxygen inducing the low $g_1$-value needs to be firmly established with, for example, deuterium, $^{57}$Fe, $^{17}$O, and $^{15}$N exchange experiments using ENDOR or ESEEM measurements, but most likely it is an exchangeable proton. Interestingly, hydrogen bonding differences in tyrosyl radicals have also been observed in the two PSII tyrosyl radicals (15).

We cannot at this point fully exclude other factors that also may influence the g-value anisotropy of the tyrosyl radicals in RNR-R2s as differences in interactions with the iron-oxygen center (5, 12, 25) or (other) charged centers. For instance, the saturation recovery EPR experiments (12) showed differences, reflected in the ratios ($k_{\text{exchange}}/k_{\text{dipole}}$)$^{1/2}$ between the exchange and dipolar coupling constants of the R2 tyrosyl radical and its neighboring dfferent iron-oxygen cluster between mouse on the one hand and E. coli and S. typhimurium on the other hand. Such interactions might possibly also influence the g-value anisotropy. The definitive demonstration of the presence of a hydrogen bonded proton must await studies with ESEEM, ENDOR, or related techniques, which are in progress.

A working model for the source of the postulated hydrogen bonded proton is that it may be the proton that is originally bound to the tyrosine-oxygen that will be lost in the formation of the deprotonated and neutral tyrosyl radical (5, 25). A possible acceptor for this tyrosyl-derived proton is the iron-ligand Asp (Asp$_{54}$ in E. coli numbering). This residue has its closest carboxylate oxygen 3.5 Å from the tyrosine-oxygen in the crys-

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$^3$ Interestingly, the resonance Raman at 406.7 nm of the tyrosyl free radical of mouse RNR-R2 shows a major vibration around 1513 cm$^{-1}$, which is more than 10 cm$^{-1}$ higher in energy than in the E. coli radical (unpublished results in collaboration with M. A. Hanson and E. I. Solomon). This may be related to the different chemical environments of the two RNR-R2 tyrosyl radicals.
tal structure of E. coli met-RNR-R2 where the radical is absent. The electron and proton transfer needed for generation of the active tyrosyl radical might be in the form of an electron + proton unit and not as previously believed as a single electron.

It has been believed that all tyrosyl radicals in RNR class 1 would have similar properties to the E. coli enzyme. Clearly this has to be revised because differences both in dihedral angles (in class Ib) (16) and in the presence of a hydrogen bond to the tyrosyl radical (in the mouse case) can be observed.

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