Characterization of a New Tyrosyl Free Radical in *Salmonella typhimurium* Ribonucleotide Reductase with EPR at 9.45 and 245 GHz

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Free radicals on tyrosine residues have been found in ribonucleotide reductase (RNR) from several different sources, as well as in photosystem II (PS II).[1] Here we present EPR spectra from a new type of tyrosyl free radical in RNR from *Salmonella typhimurium* at 9.45 and 245 GHz. Its X-band EPR spectrum is similar to that of the PS II tyrosyl radicals, but its *g*-anisotropy, when precisely determined from high-field EPR spectra, is similar that of the *Escherichia coli* RNR radical.

Several classes of RNR have been described.[2−4] The *S. typhimurium* bacteria contain an active class I RNR. In addition, two normally not expressed chromosomal genes code for a second class I RNR with proteins R1E and R2F.[5] Like usual R2 proteins, the R2F protein has a diferric iron center and a tyrosyl free radical.[2,3,5,6] The X-band EPR spectrum of the tyrosyl radical of protein R2F is strikingly similar to that observed for the Y_D tyrosyl radicals of PS II.[5−7]

The spin density in tyrosyl radicals follows an odd-alternate pattern, with large spin density at carbon C1, C3, and C5 (Figure 1B inset), as well as on the hydroxyl oxygen.[7−9] The differences in EPR spectra for different tyrosyl radicals are mainly attributed to changes in the dihedral angle, θ_H, defined by the locations of the β-methylene proton, the β-methylene carbon, the ring carbon C1, and its p_z axis (Figure 1B inset).[7−10]

It is necessary to use, for example, ENDOR or ESEEM spectroscopy in order to measure the hyperfine couplings directly.[7−9] It is also possible to evaluate those parameters from computer simulations of ordinary EPR spectra. Here we have measured *g*-values with high precision and have used this information in simulations to estimate indirectly the hyperfine coupling parameters.

The 245 GHz EPR[11,12] spectrum of a frozen solution of the R2F protein of RNR class Ib from *S. typhimurium* is shown in Figure 1A. The anisotropic *g*-tensor will dominate the spectrum at this high field of 8.7 T. The spectrum shows a rhombic Zeeman powder pattern without resolved hyperfine couplings. This spectrum was used to determine the anisotropy of the *g*-tensor. The isotropic part of the *g*-tensor, $g_{iso} = 2.005\ 17 \pm 0.000\ 07$, was determined at 9.45 GHz because of careful calibration of the magnetic field at X-band.[13] The main components of the *g*-tensor were determined with the help of a computer-simulated spectrum[14] (see Figure 1B). The result was $g_{xx} = 2.0090$, $g_{yy} = 2.0044$, and $g_{zz} = 2.0022$, with the *x*-axis parallel to the carbon−oxygen bond and the *z*-axis perpendicular to the ring plane. An isotropic line width of 21.4 G was used, together with the hyperfine coupling tensors presented in Table 1.

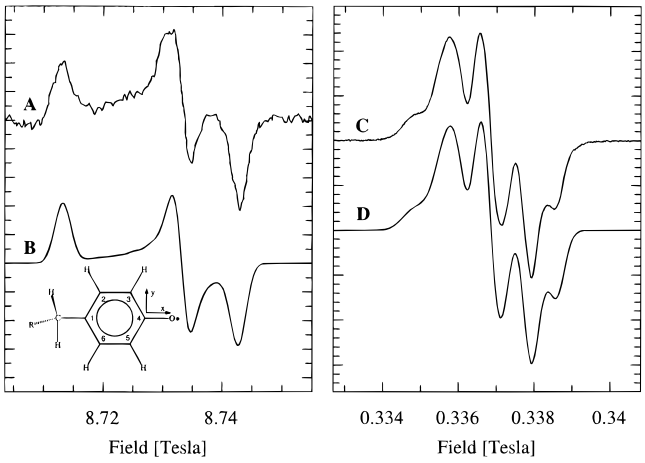

Figure 1. EPR spectra of the tyrosyl radical present in protein R2F of ribonucleotide reductase from *S. typhimurium* at 15 K. Inset: Numbering of carbon atoms in tyrosine and coordinate system for the *g*-tensor. The sample was 250 μL of 75 μM protein R2F prepared as previously reported.[5] (A) Experimental spectra acquired at 244.997 GHz[11,12] (8.7 T). Experimental parameters: Modulation amplitude 15 G, modulation frequency 10 kHz. (B) High-field simulation using the parameters listed in Table 1 and an isotropic line width of 21.4 G. (C) Experimental spectrum acquired at 9.454 GHz (X-band). Experimental parameters: Microwave power 0.63 mW, modulation amplitude 2.6 G. (D) X-band simulation using the parameters listed in Table 1 and an anisotropic line width of $LW_x = 5.8$ G, $LW_y = 5.3$ G, and $LW_z = 3.7$ G.

These values of the *g*-tensor components were used in the fitting of a simulated spectrum to the 9.45 GHz spectrum shown in Figure 1C. The hyperfine coupling tensors have the principal *y*-axis approximately parallel to the carbon−proton vector for H3 and H5 and the carbon−carbon vector for the β-proton. The principal *z*-axis is perpendicular to the ring plane. The *S. typhimurium* radical EPR spectrum was successfully simulated with hyperfine coupling tensors to the H3 and H5 protons and an almost isotropic hyperfine coupling to one β-proton, here called β1. Starting EPR parameters were obtained from the Y_D radical in PS II.[7] We have used an anisotropic line width tensor to account for unresolved hyperfine couplings to H2, H6, and the second β-proton. The simulated spectrum is shown in Figure 1D, and the corresponding parameters are given in Table 1.[15]

From the isotropic part of the hyperfine coupling tensors for H3 and H5, the spin density at C3 and C5 was calculated to be 0.28.[17] The possible range of spin density on the C1 carbon was calculated from the hyperfine couplings of the β-protons

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Table 1. EPR Parameter Values for Tyrosyl Radicals in *E. coli* R2 Protein, Photosystem II, and *S. typhimurium* R2F Protein[a]

	E. coli RNR	PS II	*S. typhimurium* RNR[b]
g_x	2.009 12[22]	2.007 45[23]	2.0089(7)
g_y	2.004 57[22]	2.004 22[23]	2.0043(7)
g_z	2.002 25[22]	2.002 12[23]	2.0021(7)
$A_{3,5x'}$[b]	−9.6[7]	−9.1[9]	−11.5
$A_{3,5y'}$[b]	−2.8[7]	−2.6[9]	−2.5
$A_{3,5z'}$[b]	−7.0[7]	−7.0[9]	−7.1
$\Phi_{3,5}$	±27[7]	±22[7]	±23
$A_{\beta 1x'}$[b]	19.6[7]	7.2[9]	10.5
$A_{\beta 1y'}$[b]	21.2[7]	10.5[9]	7.4
$A_{\beta 1z'}$[b]	19.6[7]	7.2[9]	9.5
$A_{\beta 2\|\|}$[b]	1.75[7]	5.1[9]	<3.0
$A_{\beta 2\perp}$[b]	−0.7[7]	1.9[9]	<3.0
$\rho_{C3,5}$	0.25[21]	0.24[9]	0.28
ρ_{C1}	0.38[21]	0.37[9]	0.16−0.40
θ_{H1}	33°[8]	52°[9]	−5°−51°
ρ_O^{π}	0.29[21]	0.26[9]	0.49−0.25[c]

[a] The components of the *g*-tensor and anisotropic hyperfine couplings are shown. Selected spin densities, ρ, and the methylene proton dihedral angle, θ_{H1}, derived from the EPR parameters are shown. [b] Estimated uncertainties for the present measurements are 0.000 07 for *g*-values and 1.0 G for hyperfine couplings. [c] In gauss.

to be $0.16 \leq \rho_{C1} \leq 0.40$, and the corresponding dihedral angle was $-5° \leq \theta_{H1} \leq 51°$.[18] The spin density on the oxygen was calculated to be $0.25 \leq \rho_O^{\pi} \leq 0.49$ for the *S. typhimurium* radical.[20]

Table 1 summarizes the estimated EPR and molecular parameters for the *S. typhimurium* RNR class Ib radical. The table also includes the corresponding results for the *E. coli* RNR[8,21,22] and PS II[9,23] tyrosyl radicals, including a recent reevaluation of the *E. coli* RNR spin densities.[21] The literature data are partly derived also from ENDOR, ^{2}H ESEEM, and high-field EPR studies. It is clear that the spin density distribution is almost invariant among the tyrosyl radicals,[21] and different β-methylene dihedral angles due to varying molecular conformations determine the overall EPR spectral shapes.

The present results (Table 1) show a tyrosyl radical in *S. typhimurium* with a *g*-anisotropy similar to that of the *E. coli* RNR radical[22] but with β-methylene dihedral angles similar to those of the PS II radical.[9] In previous studies of tyrosyl radicals in PS II and RNR, possible correlations between spin density distributions, *g*-value anisotropy, and phenol oxygen hydrogen bonding were discussed.[22] Theoretical[24] as well as experimental studies of model compounds suggest that (a) there is a proportionality between ρ_O^{π} and $g_x - g_e$,[26,28] and between ρ_O^{π} and g_{iso},[29,30] and (b) the presence of a hydrogen bond to the oxygen substituent reduces ρ_O^{π} and, consequently, the *g*-anisotropy.[26,31]

The present result may be interpreted as follows. First, the molecular conformations, including the β-methylene dihedral angles, for the tyrosyl radicals in *S. typhimurium* RNR and PS II are similar to those of some neutral model tyrosine radicals.[32] Therefore, these radicals may represent a "relaxed", normal state. The *E. coli* RNR tyrosyl radical has a perturbed conformation, most likely due to the local charge and steric environment. Second, an H bond to the phenolic oxygen of the tyrosyl radical is present in the dark-stable Y_D radical of PS II,[9] but not in *E. coli* RNR.[8] The relation between *g*-anisotropy, ρ_O^{π}, and H bonding may be understood within the Stone theory.[25] The balance between ρ_O^{π} and ρ_C^{π} within the C4−O fragment is shifted toward smaller ρ_O^{π} by an H bond. The shift may be on the order of 10%[21] and is generally too small to be directly resolved from hyperfine parameters without ^{17}O labeling. On the other hand, its effect on the *g*-anisotropy, when precisely determined at high magnetic fields, should be a reliable indicator of H bonding. The almost coinciding *g*-anisotropy for the *E. coli* and *S. typhimurium* RNR radicals indicates similar values of ρ_O^{π} and, consequently, a similarity in the electronic interactions, e.g., in both cases the absence of an H bond and a similar exchange interaction[6] with the iron center. The absent H bond and the probably common fate of the lost phenolic proton in the radical state of the two RNR tyrosyl radicals from different species are consistent with a possible functional significance of this proton for the enzyme.[33]

Orientation-dependent T1 relaxation has been observed for the PS II radical at high field through partial power saturation of the spectra.[23] No such effect was observed for the radical in the present study.

Several other tyrosyl radicals in various species of RNR all have hyperfine coupling patterns similar to the one in *E. coli*, reflecting a similar strained orientation of the locked tyrosyl ring. The new R2F radical represents another family of geometry of protein-bound tyrosyl radicals, similar to that in PS II. The more common class I RNR radicals, with the different type of conformation, may represent a fine tuning of the geometry and accompanying redox activity of the tyrosyl radicals.

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(15) The small anisotropy of the $\beta 1$ hyperfine coupling has an unexpected symmetry in that $A_{\beta 1y'}$ is the smallest component, in contrast to what is expected.[16] This particular result of the iterative fitting procedure may be affected by the large number of fitted hyperfine and line width parameters and is not necessarily significant. The line width was determined as follows: $LW_x = 5.8$ G, $LW_y = 5.3$ G and $LW_z = 3.7$ G.

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(18) The following equation was used: $A_{iso} \approx B_1\rho \cos^2 \theta_H$, where the dihedral angle θ_H was defined previously. B_1 is the proportionality constant determined to be 58 G.[19] The isotropic hyperfine coupling to the $\beta 1$-proton is known directly from the simulation as the average over the three main components ($A_{iso\beta 1} = 9.10$ G). For the second β-proton, we assumed that it is less than half of the measured line width, $0.0 \text{ G} < A_{iso\beta 2} < 3.0$ G, which gives the range of allowed values of ρ_{C1}.

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