High-Frequency (139.5 GHz) EPR Spectroscopy of the Tyrosyl Radical in *Escherichia coli* Ribonucleotide Reductase

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The importance of amino acid-based radicals in the catalytic transformations of a number of enzymes has become widely recognized.[1] The development of new spectroscopic techniques to identify and characterize protein free radicals is critical in order to advance the understanding of their structures and functions. Considerable structural information has been deduced from electron–nuclear hyperfine couplings elicited through EPR and related techniques at conventional frequencies (≤40 GHz).[2-10] High-frequency (≥95 GHz) EPR spectroscopy has recently been shown to facilitate the resolution of the *g*-anisotropy of free radicals;[11-14] nuclear hyperfine structure has been observed in the high-frequency EPR spectra of nitroxides.[15-18] In this communication we report the 139.5-GHz EPR study of the stable tyrosyl radical (Y_{122}) in *Escherichia coli* ribonucleoside diphosphate reductase (RDPR).[19-23] It is the first high-frequency EPR spectrum of an endogenous protein radical that exhibits both Zeeman and hyperfine structure and thus provides a detailed characterization of the radical center.

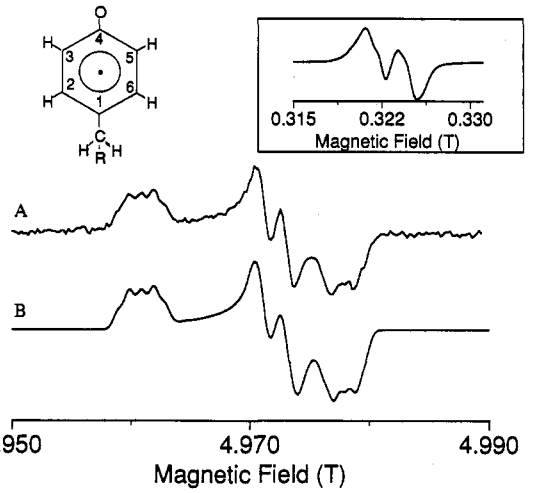

Figure 1. EPR spectra of the tyrosyl radical (Y_{122}) present in RDPR. (a) High-frequency (139.5 GHz) spectrum of 5 μL of a 200 μM solution of the B_2 subunit. The high-frequency spectrometer is described in ref 37. Instrument parameters are as follows: temperature, 90 K; microwave power, ~100 μW; modulation amplitude, 4 G; modulation frequency, 400 Hz; time constant, 1 s. (b) Simulation using the parameters listed in Table I. The inset is a 9-GHz EPR spectrum of ~0.3 mL of the same sample.

Table I. Tyrosyl Radical EPR Parameter Values[a]

g-Values

	g_{11}	g_{22}	g_{33}	g_{iso}[b]	ref
E. Coli RDPR (139.5 GHz)	2.009 12	2.004 57	2.002 25	2.005 31	this work
Y_D (PSII) (135 GHz)	2.007 52	2.004 26	2.002 12	2.004 63	14[c]
single crystal (9 GHz)	2.0067	2.0045	2.0023	2.0045	26

Hyperfine Coupling[d]

	A_{11}	A_{22}	A_{33}	A_{21}[e]	ref
β-methylene ^{1}H	20.7	19.8	18.7	0	this work
3,5-ring ^{1}H	8.6	4.7	7.9	3.4	this work

[a] Estimated uncertainties for this reference are 0.000 05 for *g*-values and 0.5 for *A*-values. [b] $g_{iso} = (g_{11}+g_{22}+g_{33})/3$. [c] These *g*-values agree well with those obtained in an EPR study of perdeuterated samples described in ref 29. [d] In gauss. [e] We assume that $A_{12} = A_{21}$ and that other off-diagonal elements equal 0, as indicated in the text.

The high-frequency (139.5 GHz) EPR spectrum of a 200 μM frozen solution of the B_2 subunit of RDPR is shown in Figure 1A; for comparison, the X-band EPR spectrum of the sample is shown in the inset. In contrast to the low-frequency spectrum, the structure of which is primarily governed by proton hyperfine interaction, the 139.5-GHz spectrum clearly exposes the Zeeman anisotropy; the principal *g*-values (Table I) are readily obtained from the canonical features of the rhombic Zeeman powder pattern.

Hyperfine structure is also apparent in the spectrum. A doublet structure is evident at each canonical position; this structure can be assigned, on the basis of prior studies,[3,24] to a β-methylene proton with a large isotropic interaction (~20 G) and small hyperfine anisotropy. Further structure is recognizable at the

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low-field edge of the spectrum, where an additional coupling to an equivalent pair of spin $^1/_2$ nuclei (assignable as the 3,5 ring protons) leads to a 1:2:2:2:1 quintet. Hyperfine structure from this pair of nuclei is less well evidenced at the high-field edge of the spectrum and is essentially absent at the central position,[25] reflecting the substantial anisotropy of this interaction. Guided by these observations and by comparisons with prior EPR and ENDOR measurements,[3,26,27] we constructed the simulation, presented in Figure 1B, employing the parameters shown in Table I. The simulation reproduces the experimental spectrum extremely well.

The hyperfine coupling values obtained in this study are in general agreement with the more precise values determined by ENDOR;[3] their implications regarding the electronic and molecular structure of the tyrosine have been extensively discussed. It is worth noting, however, that the quintet structure observed in the high-frequency EPR spectrum effectively rules out ortho-substitution of the RDPR tyrosyl radical. Although this lack of an ortho substituent has been established previously in the case of RDPR tyrosyl radical,[3,23,24] the application of high-frequency EPR may prove useful in studies of systems known or believed to have ortho-substitution, such as the tyrosyl radical generated in apogalactose oxidase.[7]

The resolution of hyperfine structure at the canonical positions in the high-frequency EPR spectrum provides a measure of the components of the hyperfine matrix within the molecule-based, g-axis frame. In our simulation of the high-field spectrum, we assumed a McConnell-type relation between the hyperfine principal axis orientations and ring structure[28] and tested various alternative settings of the g-axes along the local symmetry axes of the tyrosyl ring. In order to achieve agreement with the structure of the experimental spectrum, we found that the axis belonging to g_{11} must lay parallel to the C_4–O bond and that the principal axis belonging to g_{33} is normal to the ring. This disposition of the g-axes conforms precisely to that found in the tyrosyl radical single-crystal rotation study.[26] Conversely, the assumption of such g-axis orientations could similarly enable the determination of hyperfine axis orientations from high-frequency EPR spectra of protein tyrosyl radicals.

Included in Table I are g-values of other tyrosyl radicals accurately determined by either high-frequency[14] or single-crystal rotation[26] EPR studies. In each case, the values of g_{22} and g_{33} are nearly identical, but significant differences occur in the g_{11} values. This same behavior was also observed by Lebedev and co-workers[15] in the 140-GHz EPR spectra of a series of phenoxy radicals and was attributed to variations in spin density of the oxygen substituent (ρ_O). This is in agreement with Lee and Box,[30] who derived a direct proportionality relation between ρ_O and $g_{11}-g_e$ (g_e, the free-electron g-value). An analogous relation between ρ_O and g_{iso} has been documented for phenoxy radicals;[31-33] high-frequency EPR reveals that ρ_O in the RDPR tyrosyl radical mainly affects a single g-value (g_{11}).

The tabulated g-values suggest that the radical in RDPR has a ~30% greater oxygen spin density than the neutral tyrosyl radicals in PSII (Y_D) and in irradiated tyrosine crystals. This result is at variance with the analysis of proton hyperfine couplings by Hoganson and Babcock.[34] Theoretical and experimental studies of ^{17}O-labeled quinone radicals have shown that hydrogen bonding to the oxygen substituents reduces their ^{17}O spin density.[35] Accordingly, our conclusion is supported by ENDOR and ESEEM studies of Y_D and RDPR, which reveal the presence of a proton participating in a hydrogen bond with the oxygen of Y_D[4,34] but suggest the absence of such an interaction in RDPR.[3] The determination of g_{11} values by high-frequency EPR may prove to be an ideal probe of hydrogen-bonding interactions of tyrosyl radicals.

The resolution of hyperfine structure within the 139.5-GHz EPR spectrum of the RDPR tyrosyl radical has one further implication, which we note in closing. The powder pattern line-broadening function, which subsumes both homogeneous and inhomogeneous effects, clearly cannot involve a width greater than the minimal resolved hyperfine splitting. In the simulation presented in Figure 1B, the EPR spectrum is convolved with a 8.2-G fullwidth at half maximum Gaussian function. Since the Zeeman dispersion is 2 orders of magnitude larger than this line-broadening, a clear link between EPR line position and orientation within the g-axis frame is made, and the prospects for high-frequency orientation selective spectroscopy are excellent. Indeed, Burghaus *et al.*[36] have very recently demonstrated orientation-selective effects in the ENDOR spectrum of a quinone radical at 95 GHz.

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