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Published on Web 12/10/2002

Very High-Field EPR Study of Glycyl Radical Enzymes

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Since the discovery of the catalytically essential stable tyrosyl radical in ribonucleotide reductase (class I), a growing number of proteins have been identified as radical proteins.[1] Most of them are produced by a one-electron oxidation of protein side chains, mainly on tyrosine, tryptophan, and cysteine residues. There is one example of a radical located on the main chain of the protein, the dioxygen-sensitive glycyl radical. Presently, only three enzymes, all of them in anaerobically growing microorganisms, have been found to carry a glycyl radical: the anaerobic class III ribonucleotide reductase (RNR),[2] the pyruvate formate lyase (PFL),[3] and the benzylsuccinate synthase (BSS).[4] For the first two systems, the three-dimensional structures have revealed that the radical is at the active site and is expected to play an essential role during initiation of the catalysis. On the basis of the sequence homologies at the glycine site, it is very likely that a much larger number of enzymatic systems depend on a glycyl radical for activity.[5]

All of the reported glycyl radicals have common EPR properties. Their X-band EPR spectra are dominated by the isotropic hyperfine coupling interaction of the spin with the α-hydrogen atom of glycine, giving rise to a doublet at $g_{iso} = 2.0033-2.0037$.[2-4] Under these conditions, the *g*-anisotropy of the radical is very small and cannot be determined. Saturation studies done on the RNR or PFL glycyl radical demonstrated that the radical does not interact with another paramagnetic center. The BSS radical has been much less studied.

In this paper, we report the first complete analysis of the *g*-tensor of glycyl radical enzymes that we obtained by high-field EPR spectroscopy, thus providing new information on their EPR properties. High-field EPR (HF-EPR) is a unique technique for measuring small *g*-anisotropies of organic radicals with a good precision, allowing their identification. The *g*-anisotropy may also be indicative of the local environment of the radical. For example, as in the case of RNR (class I) and photosystem II, the hydrogen bonding state of a tyrosyl radical can be assessed by the accurate determination of its *g*-tensor.[6] Under high magnetic field conditions, the *g*-matrix may be solved if $\Delta g/g_{iso} > \Delta B^{hfi}_{1/2}/B_0$, with g_{iso} as the isotropic *g*-factor, $\Delta B^{hfi}_{1/2}$ as the hyperfine-broadened EPR line width, and B_0 as the externally applied magnetic field.

Figure 1 displays 9 GHz (A) and 285 GHz (B and C) EPR spectra of two different frozen preparations of the glycyl radical-containing form of RNR (class III). The 9 GHz spectrum is characterized by a resolved 2-fold splitting of about 1.4 mT as reported previously.[2a] At high frequency, the anisotropy of the *g*-tensor dominated the spectra, which display a rhombic Zeeman powder pattern without the resolved hyperfine coupling observed at lower frequency. The isotropic part of the *g*-tensor ($g_{iso} = 2.0033$)[2a] was used as a calibration for the simulation of the 285 GHz EPR spectra. The main components of the *g*-tensor were determined from the diagonalization of the full spin Hamiltonian matrix ($\mathbf{H} = g\beta\mathbf{B}\cdot\mathbf{S}$) (Table 1).[7] Both high-field EPR spectra (Figure 1B-1C) were simulated with the *g*-values, taking into account the error barriers.[8]

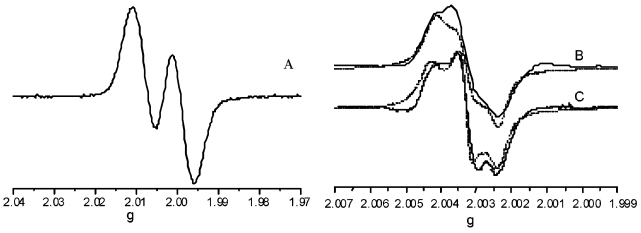

Figure 1. X-band (A) and high-field (285 GHz; $g = 2$ at 10.2 T) (B and C) EPR spectra of the RNR glycyl radical generated as described in 2a by incubation of RNR with *S*-adenosylmethionine, K^+, dithiothreitol, NADPH, flavodoxin, flavodoxin reductase (B), or with photochemically reduced deazaflavin substituting for NADPH and the flavodoxin system (C) (solid line, experimental measurement; dashed line, simulation). In the first case, the contribution of the FMN radical was subtracted. HF-EPR spectra were recorded at 20 K and under nonsaturating conditions. A field modulation of 0.8 mT and a modulation frequency of 1.2 kHz were used. The doublet line (A) corresponds to a peak-to-trough splitting of 1.4 mT. The total spectra width (B and C) is equal to about 10.0 mT.

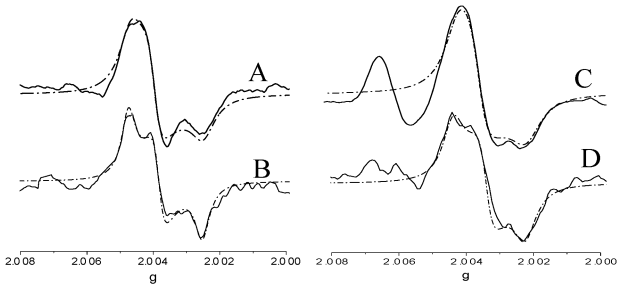

Figure 2. High-field EPR spectra of the PFL glycyl radical recorded at 285 GHz ($g = 2$ at 10.2 T) (A) and at 525 GHz ($g = 2$ at 18.8 T) (B) and of the BSS glycyl radical recorded at 285 GHz (C) and at 525 GHz (D) (solid line, experimental measurement; dashed line, simulation). A field modulation of 0.8 mT (A and B) or 1.0 mT (C and D) and a modulation frequency of 1.2 kHz (A and B) or 1.0 kHz (C and D) were used. All spectra were recorded at 20 K and under nonsaturating conditions.

The HF-EPR study of PFL was done on whole *E. coli* cells grown anaerobically,[9] whose X-band EPR spectrum only displays the PFL radical ($g_{iso} = 2.0037$).[3] At 285 GHz, the *g*-anisotropy of the signal was not completely resolved with only two components distinguishable (Figure 2A). However, complete resolution of the *g*-tensor into its three components was obtained at 18.8 T with the help of a resistive magnet providing up to 20 T (Figure 2B − Table 1). The frequency of 525 GHz was generated using a laser source.

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10.1021/ja026690j CCC: $25.00

Table 1. Comparison of *g*-Values and Isotropic Hyperfine Values of the RNR, PFL, and BSS Glycyl Radical with Those of the *N*-Acetyl Glycine Radical, Tyrosyl Radical, and Tryptophan Radical

	g_x	g_y	g_z	$\Delta(g_z-g_x)$	ref
RNR (Figure 1A)[a]	2.0042	2.0033	2.0023	0.0019	this work
RNR (Figure 1B)[a]	2.0043	2.0033	2.0023	0.0020	this work
PFL[a]	2.0047	2.0039	2.0025	0.0022	this work
BSS[a]	2.0045	2.0036	2.0022	0.0023	this work
N-acetyl glycine radical	2.0045	2.0031	2.0020	0.0025	11a
	2.0042	2.0032	2.0027	0.0015	11b
tyrosyl radical	2.0091−2.0076	2.0046−2.0044	2.0021	0.0070−0.0055	6
tryptophan radical	2.0035−2.0033	2.0024−2.0025	2.0023−2.0021	0.0018−0.0011	14

[a] The *g*-values are given with an error of $\pm 4 \times 10^{-4}$ and $\Delta(g_z-g_x)$ values with an error of $\pm 2 \times 10^{-4}$.

Cell free extracts from *Azoarcus* sp. strain T growing anaerobically on toluene were used for the HF-EPR studies of BSS.[4a] The isotropic *g*-value ($g_{iso} = 2.0034$)[4b] determined by X-band EPR is different from that of a previous study ($g_{iso} = 2.0021$).[4c] At 285 GHz, two signals were observed, one associated with the glycyl radical with two distinct components, the other characterized by an isotropic signal centered at $g = 2.0068$, still unidentified (Figure 2C). At higher frequency (Figure 2D), only the signal associated with the BSS glycyl radical was observed, and the three components of the *g*-tensor were completely resolved.

The *g*-anisotropy of the glycyl radical is small as expected for organic radicals, especially for carbon-centered radicals characterized by spin−orbit coupling values lower than those for nitrogen- or oxygen-centered radicals. It is similar in the three enzymes with a comparable total width of the spectrum ($\Delta(g_z-g_x) = 0.0019-0.0023$). Such a similarity suggests that there are related local environments and structures around the radical, in agreement with the fact that the glycyl radical site is located at the C-terminal part of each enzyme in a rather conserved sequence. Furthermore, as shown from the available three-dimensional structures of RNR and PFL, the glycine site is in both cases located at the tip of a loop directed toward the inside of a barrel structure, facing the tip of a second loop carrying an essential cysteine of the active site.[10] On the other hand, much higher field is required to separate the g_x and g_y components in the case of PFL and BSS, while the line widths are similar for each enzyme. Whether this reflects a small difference in the environment along the direction associated to g_y remains to be investigated.

The only previously available information on glycyl radicals has been established from EPR studies on irradiated *N*-acetylglycine crystals showing that the C_α, N, and O atoms in the molecule and the α-hydrogen atom are almost in the same plane.[11] The sp^3 C_α atom of *N*-acetylglycine is replaced by a nearly sp^2 radical center with the unpaired spin density mainly in a $2p\pi$ orbital perpendicular to the molecular plane on the C_α carbon (72−76%; $A_{iso} = 1.82-1.74$ mT). The remaining spin density is delocalized over the entire molecule. Two sets of slightly different spin Hamiltonian parameters can be found in the literature (Table 1). In both cases, the g_x and g_y components are coplanar or quasi-coplanar with the molecular plane, whereas g_z is perpendicular to this plane as expected for a π-system.

The *g*-anisotropy for the glycyl radical enzymes is comparable to that for irradiated crystals of *N*-acetylglycine, suggesting a planar conformation and similar magnetic axes for the protein radicals as well. This is also consistent with a quantum mechanical study of a glycyl radical engaged in a model dipeptide, showing that planar or quasi planar conformations for the glycyl radical are energetically favorable. This is consistent with the low nuclear hyperfine constant for H_α due to the π character of the electron that is delocalized along the peptide chain.[12]

To our knowledge, only one other study at such very high fields >18 T has been reported (above the limits of superconducting magnets).[13] Although examples are known in which the *g*-tensor resolution is then impaired by the line broadening,[13a] in our case, the increase of the field had no effect on the line widths of the transitions, implying that the *g*-strain is negligible and suggesting a remarkable structural homogeneity for the three glycyl radicals.

In summary, HF-EPR spectroscopy may allow a determination of the *g*-tensor anisotropy of protein radicals, including C-centered ones and detection of small but significant differences in *g*-anisotropy. This parameter and, in particular, the total width of the spectrum ($\Delta(g_z-g_x)$) may be used as a probe for distinguishing a tyrosyl-, a glycyl-, or a tryptophanyl-radical because, as shown in Table 1, the *g*-anisotropy is in the order tyrosyl → glycyl → tryptophanyl radical.

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JA026690J