Determination of the Electron Spin Density on the Phenolic Oxygen of the Tyrosyl Radical of Photosystem II

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We have determined, through ^{17}O-labeling of the tyrosyl radical, $Y_D^{\bullet}$, of Photosystem II, the ^{17}O hyperfine coupling constant. This measurement has allowed us to estimate the unpaired electron spin density on the phenolic oxygen of the radical and to show that the spin density is only very weakly dependent on hydrogen bonding. In so doing, we resolve one of the uncertainties in the electronic structure of the radical and provide new insights into radical behavior.

A number of enzymes that have been recently shown to contain redox-active tyrosines that participate in proton-coupled electron transfers that are integral to the catalytic function of the protein. Among the best characterized of these are Y122 of *Escherichia coli* ribonucleotide reductase (RNR)[1] and $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ of Photosystem II (PSII),[2,3] each of which is oxidized to the neutral radical. Their common structures notwithstanding, each of these radicals has a unique function. The tyrosyl radical in RNR initiates events that convert ribonucleotides to deoxyribonucleotides. Y_D may act to maintain proper metal valence in the oxygen-evolving complex during prolonged dark periods. Y_Z increasingly appears to be an integral participant with the Mn cluster in photosynthetic water oxidation, and a number of models have recently appeared that propose specific mechanisms for this participation.[4,5] Consistent with their differing functions, the reduction potentials of $Y_D^{\bullet}$ and $Y_Z^{\bullet}$ have been estimated to differ by as much as 250−300 mV,[6,7] indicating that the protein environment has major consequences for their redox behavior. Hydrogen bonds are one way in which the protein environment may alter the redox behavior of tyrosine, and hydrogen bonds have also been proposed to influence the distribution of unpaired electron spin density of tyrosyl radicals.[8,9] A comparison of the spin density on the tyrosyl radical in the presence and

absence of a hydrogen bond would allow an appreciation of its influence on the electronic structure of the radical. It would also allow a more accurate calculation of the length and therefore the strength of the hydrogen bonds to the tyrosyl radicals.

We and others have been examining hydrogen bonding and unpaired electron spin densities on the tyrosine radicals using ESEEM, ENDOR, and high-field EPR spectroscopies.[1,10−18] This work has shown that both of the PSII radicals are coordinated by hydrogen bonds,[10,11,15−18] but that of *E. coli* RNR is not.[1] The electron spin density distribution and the orientation of the methylene group have been determined for $Y_D^{\bullet}$ by Hoganson and Babcock,[8] Warncke et al.,[12] and Rigby et al.,[13] for $Y_Z^{\bullet}$ by Tommos et al.,[14] and for RNR by Bender et al.[1a] These studies showed an odd-alternant pattern of electronic spin density with the following assignments: C1 (0.37), C2,6 (−0.06), C3,5 (0.25), and O + C4 (0.25). The remaining uncertainty in these values lay with the spin density distribution between the phenolic oxygen and C4 to which it is bonded. Hoganson et al.[19] have recently used ^{17}O-labeling to determine the electronic spin density on the phenolic oxygen of the non-hydrogen-bonded tyrosyl radical of *E. coli* RNR. We determine here the spin density on the phenolic oxygen of the hydrogen-bonded $Y_D^{\bullet}$ and find that the oxygen spin densities in $Y_D^{\bullet}$ and RNR are very similar.

Figure 1 (parts a and b) shows the $Y_D^{\bullet}$ EPR spectra in [^{16}O]- and [^{17}O]tyrosine-labeled PSII core complexes, respectively.[20] Four sets of lines due to the $m_I = \pm^3/_2$, $\pm^5/_2$ nuclear spin states are clearly visible in the EPR spectrum. The lines due to $m_I = \pm^1/_2$ states are partially obscured by the spectrum of $Y_D^{\bullet}$ containing nonmagnetic isotopes of oxygen. The ^{17}O hyperfine constant (A_Z), determined from the six distinct lines is 4.32 ± 0.05 mT. Using the relationship, $A_Z = -15.4\rho_O$, as applied to Y122$^{\bullet}$ of *E. coli* RNR,[19] we arrive at a value of 0.28 ± 0.01 for the electron spin density of the phenolic oxygen. Given previous estimates[12,13] of the overall spin density within the C4−O of 0.25, this leaves a spin density of −0.03 at C4.

An ^{17}O−^{16}O difference spectrum (Figure 1, part c) was calculated from the EPR spectra shown and represents the spectrum of the ^{17}O-labeled radical alone. This was done by

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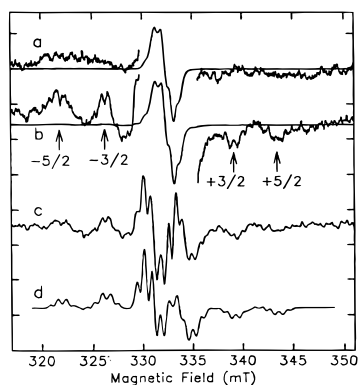
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(20) Wild-type *Synechocystis* PCC 6803 was grown photoautotrophically in a 10 L culture of BG-11 for 1 week in the presence of 0.25 mM tryptophan, 0.50 mM phenylalanine, and either 0.25 mM [^{16}O]tyrosine or [^{17}O]tyrosine (isotope enrichment 35−40%) according to Barry and Babcock.[21] PSII core complexes were isolated as previously described.[22] $Y_D^{\bullet}$ was trapped by illuminating the concentrated core complexes for 35 s at 0 °C in the presence of 0.3 mM K$_3$Fe(CN)$_6$. After a 15 min incubation at 0 °C in the dark, the samples were frozen in liquid nitrogen and stored in liquid nitrogen until use. X-band EPR spectra were recorded on a Bruker ESP300 spectrometer equipped with a Bruker ER035M NMR gaussmeter, a Hewlett-Packard 5350B microwave frequency counter, and an Oxford liquid nitrogen cryostat.
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Figure 1. EPR spectra of the $Y_D^\bullet$ radical with the phenolic oxygen either (a) ^{16}O or (b) ^{17}O (ca. 30% labeled). The wings have been expanded vertically ×32. Computed spectrum of pure ^{17}O-labeled Y_D (c) and a simulation of the same (d). The following parameters were used in the simulation: *g*-tensor (x,y,z), 2.00745, 2.00422, 2.00212; hyperfine tensors ^{17}O, 0.45, 0.55, −4.32 mT; methylene ^1H, 1.02, 0.89, 0.89 mT, ring ^1H (H3 or H5), −0.99, −0.28, −0.72 mT, ring ^1H (H5 or H3), −0.92, −0.28, −0.67 mT. The *Z* axes of the ring proton tensors are colinear with the g_z axis, while the *X* axes are 25 and −25° from the g_x axis. The axes of the other two hyperfine tensors are aligned with the *g*-tensor. Experimental conditions: temperature 160 K, microwave frequency 9.331 GHz, microwave power 0.1 mW, field modulation 0.2 mT at 100 kHz, time constant 160 ms, 512 scans each, scan time 80 s.

subtracting from spectrum b an amount of spectrum a equivalent to 71% of the spins of spectrum b, based on the double integrals of the spectra, which eliminates the unlabeled component of the spectrum. Thus, the labeled sample was about 29% $[^{17}O]$-tyrosine, indicating that about 75% of the tyrosine was derived from the 35−40% ^{17}O-labeled tyrosine of the growth medium. The simulation, shown as Figure 1 (part d), was performed using a modified version of the program described in ref 8. The spectrum of Figure 1 (part d) was obtained by using the ^{17}O A_Z hyperfine component determined above with values estimated for the corresponding X and Y tensor components and with the three large proton hyperfine coupling tensors determined in earlier work.[8,12,13] The simulation compares very well with the difference spectrum and is further evidence in support of our estimates of the oxygen spin density and the use of the *Q* and *B* values of −4.0 and −5.7 mT, respectively, to estimate the contact $(A_{iso} = Q\rho_O)$ and dipolar $(A_{dip,||} = 2B\rho_O, A_{dip,\perp} = -B\rho_O)$ ^{17}O hyperfine couplings. Additional EPR simulations (not shown) indicate that the lines in the center of the simulation are due mainly to the ^{17}O A_Z hyperfine interactions with the $m_I = \pm^1/_2$ spin states.

The ^{17}O-labeled spectrum shows no resolved features that can be attributed to coupling with ^{17}O A_X or A_Y hyperfine tensor components. From this lack of additional lines, we can set an upper limit on the value of A_X and A_Y equal to 0.7 mT. This estimate is somewhat larger than the 0.49 mT estimated from spectroscopic data on RNR, but it is much smaller than the value of 1.3 mT estimated from recent density functional calculations,[9] which appear to have suffered from spin contamination of the ground state wave function.

From the measured hyperfine coupling to the hydrogen-bonded proton equal to 3.1 MHz[10,23] in *Synechocystis*, and the knowledge that such hyperfine interactions are predominantly dipolar,[16,24] we estimate the O···H hydrogen bond distance to be 0.192 nm. While earlier evidence had shown D2-histidine189 to be important for the observation of hydrogen bonding to $Y_D^\bullet$,[23,25] we have recently determined, using pulsed ENDOR and ^{15}N-isotopic labeling in wild-type and site-directed mutant

strains of *Synechocystis*, that this residue is the direct hydrogen-bond donor to $Y_D^\bullet$.[26]

The ^{17}O hyperfine coupling value determined here for $Y_D^\bullet$ shows a small but experimentally significant difference from that determined for $Y122^\bullet$ of *E. coli* RNR, 4.47 ± 0.03 mT. Both the amplitudes of the ^{17}O hyperfine coupling values and the small decrease that arises from hydrogen bonding are consistent with recent density functional calculations of O'Malley and Ellson[27] on both hydrogen-bonded and non-hydrogen-bonded *p*-methylphenoxyl radicals. From the present measurements, we estimate the difference in phenolic oxygen spin densities to be $(4.47 − 4.32)15.4 = 0.010 ± 0.004$, with the hydrogen-bonded radical having the smaller spin density. Carbon atom densities also differ only slightly between the two radicals.[19] These rather small changes in spin density contrast with the behavior of semiquinone radicals, where hydrogen bonding has considerable influence on the spin density.[24,28]

The g_x component of the anisotropic *g*-tensor of the radical has been previously reported to be influenced by the presence of a hydrogen bond to $Y_D^\bullet$,[15,17] with $g_x = 2.00740$ for the hydrogen-bonded wild-type and $g_x = 2.00832$ for the non-hydrogen-bonded, D2-histidine189 to glutamine mutant[23] and 2.00868 for $Y122^\bullet$ of *E. coli* RNR. The g_x component is dependent on the electron spin density (ρ_z) of the oxygen p_z orbital in the ground state singly occupied molecular orbital (SOMO) of molecular orbital π^*, on the spin density (ρ_y^*) of the O p_y orbital in the excited state SOMO of a nonbonding molecular orbital centered on the O, and on ΔE, the energy difference between the ground and excited state SOMOs[15,17,29]

$$\Delta g_x = g_x - g_e \approx \zeta_o \rho_z \rho_y^* / \Delta E$$

where ζ_o is the oxygen spin−orbit coupling constant. Thus, as the variation in the ground state spin density is extremely small, the dependence of g_x on the hydrogen bonding likely comes from either variations in ρ_y^* or variations in ΔE.

For the tyrosyl radicals considered here, hydrogen bonding has a very minor impact on the ground state spin distribution. More likely, electrostatic interactions arising from hydrogen bonding and/or neighboring charges have greater influence on their redox behavior. This point is further strengthened by similar studies (manuscript in preparation) that we have performed on ^{17}O-labeled $Y_Z^\bullet$, which show only small differences with the O spin density of $Y_D^\bullet$. Considering the different functions and reduction potentials of $Y_D^\bullet/Y_D$, $Y_Z^\bullet/Y_Z$, and the tyrosyl radical of RNR, we conclude that protein control of radical activity is modulated by these more subtle electrostatic interactions and/or by excited state behavior of the radical and is not achieved by direct perturbation of the ground state radical spin density.

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