Rapid Freeze−Quench ENDOR of the Radical X Intermediate of *Escherichia coli* Ribonucleotide Reductase Using $^{17}O_2$, $H_2^{17}O$, and ^2H_2O
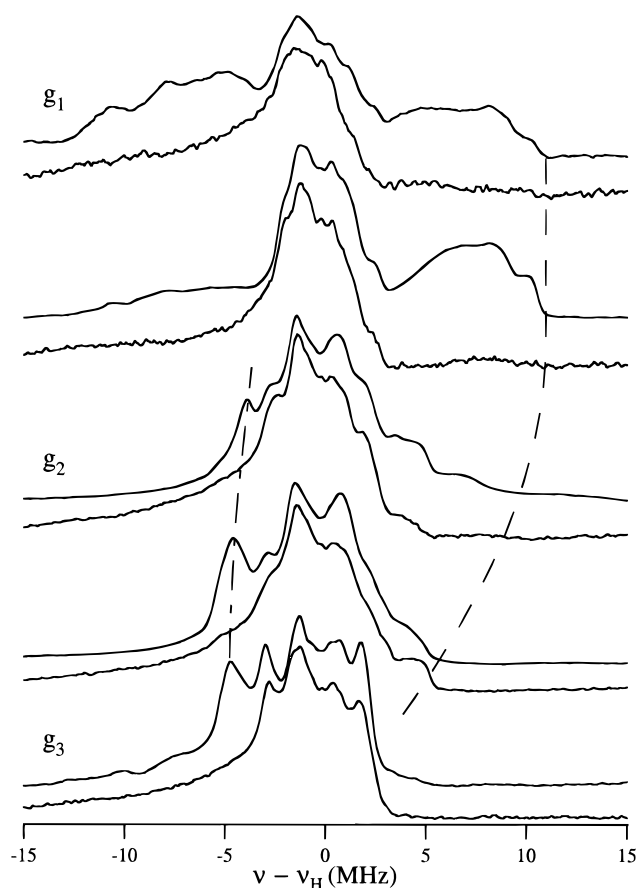
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Proteins containing diferrous clusters are capable of carrying out reactions with O_2, whose diversity is comparable to that shown by the well-characterized heme systems.[1,2] In particular, the diiron center in ribonucleotide reductase (RNR) is important because its formation is accompanied by the oxidation of a tyrosine residue 122 to the tyrosyl radical that initiates the catalytic nucleotide reduction process.[3,4] A variety of rapid-kinetics methods, including stopped-flow absorption, rapid freeze−quench (RFQ) EPR, and Mössbauer spectroscopies, have been used to investigate the mechanism by which the incubation of the apo R2 subunit of *Escherichia coli* RNR with Fe^{2+} and O_2 leads to the self-assembly of its diferric cluster and the formation of the radical cofactor (•Y122-R2).[5] These studies revealed the formation of a diiron intermediate (X) that directly "ignites the pilot light" of RNR by oxidizing Y122-R2 to the tyrosyl radical. This intermediate has a spin of $S = {}^1/_2$ and a formal [Fe(III),Fe(IV)] oxidation state, although it has been described in terms of two high-spin Fe^{3+} ions spin-coupled with a ligand radical.[6,7] The present paper reports the use of RFQ Q-band ^{17}O and ^1H electron−nuclear double resonance (EN-DOR) spectroscopy[8,9] to study the intermediate X generated in the presence of either $^{17}O_2$ or $H_2^{17}O$, or ^2H_2O. The ENDOR measurements allow us to examine the fate of the two atoms from O_2 and to test for solvent-derived H_xO ligands to the irons.

Proton ENDOR spectra[10] of X in H_2O and D_2O show signals from strongly-coupled protons that are lost upon exchange in D_2O buffer (Figure 1) and that are undoubtedly associated with protonated oxygenic ligand(s). The EPR spectrum of X does not show resolved g-anisotropy at X-band,[5,6] but at Q-band it is characteristic of a rhombic g-tensor[11] and exhibits sufficient anisotropy ($g_1 \approx 2.007$, $g_2 \approx 1.999$, $g_3 \approx 1.994$) to permit ENDOR to estimate full hyperfine tensors from orientation-

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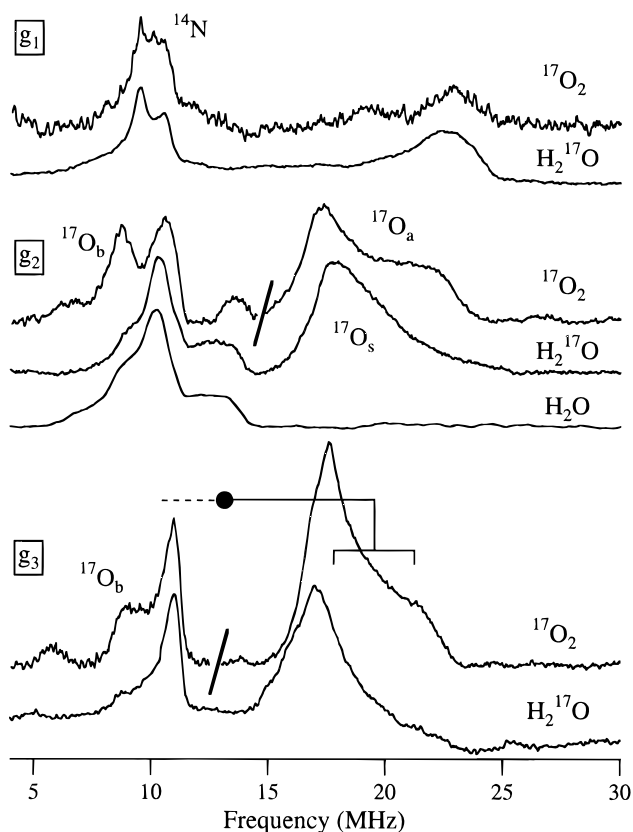(7) Ravi, N.; Bominaar, E. L. *Inorg. Chem.* **1995**, *34*, 1040−1043.
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(10) Q-band CW ENDOR spectra were collected as described previously (Werst, M. M.; Davoust, C. E.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 1533−1538), using bandwidth broadening (100 kHz) of the radio frequency as described (Hoffman, B. M.; DeRose, V. J.; Ong, J.-L.; Davoust, C. E. *J. Magn. Reson., Ser. A* **1994**, *110*, 52−57).
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Figure 1. Representative Q-band, CW ^1H ENDOR spectra for X, centered at the proton Larmor frequency, $\nu_H \sim 53$ MHz. At each field, the upper spectrum is for a sample in H_2O, the lower for D_2O. Conditions:[10] ν(microwave) ≈ 35.3 GHz; 100 kHz modulation amplitude, 8 Gpp; rf scan speed, 1 MHz/s; $T = 2$ K.

selective spectra taken at numerous fields throughout the EPR envelope.[8] Orientation selection is illustrated by the changes in the ^1H pattern as the field of observation is moved across the EPR envelope (Figure 1). For $g \gtrsim 1.996$, the breadth of the pattern is determined by a class of exchangeable proton with a highly anisotropic coupling. Between g_1 and $\sim g_2$, the breadth corresponds to a hyperfine splitting $A(^1H1) \approx 21$ MHz, with the $\nu_- = \nu_H - A(^1H1)/2$ branch of the signal showing a lower intensity than the $\nu_+ = \nu_H + A(^1H1)/2$ one, except at the low-field g_1 edge of the EPR envelope. As the field is increased toward $\sim g_2$, the coupling begins to decrease, tending toward $A(^1H1) \approx 0$ MHz at g_3. An additional exchangeable feature, most clearly seen from $\sim g_2$ to g_3 in the ν_- branch ($\nu < \nu_H$) is tentatively assigned to a second class of exchangeable proton, with $A(^1H2) \approx 10$ MHz at g_3.[12]

Q-band CW ENDOR spectra of X without ^{17}O enrichment show resonances from the ^{14}N of histidine ligands to Fe in the region $\nu \lesssim 15$ MHz, as illustrated by the g_2 signal in Figure 2. When X is prepared with $^{17}O_2$ gas (86% enriched),[13] a new signal appears in the range $15 \lesssim \nu \lesssim 25$ MHz (Figure 2), which we assign as the ν_+ branch of an ^{17}O ENDOR pattern for a site denoted O_a. In a spectrum taken at g_3, it is centered at $\nu_+ = A_3/2 + \nu(^{17}O) \approx 20$ MHz, corresponding to $A_3 \approx 25$ MHz; no additional ^{17}O signals are seen at higher frequency. The ~ 4.5 MHz breadth of the pattern is assigned to unresolved ^{17}O

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(12) Efforts are under way to explore similarities of these signals with those of the hydroxo bridge and water of mixed-valence diiron centers (DeRose, V. J.; Liu, K. E.; Kurtz, D. M., Jr.; Hoffman, B. M.; Lippard, S. J. *J. Am. Chem. Soc.* **1993**, *155*, 6440−6441. DeRose, V. J.; Liu, K. E.; Lippard, S. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1996**, *118*, 121−134.

Figure 2. Representative CW ENDOR data for X prepared[13] with $^{17}O_2$, $H_2^{17}O$, and H_2O. For the $^{17}O_2$ sample, g_1 and g_2 data are scaled by $^1/_2$ for $\nu > 14$ MHz. At g_3, ● represents $A(^{17}O_a)/2$; the line, $\nu(^{17}O)$; the final brace, the quadrupole breadth. Conditions:[10] as in Figure 1 except scan speed, 0.5 MHz/s.

quadrupole splittings.[8,14] The pattern's center does not shift as the field is lowered to g_2, giving $A_2 \approx 25$ MHz; it then increases in frequency, and by g_1, the hyperfine coupling is $A_1 \approx 31$ MHz, with some structure also appearing that may reflect quadrupolar effects or site heterogeneity. This signal is assigned to a single ^{17}O, because EPR simulations[15] indicate that contributions from two ^{17}O nuclei with similar couplings would give substantially greater EPR line-broadening than observed.[5]

The ENDOR experiment was repeated with the sample in $H_2^{17}O$ (32% enriched),[13] and at all fields (g-values), we obtained

(13) Preparation of X in the presence of $^{17}O_2$ or $H_2^{17}O$ for analysis by ENDOR spectroscopy: The RFQ experiments were carried out by modifications of those previously described (Bollinger, M. J., Jr.; Tong, W. H.; Huynh, B. H.; Edmondson, D. E.; Stubbe, J. *Methods Enzymol.*, in press). An argon-saturated solution of apo-Y122F-R2 (600 μM) in 100 mM Hepes, pH 7.7, was transferred to one drive syringe attached to an Update Instruments System 1000. A solution of FeSO$_4$ (3.10 mM in 5 mN H$_2$SO$_4$) was attached to a vacuum manifold and degassed by standard procedures. The solution was then equilibrated with $^{17}O_2$ (85.4% ^{17}O; from Isotec) at 5 °C and then transferred to the second syringe. The reaction was initiated by mixing equal volumes of these solutions at 5 °C and quenched at 610 ms into an ENDOR tube. For the $H_2^{17}O$ experiments, apo-Y122F-R2 (536 μM) was prepared in 100 mM Hepes, pH 7.7, which contained 26% $H_2^{17}O$ and the solution was saturated with $^{16}O_2$ and placed in a drive syringe. A solution of FeSO$_4$ (2.82 mM in 5 mN H$_2$SO$_4$) was prepared in $H_2^{17}O$ (37 atom % ^{17}O), saturated with $^{16}O_2$, and loaded into a second syringe. The reaction was carried out as described above, yielding a final enrichment of ~32% $H_2^{17}O$.
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spectra from a strongly-coupled ^{17}O site, denoted O_s, that exhibits ν_+ patterns similar to those of O_a in the $^{17}O_2$ sample (Figure 2). The shapes of the signals from the two samples are not identical in detail, with the high-frequency edges of the pattern for $H_2^{17}O$ being less resolved at g_2 and g_3; this likely reflects differences in quadrupole tensors. Nonetheless, the values of $A(^{17}O)$ for the $^{17}O_s$ species derived from water are quite similar to those of the $^{17}O_a$ species derived from dioxygen gas at all observed fields.

It might be suggested that X does not contain two oxygenic species with separate sources, but rather that an oxygen from $H_2^{17}O$ solvent exchanges into a site that is initially derived from O_2 gas, and that the spectral differences in the figure reflect differences in the spin-relaxation properties of the two samples, not in the ^{17}O couplings themselves. However, this alternative is countered by resonance Raman studies which show that the oxo bridge of the diferric cluster is derived from O_2.[16] Indeed, the ENDOR data argue against exchange: the height of the ^{17}O signal at g_3 for the $^{17}O_2$ sample, as normalized to the sharp ^{14}N peak at 10 MHz, is ~2.5 times more intense than that for the $H_2^{17}O$ signal, consistent with the ~2.7-fold higher enrichment of $^{17}O_2$.[13]

One of the atoms of O_2 is detected by ENDOR as $^{17}O_a$, but what about the second? Close examination of the low-frequency region of the ENDOR spectra at g_2 (Figure 2) shows a well-defined peak at ~9 MHz for the sample prepared from $^{17}O_2$, but not for the unenriched or $H_2^{17}O$ samples; furthermore, at g_3, the intensity in that region is greater in the $^{17}O_2$ than in the $H_2^{17}O$ spectrum. We assign these features as part of the ν_+ branch of a second oxygen derived from $^{17}O_2$, denoted $^{17}O_b$, whose hyperfine couplings are much smaller than those of $^{17}O_a$; preliminary studies on a sample doubly labeled with ^{17}O and ^{15}N support the assignment.[17]

The ^{17}O ENDOR results thus have detected three exogenous O atoms associated with X. Both atoms of the reactant O_2 remain bound, and one of these, O_a, has properties that are similar to those of the solvent-derived $^{17}O_s$. The ^1H data show strongly-coupled, exchangeable proton(s) associated with one or more of these sites. These results for X could be discussed in terms of a model that involves a diferric center coupled to an O radical or an oxo (hydroxo)-bridged, trapped-valence [Fe^{III}-$(S = ^5/_2)$,Fe$^{IV}(S = 2)$] center. ^{57}Fe ENDOR measurements in progress show that one of the Fe sites of X has an anisotropic hyperfine tensor, and therefore at least some Fe^{IV} character.[17] In addition, a model compound that exhibits a di-μ-oxo-bridged trapped-valence center has been shown to exhibit many of the properties of X.[18] Following this model, two of the three exogenous O atoms shown here to be part of X would correspond to bridging oxo (hydroxo) ligands, and one a terminal aquo/hydroxo. Experiments under way[17] will permit a definitive description of X.

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