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Direct EPR Observation of a Tyrosyl Radical in a Functional Oxidase Model in Myoglobin during both H_2O_2 and O_2 Reactions

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Supporting Information

ABSTRACT: Tyrosine is a conserved redox-active amino acid that plays important roles in heme−copper oxidases (HCO). Despite the widely proposed mechanism that involves a tyrosyl radical, its direct observation under O_2 reduction conditions remains elusive. Using a functional oxidase model in myoglobin called F33Y-Cu_BMb that contains an engineered tyrosine, we report herein direct observation of a tyrosyl radical during both reactions of H_2O_2 with oxidized protein and O_2 with reduced protein by electron paramagnetic resonance spectroscopy, providing a firm support for the tyrosyl radical in the HCO enzymatic mechanism.

As a redox active amino acid, tyrosine (Tyr) plays important roles in a number of enzymes.[1−5] Of particular interest is the Tyr in heme−copper oxidases.[6−8] In this class of proteins, O_2 is reduced to water at the binuclear heme−copper active site with a conserved Tyr next to one of the histidines that coordinates to the Cu_B center. The Tyr is proposed to donate an electron and a proton in the reaction process, forming a radical intermediate.[7−11] To confirm the hypothesis, Babcock and co-workers have used radioactive iodine to label the tyrosyl radical during the oxygen reduction reaction and mapped the radical to the peptide corresponding to the active-site tyrosine.[12] However, due to the transient nature of the tyrosyl radical and $\mathbf{P_M}$ intermediate associates with it, the tyrosyl radical has not been directly observed during the oxygen reduction reaction.[5] Instead, a number of groups have treated the oxidized HCOs with H_2O_2, and observed a radical formed in this process.[13−18] Recently, Gerfen and co-workers have performed high-frequency EPR on H_2O_2-reacted bovine C*c*O and separated two tyrosyl radical signals, one of which has been assigned to an active-site Tyr (Tyr244).[19] Even though the tyrosyl radical was observed under this "peroxide shunt" pathway, direct observation of such a radical under physiological conditions during the O_2 reduction remains elusive; it is an important piece of the puzzle of the HCO reaction mechanism that is still missing.

One contributing factor in the lack of the proof for the tyrosyl radical in O_2 reduction in HCO is the difficulty in preparing and studying large membrane proteins such as HCO that contains several redox active centers that may interfere with spectroscopic characterizations. To overcome this limitation, synthetic models of HCOs have been prepared, and those have contributed to understanding the structure and function of HCOs.[20−22] Phenoxyl radical has been generated in a model complex by UV laser irradiation.[22] However, such a radical has not been observed as an intermediate in any O_2 or H_2O_2 reactions that are related to the biological function of HCOs.[20−24]

As a complementary approach for studying native enzymes or synthetic models, we have previously reported designs of functional protein models of HCOs by introducing one Tyr and two additional His into sperm whale myoglobin.[25−34] Interestingly, we found that the presence of a Tyr in the HCO protein model called F33Y-Cu_BMb (Figure 1) is critical in conferring the oxidase activity (turnover rate 0.09 s^{-1} with 258 μM O_2 at 25 °C, while native HCOs from different organisms display turnover rates in a range of 50−1000 s^{-1} with respect to O_2 consumption[35,36]) that reduces oxygen to water with minimal release of other reactive oxygen species such as superoxide and peroxide and hundreds of turnovers.[30] Here we report that such a functional protein model of HCO allows direct observation of a tyrosyl radical not only in the peroxide

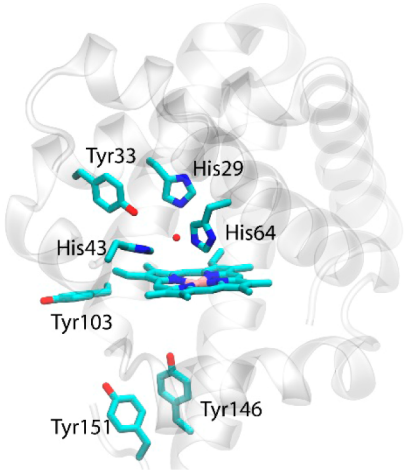

Figure 1. X-ray crystal structure of F33Y-Cu_BMb. Heme *b* and side chains of His29, His43, His64, and Tyr33 in Cu_B site, as well as Tyr103, Tyr146, and Tyr151 are shown in the licorice diagram. A water molecule, shown as a red sphere, is present at the Cu_B site. For purposes of clarity, propionate side chains of heme are omitted. PDB ID: 4FWX.[30]

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shunt pathway but also during the O_2 reduction, confirming the active role of the tyrosyl radical in the oxidase reaction.

The F33Y-Cu_BMb was expressed and purified without a copper ion at the Cu_B site, as previously described.[30] No copper ion was added in this work, as previous studies have shown the presence of copper does not have any influence on the oxidase activity,[30] similar to the Cu_B-independent cytochrome *bd* oxidase.[37−39] The protein displays an EPR signal typical of water-bound high-spin ferric myoglobin (Figure 2). Upon

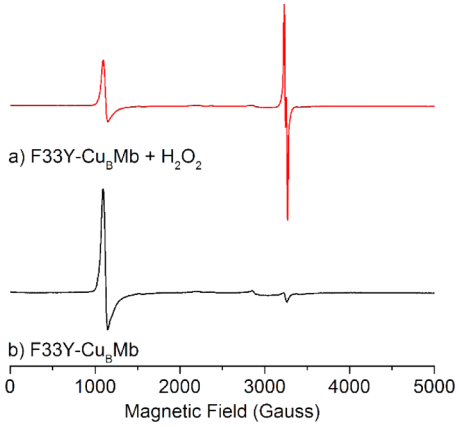

Figure 2. X-band EPR spectra of 0.2 mM F33Y-Cu_BMb in the absence (black line) and in the presence of (red line) 1 equiv of H_2O_2 in 100 mM potassium phosphate buffer. Experimental parameters: frequency: 9.05 GHz; power: 5 mW; gain: 2000; temperature: 30 K; modulation: 10 G.

reaction with one equivalent of H_2O_2 for 30 s when the reaction was stopped by flash freezing in liquid N_2, the high-spin heme signal decreased by 80% and a $g \approx 2$ signal grew in, accounting for 54% of the total protein concentration (Figure S1, Table S1 in Supporting Information [SI]). To further characterize the $g \approx 2$ signal, an EPR spectrum was collected in this region using a smaller modulation at 2 G. Under such conditions, an exquisite hyperfine structure was observed (Figure 3a), which is similar to that of tyrosyl radical reported in heme−copper oxidase[14] and ribonucleotide reductase.[40−42]

In addition to tyrosine, another redox active amino acid, tryptophan, could also generate a radical with a similar shape in X-band EPR, as both wild-type myoglobin (WTMb) and F33Y-Cu_BMb contain two Trp residues.[2,40,43,44] To improve the accuracy in EPR fitting, we collected the Q-band EPR spectrum of a sample prepared in the same way (Figure 3b) and performed simulation[45] of both X- and Q-band spectra to extract *g*-values and hyperfine coupling constants (Tables 1 and S2 in SI). Simulation of both X- and Q-band spectra (Figure 3 and Figures S2, and S3 in SI) revealed two species. One species shows a well-defined proton-hyperfine coupling pattern that can be simulated as a radical split by four nuclei, presumably from 3, 5-^{1}H and two β-^{1}Hs of a tyrosine. The other species has an unresolved hyperfine structure whose width and intensity vary with preparation such as tube size and mixing (Figure S2 and Table S2 in SI), in contrast to the first species. The width of the second species also differs from that of the radical formed in Cu_BMb (Figure S4B,E in SI). The *g* values of the first species obtained from simulations of both X- and Q-band EPR spectra are 2.0091, 2.0044, and 2.0021. This large *g* anisotropy is very similar to that of a typical tyrosyl radical (2.0076, 2.0044, 2.0021) and different from more isotropic values (2.0033,

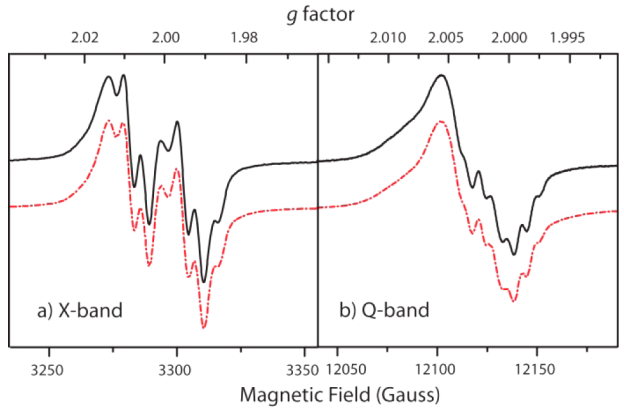

Figure 3. X-band (a) and Q-band (b) EPR spectra of F33Y-Cu_BMb after addition of 1 equiv of H_2O_2. Experiment parameters for X-band: frequency: 9.23 GHz; power: 2 mW; gain: 4000; temperature: 30 K; modulation: 2 G. Experiment parameters for Q-band: frequency: 34.0 GHz; power: 50 μW; temperature: 50 K; modulation: 3.2 G. Spectrum simulated by SIMPOW6[45] is red dashed line. Both temperature-dependent (Figure S6 in SI) and microwave power-dependent (Figure S7 in SI) EPR studies indicate no spectral saturation occurred under the EPR condition used.

Table 1. Simulated EPR Parameters for Tyrosyl Radical in F33Y-Cu_BMb and RNR

	nucleus	A_x/MHz[e]	A_y/MHz[e]	A_z/MHz
F33Y-Cu_BMb[a,b,c]	3-^{1}H[d]	26	11	19
	5-^{1}H[d]	26	11	19
	β-^{1}H	51	60	59
	β-^{1}H	24	8	16
RNR[f,g,h]	3-^{1}H	25	12	19
	5-^{1}H	21	14	16
	β-^{1}H	60	53	60
	β-^{1}H	27	7	16

[a] *g* values of 2.0091, 2.0044, and 2.0021 were used. [b] Line width (peak-to-peak) of 7.5, 4.7, 3.2 G were used. [c] *g* strain of 0.0005, 0.0001, and 0.0000 were used. [d] Euler angles of $[\alpha, \beta, \gamma] = [\pm 22°, 0°, 0°]$[46] were used. Hyperfine for 3-H and 5-H assumed equivalent. No significant improvement if they are allowed to be inequivalent. [e] The assignment of *x* vs *y* is arbitrary. [f] From ref 40. [g] *g* values of 2.0076, 2.0044, and 2.0021 were used. [h] Line widths of 6.0, 6.0, 6.0 G were used.

2.0024, 2.0021) of a tryptophan radical reported previously.[40,42,46] The hyperfine coupling constants of this species are also very similar to those of a tyrosyl radical in ribonucleotide reductase (RNR) from Table 1.[40,42,46] These similarities, especially for the more conserved hyperfine coupling constants of the ring protons,[42,46,47] also suggest the observed radical species is based on tyrosine. The yield of pure radical species is 29%, comparable to yields of the radical in different HCOs (3−20%).[13,14,19]

In addition to the engineered Tyr33, there are three other tyrosines (Tyr 103, 146, and 151) in the F33Y-Cu_BMb. To identify the location of the tyrosyl radical, we carried out several control experiments. First, under the same conditions, reaction of 1 equiv of H_2O_2 with WTMb, which contains the above three tyrosines, but not Tyr33, resulted in no radical EPR signal in this region (Figure S4A,D in SI). Second, the same reaction with Cu_BMb which contains the three tyrosines and the histidines for the Cu_B site showed a broad signal with unresolved hyperfine structure (Figures S4B,E in SI). Finally, when the three other tyrosines in F33Y-Cu_BMb were mutated

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into Phe, the same reaction with H_2O_2 under the same conditions resulted in a radical signal that is almost identical to that without the above tyrosine mutations (Figure S5 in SI). Together these results indicate that the location of the radical with defined hyperfine coupling pattern (Figure 3) is at Tyr33.

After demonstrating a protein radical, most probably formed on Tyr33, in the "peroxide shunt" pathway, we next investigate whether the Tyr radical can form during the O_2 reduction reaction. Deoxy−F33Y-Cu_BMb was mixed with O_2 saturated buffer using a rapid freeze quench apparatus to trap reaction intermediates at ∼20 ms. An EPR spectrum was then collected and compared with the corresponding EPR signal from oxidized protein (Fe(III)−F33Y-Cu_BMb) treated with H_2O_2. As shown in Figure 4 and Figure S8 in SI, the radical signal of

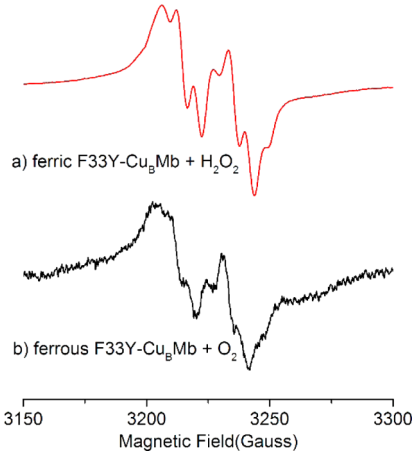

Figure 4. EPR spectra of ferric F33Y-Cu_BMb reacted with 1 equiv of H_2O_2 (a) and ferrous F33Y-Cu_BMb with O_2 (b). Ferrous F33Y-Cu_BMb was mixed with O_2 saturated buffer and rapidly freeze−quenched to capture the reaction intermediate after 20 ms. Experiment parameters for X-band EPR: frequency: 9.04 GHz; power: 2 mW; gain: 4000 for H_2O_2-reacted and 12500 for O_2-reacted samples; temperature: 30 K; modulation: 4 G.

the freeze-quenched sample from direct O_2 reduction is similar to that from the H_2O_2 reaction, with a similar hyperfine splitting pattern. The highly specific hyperfine splitting pattern suggests the same tyrosyl radical is generated through the O_2 reduction reaction. The observed signal at the shortest time point (20 ms) of freeze quench represents ∼7% of total protein based on spin quantification (Table S1 in SI), and the signal intensity decreases further at 50 ms and disappears completely at 100 ms (Figure S9 in SI), indicating that this tyrosyl radical is transient and unstable, consistent with difficulty in observing it in native HCOs under turnover conditions. Given the fast decay of the radical in comparison to the relatively slow turnover rate, the radical formation and decay are most likely not a rate-determining step. Instead, other steps, such as electron injection to the active site, may be limiting and will be investigated.

In summary, using EPR spectroscopy, we have detected the same radical species in the reaction of the ferric F33Y-Cu_BMb with H_2O_2 and of the ferrous F33Y-Cu_BMb with O_2. Simulation of the radical EPR spectra collected at both X- and Q-bands showed it is consistent with a tyrosyl radical signal, while control experiments using WTMb, Cu_BMb, and mutations of other native tyrosines in the protein established that Tyr33 is the active-site Tyr involved in the radical formation. The direct observation of the tyrosyl radical in a functional oxidase model, especially during O_2 reduction, provides strong support for the role of such a radical in the oxidase reaction mechanism and thus fills an important missing piece in the puzzle of HCO bioenergetics.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, EPR of F33Y-Cu_BMb, Cu_BMb, and WT Mb, spin counting, EPR parameters from simulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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