SUPPORTING INFORMATION

Two tyrosyl radicals stabilize high oxidation states in cytochrome *c* oxidase for efficient energy conservation and proton translocation

Michelle A. Yu[a], Tsuyoshi Egawa[a], Kyoko Shinzawa-Itoh[b], Shinya Yoshikawa[b], Victor Guallar[c], Syun-Ru Yeh[a], Denis L. Rousseau[a], and Gary J. Gerfen[a]

[a]Department of Physiology and Biophysics
Albert Einstein College of Medicine
1300 Morris Park Ave.
Bronx, NY 10461

[b]Department of Life Science
University of Hyogo
3-2-1 Kouto
Kamigori, Ako, Hyogo 678-1297, Japan

[c]ICREA Joint BSC-IRB Research Program in Computational Biology.
Barcelona Supercomputing Center,
Jordi Girona 29, 08034 Barcelona SPAIN

Radicals in Model Complexes and other Proteins. The principal g-values are characteristic of the properties of the specific amino acid residue on which the radical resides. Dependent on the electrostatic environment, unmodified neutral tyrosyl radicals in proteins generally display the following g-value ranges: $2.010 > g_x > 2.006$; $2.005 > g_y > 2.004$; and $g_z \sim 2.002$.[1-5] However, very specific changes occur in modified tyrosines. In apogalactose oxidase, a catalytic tyrosyl radical, which is cross-linked to the sulfur of a cysteine in the *ortho*-position, gives rise to principal *g*-values of 2.00741, 2.00641, and 2.00211, yielding $|g_x - g_y| = 0.0010$)[6] (See Table S2). In addition, the model compound *o*-(methylthio)-cresol exhibits the same convergence of g_x and g_y, which was not seen in unsubstituted tyrosyl species.[6] The near-axial symmetry of the g-tensor, compared with the greater degree of rhombicity normally displayed by tyrosyl radials, is due to electron spin delocalization onto the cross-linked sulfur.[7,8] More convincingly, model complexes exhibit the same decreased g_x and increased g_y relative to unmodified tyrosyl radicals.[9-11] A study on the radical formed the Tyr-His model compound 2-(4-methyl-1*H*-imidazol-1-yl)-

4-methyl phenol reports *g*-values of 2.0065, 2.0050, and 2.0023,[10] resulting in a $|g_x - g_y|$ difference is 0.0015. Therefore, the axially-symmetric powder pattern of the narrow radical follows the trend in the literature for modified tyrosyl species, containing an S or N nucleus in the *ortho* position.

We further investigated this trend by characterizing phenoxyl radicals generated on L-Tyr (Figure S1) and 2-amino-p-cresol (Figure S2). The X-band CW-EPR spectrum of irradiated L-Tyr is 20 G wide and well-fit by two ortho protons, two β-methylene protons, and two meta protons. The hyperfine couplings (Table S1) are consistent with free L-Tyr and unmodified p-cresol radicals measured in other studies.[11,12] The g_x, g_y, g_z-values for L-Tyr, determined by D-band EPR, are 2.0069, 2.0042, and 2.0020 respectively (Figure S2B). The data can be simulated with a Gaussian distribution of g_x with a half-width at half-height equal to 0.0013; a distribution of g_x is characteristic of unmodified tyrosyl radicals. In contrast, 2-amino-p-cresol has g-values that are perturbed although at X-band (Figure S2A), the modified model looks similar to L-Tyr (Figure S1A). We assign g_x, g_y and g_z values of 2.0050, 2.0038, and 2.0017, respectively, in the 2-amino-p-cresol radical and note that the $|g_x - g_y|$ of 0.0012 consistently trends toward that observed for the narrow radical in the bC*c*O data. In addition, the downshift in the g_x value from 2.0069 in free tyrosine to 2.0050 in the 2-amino-p-cresol is similar to the g_x of the 12 G radical (2.0059), consistent with the substitution of a nitrogen atom at the ortho position in Y244 of bCcO. Controls of irradiated HEPES/Acetonitrile showed no radical.

The initial assignment of the narrow radical as a modified tyrosyl radical originates from the g-values determined at D-band. Using these values and the assumption of a tyrosyl radical, the width (12 G peak to trough) of the X-band EPR spectrum can only be reproduced by including only one ortho proton, decreasing $\rho^{\pi}(C_4)$ relative to unmodified tyrosyl radicals, and minimizing the combined hyperfine splitting from the beta protons by setting $\theta = 60°$ for both of them. All of these parameters are consistent with the assignment of the radical to Y244.

The elimination of the hyperfine coupling from an ortho proton in a modified tyrosyl radical or ortho-substituted model compound does not necessarily lead to a 12 G X-band spectrum as observed here for the narrow radical: the overall peak-to-trough width is also influenced by the number and orientation of the β-methylene protons. In apogalactose oxidase, a ~25 G radical with axially symmetric *g*-values forms on the Tyr-Cys moiety. The X-band CW EPR spectrum lacks hyperfine splittings expected for ortho protons, as seen in CcO, but large couplings to beta-methylene protons (determined by their relative orientation to the phenol ring plane normal) yield a wider spectrum compared to CcO. In addition, the width of the X-band CW-EPR spectrum resulting from the radical on 2-amino-*p*-cresol is approximately 20 G. Here, the narrowing effect of removing an *ortho* proton is offset by hyperfine couplings to three β-methylene protons instead of two. However, while an ortho-substituted tyrosyl radical may be broader than the narrow radical observed here, an unmodified tyrosyl radical with typical hyperfine couplings to two ortho protons and two β-methylene protons cannot be as narrow as the X-band spectrum of the radical observed here. This degree of narrowness is best explained

by the reduction in spin density at C4, the orientation of the two β-methylene protons and the elimination of one orthro-proton, as would be expected for a radical on Y244.

The hyperfine coupling values assigned to the two two β-methylene protons in the wide radical (pH 6, table S1) are similar to those determined for the radical observed from *Paracoccus denitrificans* C*c*O (PdC*c*O) (Fig. 5e). [13,14] Following the procedure described previously,[14] two equations for can be solved simultaneously (Eq. 2) for θ and θ-*120* using the A_{iso} values in Table 1. Of the possible solutions, the most physically reasonable gives $\theta = 26°$ and 34°, both of which are approximately equal to 30° and thus assigned to Y129 (Y167 in PdC*c*O),[14] as discussed in the text.

Table S1. Parameters used to simulate X-band and D-band for L-Tyr and 2-amino-*p*-cresol. The absolute values of hyperfine coupling constants are given in Gauss.

2-amino-*p*-cresol				L-Tyrosine			
Component	$H^{\beta 1,2,3}$	H^{ortho1}	$H^{meta1,2}$	$H^{\beta 1}$	$H^{\beta 2}$	$H^{ortho1,2}$	$H^{meta1,2}$
A_x	11.2	7.2	1.7	10.5	10.5	9.6	1.7
A_y	11.2	2.2	2.7	9.8	9.8	2.8	2.7
A_z	11.2	5.5	0.4	9.8	9.8	7.0	1.6
A_{iso}	11.2	5.0	1.6	10.0	10.0	6.5	2.0
α^a	-	+30	±30	-	-	±30	±30

[a]Angle between A_x and g_x.

Table S2. Trends of *g*-values in modified versus unmodified tyrosyl radicals.

	g_x	g_y	g_z	$\lvert g_x-g_y \rvert$	Ref.
Apogalactose oxidase	2.00741	2.00641	2.00211	0.0010	Gerfen *et al*. [6]
Thiyl-subst model	2.00720	2.0620	2.00219	0.0010	Gerfen *et al*.[6]
Imidazole-subst model	2.0065	2.0050	2.0023	0.0015	Kim *et al*.[10]
Amino-subst model	2.0050	2.0038	2.0017	0.0012	This work
CcO pH 8 radical	2.0059	2.0051	2.0017	0.0008	This work
CcO pH6 radical	2.0072	2.0041	2.0025	0.0031	This work
L-Tyr (unmodified)	2.0069	2.0042	2.0020	0.0027	This work
RNR (H-bonded)	2.0076	2.0044	2.0020	0.0032	Bleifuss *et al*.[15]
RNR (unmodified)	2.0091	2.0046	2.0021	0.0045	Bleifuss *et al*.[15]

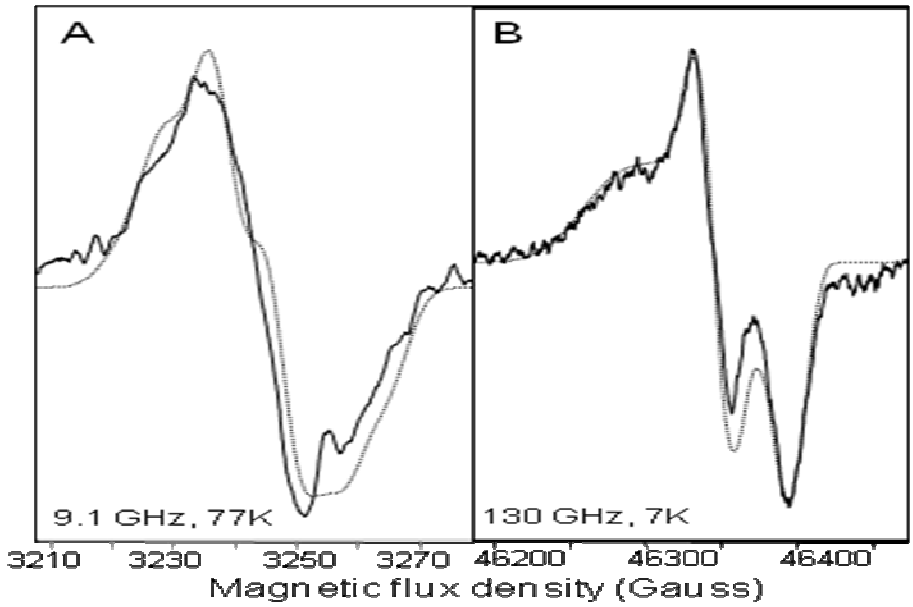

Figure S1. X-band and D-band EPR spectra of L-Tyr (unmodified) irradiated by a mercury arc lamp for 2 min. 200 μM L-Tyr was frozen at 77 K and then irradiated by UV light for 2 minutes. (A) The X-band and (B) D-band measurements are shown (solid lines) and simulated (dotted lines) with parameters given in Table S1.

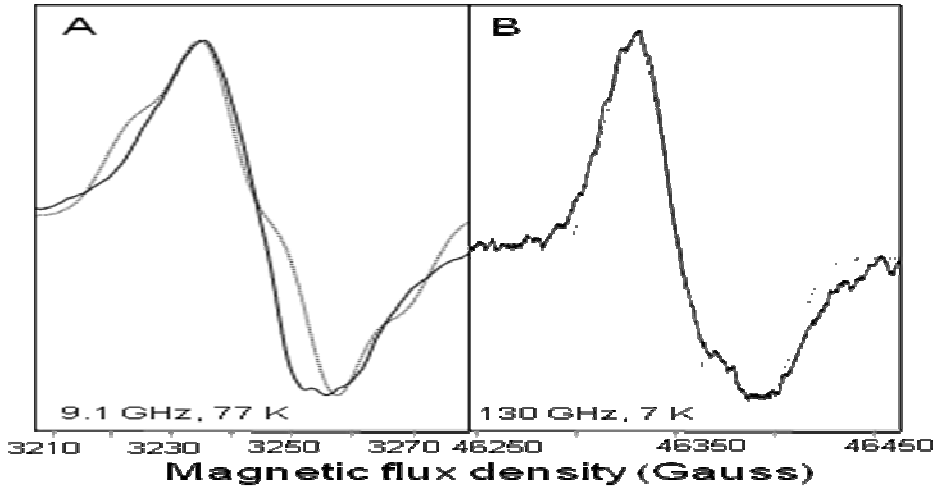

Figure S2. X-band and D-band EPR spectra of 2-amino-p-cresol irradiated by a mercury arc lamp for 2 min. 100 mM 2-amino-p-cresol was frozen at 77 K and then irradiated by UV light for 2 minutes. (A) The X-band and (B) D-band measurements are shown (solid lines) and simulated (dotted lines) with parameters given in Table S1.

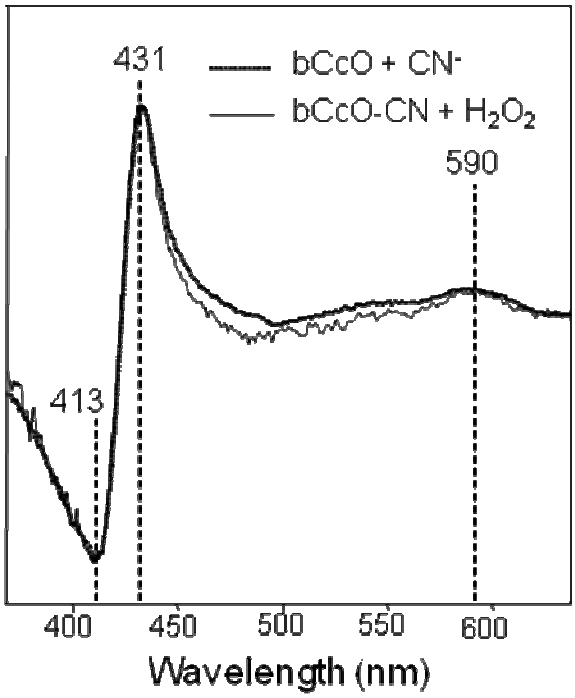

Figure S3. Optical absorption difference spectra for the cyanide-adducts of bC*c*O. The resting enzyme was the reference spectrum. The dotted line corresponds to the cyanide adduct of the oxidized enzyme and the thin solid line was obtained upon the addition of H_2O_2.

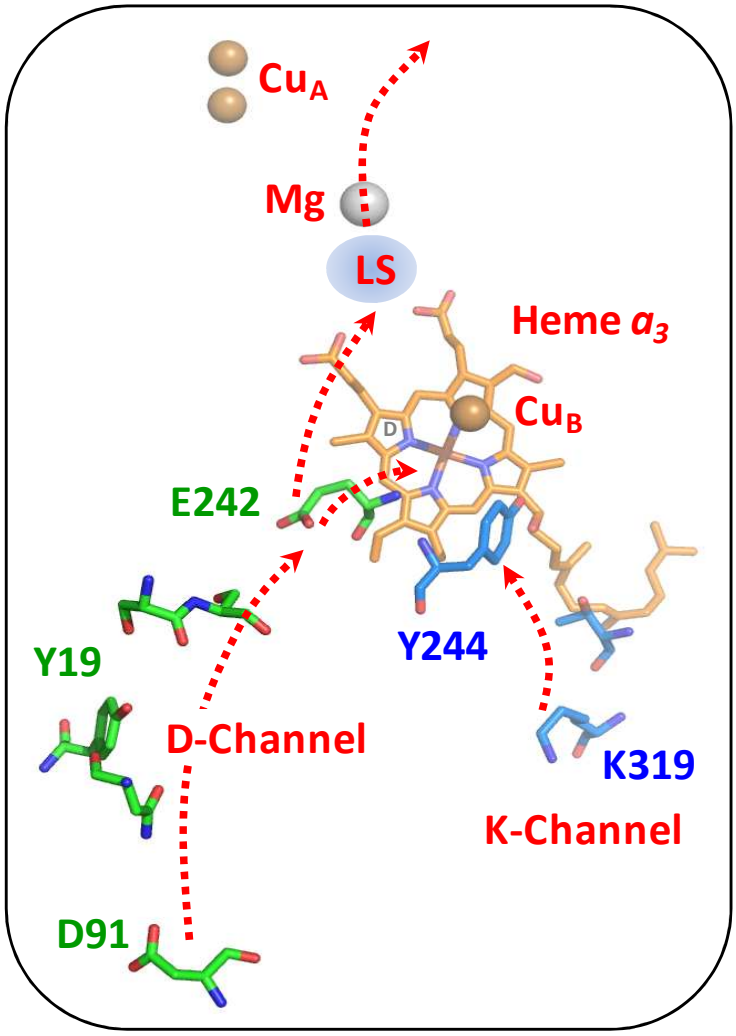

Figure S4. The D- and K-pathways in bC*c*O. The K-pathway terminates at Y244 and the D-pathway terminates at E242, where protons can be gated to pass into the binuclear center for the production of water or to the proton loading site above the heme a_3 propionates. The figure was made from PDB ID: 3AG3 with PyMol Molecular Graphics Software (Delano Scientific, LLC).

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