Supplementary Information

Characterization of an O_2 Adduct of an Active Cobalt-Substituted Extradiol-Cleaving Catechol Dioxygenase

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Experimental Methods

Reagents.

Reagents and buffers were purchased from Sigma and were used as received. All reagents and media were prepared using water treated with a Millipore Milli-Q water system to minimize trace metal-ion contamination.

Preparation of metal-substituted wt-HPCD and H200N-HPCD.

Cobalt-substituted HPCD and H200N-HPCD mutants were overexpressed in *E. coli* strain BL21 (DE3) and purified as previously described.[1-3] Protein concentrations were measured using the absorbance at 280 nm (ε_{280} = 45,000 M^{-1} cm^{-1}).[2] Metal incorporation was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) metal analysis at the Soil Research Analytical Laboratory (College of Food, Agriculture and Natural Resources Sciences, University of Minnesota). Samples were prepared for ICP-AES by digesting 2 ppm protein in 5% HNO_3 overnight and then removing precipitated protein by centrifugation.

EPR sample preparation and spectroscopic methods.

EPR samples were made to a volume of 300 μL, with 300 μM Co-HPCD in 50 mM pH 7.5 MOPS buffer. Samples were frozen by slow immersion in liquid nitrogen. Anaerobic samples were prepared by purging Co-HPCD and substrate separately by repeated cycling under argon and vacuum before mixing, followed by anaerobic transfer of samples into EPR tubes in a glovebox. [H200N-Co-HPCD(4NC/HPCA)O_2] complexes were prepared by mixing anaerobic enzyme-substrate complex with O_2-saturated buffer or by placing the enzyme substrate complex under the desired pressure of O_2 for 10 minutes before transferring the protein to EPR tubes to be frozen. For high O_2 pressure samples, the EPR tube and sample were put under the desired pressure of O_2 for 10 minutes and then rapidly frozen in an isopentane bath (-160 °C). EPR spectra were recorded on a Bruker Elexsys E-500 spectrometer equipped with an Oxford Instruments ESR-10 liquid helium cryostat at X-band (9.64 GHz). Spectra of Co-HPCD samples were acquired at 20 K over a magnetic field range of 100–6000 G using 2 mW power, 10 G modulation amplitude, and 100 kHz modulation frequency.

Determination of the dissociation constant for 4NC.

The K_D for 4NC binding to Co-HPCD (Figure S1) was determined by monitoring changes in the optical spectrum of 4NC at 600 nm (ε_{600} = 3,000 M^{-1} cm^{-1}) upon binding, as previously described.[4]

Kinetic experiments.

Enzyme used for 4NC experiments was first incubated with 2 mM H_2O_2 to inactivate any contaminating Fe-HPCD. Residual H_2O_2 was then removed by buffer exchange to prevent oxidation of 4NC to 4-nitroquinone. Single turnover reactions of 4NC by Co-HPCD were performed by rapidly mixing anaerobic [Co-HPCD(4NC)] in a sealed cuvette with O_2-saturated buffer (50 mM MES pH 6.0 at 22 °C) to yield the final desired O_2 concentration and then placing the reaction under the desired O_2 pressure. For reactions above 1 atm O_2, Co-HPCD was first equilibrated in a sealed cuvette under the desired O_2 pressure to which 4NC was added, and the reaction solution was rapidly mixed. High dissolved O_2 concentrations were achieved by using a sealable cuvette with screw cap lid from Strana with Teflon/Silcon disc septum from Thermo Scientific. The cuvette was held under pressure using a 19 gage needle connected to an O_2 cylinder using vacuum hosing. The O_2 pressure in the cuvette was set

by adjusted the pressure on the O_2 tank regulator. Dissolved O_2 concentrations were calculated using Henrys Law and the van't Hoff equation.[5] The reactions were monitored by UV-Vis absorption spectroscopy on a photodiode array spectrophotometer.

Global analysis.

The EPR and UV-Vis reaction progress curves (Figures 3 and S4) were fit simultaneously by global analysis to rate equations based on reaction Scheme S1 using the program DYNAFIT.[6] The O_2 concentration dependence data was obtained by monitoring the decay of the 4NC chromophore of the enzyme-substrate (ε_{516} = 8,700 M^{-1} cm^{-1}, ε_{390} = 5,600 M^{-1} cm^{-1}) and formation of the extradiol ring-cleaved product (ε_{516} = 1,600 M^{-1} cm^{-1}, ε_{390} = 16,500 M^{-1} cm^{-1}) complex. The EPR 4NC single turnover experiment has the advantage of being able to monitor the formation and decay of the intermediate directly.

$$\mathbf{ES + O_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathbf{ESO_2} \xrightarrow{k_2} \mathbf{E + P}$$

$$d[ES]/dt = -k_1[ES][O_2] + k_{-1}[ESO_2]$$

$$d[O_2]/dt = -k_1[ES][O_2] + k_{-1}[ESO_2]$$

$$d[ESO_2]/dt = k_1[ES][O_2] - k_{-1}[ESO_2] - k_2[ESO_2]$$

$$d[E]/dt = k_2[ESO_2]$$

$$d[P]/dt = k_2[ESO_2]$$

Scheme S1. Reaction scheme and corresponding rate equations used to simultaneously fit the EPR freeze-quench experiments and UV-Vis experiments (Figure 3 and S4) by global analysis for the single turnover reactions of [Co-HPCD(4NC)] with O_2.

pH-Activity profiles.

pH-Activity experiments were performed in a buffer mixture consisting of MES, bis-Tris, MOPS, Tris, CHES, and CAPS all at 25 mM concentrations with pHs ranging from 5.5–8.5. The final conductivity of each buffer was then adjusted to 6.4 mS using 2 M NaCl. The pH activity profile of Co-HPCD for the native substrate HPCA (180 nM Co-HPCD and 2 mM HPCA) was monitored in O_2-saturated buffer

at 22 °C, at the pH-independent isosbestic point for the colored product (ε_{350} = 13,900 M^{-1} cm^{-1}). The background rate of the base-catalyzed substrate auto-oxidation reaction monitored at 350 nm was subtracted for pH > 8.[7] The pH activity profile data (Figures S9 and S10) were fit using equations 1 or 2 to determine apparent pK_a values.[8] The b variables in Equations 1 and 2 accounts for basal activity at high or low pHs observed in pH activity profiles (Figure S9 and S10) in which the bulk solvent may be acting as the proton donor or acceptor.

Equation 1 $$k_{obs} = k - \left(\frac{k \cdot 10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \right) + b$$

Equation 2 $$k_{obs} = \left(\frac{k \cdot 10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \right) + b$$

EPR freeze quench kinetic experiments.

EPR freeze quench experiments were performed by rapidly mixing the anaerobic [Co-HPCD(4NC)] enzyme-substrate complex (pH 6.0 at 22 °C) with O_2-saturated buffer, and then stirring the reaction under 2 atm of O_2. Aliquots from the reaction were then taken every few minutes over the course of 120 minutes and rapidly frozen in EPR tubes. Initial concentration of reactants were 0.5 mM [Co-HPCD(4NC)] and 1.18 mM O_2.

Spin quantification.

To quantify the yield of the S = 1/2 species, the spectra of the anaerobic enzyme-substrate complex and the final time point sample were each integrated to get the total cobalt concentration. For the O_2 adduct, the g = 2 region (3150–3550 G) was integrated separately from the rest of the spectrum. The yield of the O_2 adduct was quantified according to the procedure of Aasa and Vänngård, which corrects the intensity of the integrated areas of highly anisotropic EPR spectra by multiplying by a correction factor that scales the area based on the g-values of each species.[9] The yield of the S = 1/2 species was also checked by simply monitoring the change in intensity of EPR features unique to each species (g = 4.8 for [Co-HPCD(4NC)], at g = 2.04 and 1.975 for [Co-HPCD(4NC)O_2], and at g = 8.9 for [Co-HPCD]). This procedure yields the normalized concentration of these two species at each time point, allowing the

yield to be calculated for the intermediate. (Normalized yield of ESO_2 = 1 - ES - E) This gave the same yield of the O_2 adduct as from the spin quantification method.

Supplementary Table and Figures:

Table S1. Electron paramagnetic resonance data for Co(II)-complexes and their Co(III)-superoxide adducts.

Sample	S =	g values	^{59Co}A (G)	^{17O}A (G)	References
Co-HPCD	3/2	6.7, 3.4, 2.4	80		[2]
[Co-HPCD(HPCA)]	3/2	7.6, 2.5, 1.9	98		[2]
[Co-HPCD(4NC)]	3/2	5.6, 3.5, 2.1	75		Figure S3
[Co-HPCD(4NC)O_2]	**1/2**	**2.10, 2.02, 1.99**	**24**		**Figure 4**
[H200N-Co-HPCD]	3/2	6.9, 3.7, 2.6	90		Figure S3
[H200N-Co-HPCD(4NC)]	3/2	6.3, 3.2, 1.9	53		Figure S3
[H200N-Co-HPCD(4NC)O_2]	**1/2**	**2.10, 2.02, 1.99**	**22**		**Figure 4**
Co-Mb	1/2	2.33, 2.32, 2.02	79, 6		[10]
[Co-Mb(O_2)] Species 1	1/2	2.081, 2.000, 1.99	-13.1, -23.5, -8.8	12, -72.5, 20	[10, 11]
[Co-Mb(O_2)] Species 2	1/2	2.079, 2.013, 1.985	-9.33, -16.7, -5.95	O_α5.0, -67.5, 2.4 O_β 5.4, -83.3, 30.3	[10, 11]
[Co-salMeDPT]	3/2	~ 4.3			[12]
[Co(salMeDPT)O_2]	1/2	2.086, 2.005, 1.999	18.7, 12.0, 13.6		[12-14]
[Co(saldpt)O_2]	1/2	2.085, 2.007, 2.000	22.7,13.3, 13.5		[15]
[Co(salen)THF]	1/2	2.4, 2.013	51, 127		[16, 17]
[Co(salen)O_2]	1/2	2.082, 2.024 1.996	25, 18		[16, 17]
[Co-(TPFC)O_2]	1/2	2.113, 2.017, 1.979	31.5, 10.3, 12.9		[18]
Vitamin B_{12r}	1/2	2.310, 2.190, 2.004	20, 26, 107		[19]
[Vitamin B_{12r}(O_2)]	1/2	2.089, 2.013, 1.993	-6.04, -23.0, -10.8	O_α43.3, 21.6, 21.6 O_β51.1, 25.6, 25.6	[19]
[Co(bzacen)pyO_2]	1/2	2.000, 2.085, 2.024	18, 19.2	60, 80	[17, 20-22]

[a]Abbreviations used: HPCD, Homoprotocatechuate 2,3-dioxygenase from *Brevibacterium fuscum*; HPCA, homoprotocatechuate; 4NC, 4-nitrocatechol; Co-Mb, cobalt-substituted sperm whale myloglobin; saldMeDPT, bis(salicy1idene-yiminopropyl)methylamine; saldpt, N,N'-bis(salicylidene)dipropylenetriamine; salen, N,N'-ethylenebis(salicylimine); THF, tetrahydrofuran; TPFC, 5,10,15-trispentaflurophenylcorrolato; bzacen, N,N′-ethylenebis(1-phenyl-3-imino-1-butanonato); py, pyridine.

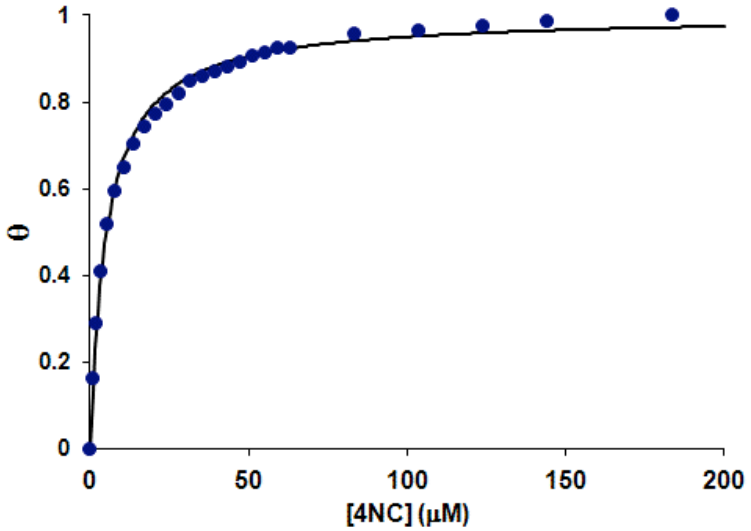

Figure S1. 4NC-binding curve from anaerobic titration of Co-HPCD with 4NC in 50 mM MES pH 6.0. Solid line represents a hyperbolic fit to data (K_D^{4NC} = 5 ± 2 μM).[4] The y-axis (θ) is the fraction 4NC bound.

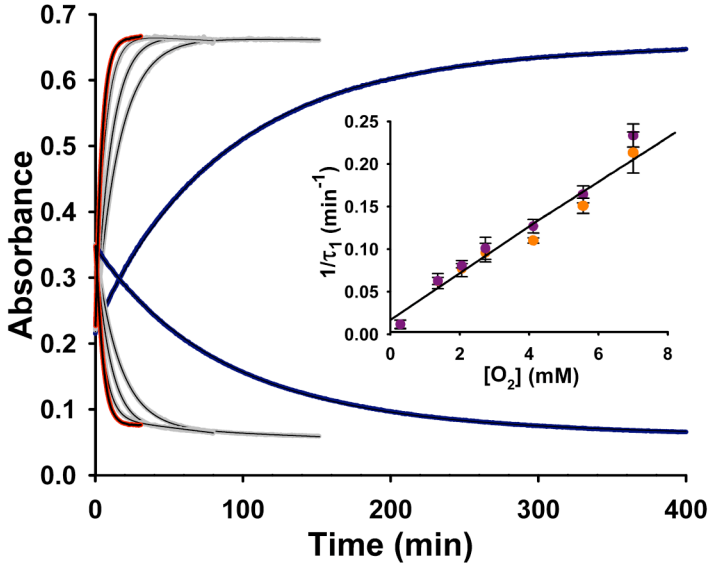

Figure S2. Single exponential fit of single wavelength traces for the single-turnover reactions of [Co-HPCD(4NC)] (30 μM 4NC and 150 μM Co-HPCD) with O_2 concentrations ranging from 0.29 mM (air-saturated buffer, blue lines) to 6.86 mM O_2 (5 atm O_2, red lines). Reaction conditions: 50 mM MES, pH 6.0 and 22 °C (K_D^{4NC} = 5 ± 2 μM, Figure S1). The reaction was monitored by following the decay of the 516 nm band and the growth of the 390 nm band. Thin black lines are the single exponential fit of experimental data. **Inset.** O_2 dependence of average 1/τ values from single exponential fits of data at both 516 (purple points) and 390 nm (orange points). The thin black line represents a linear fit of 1/τ data.

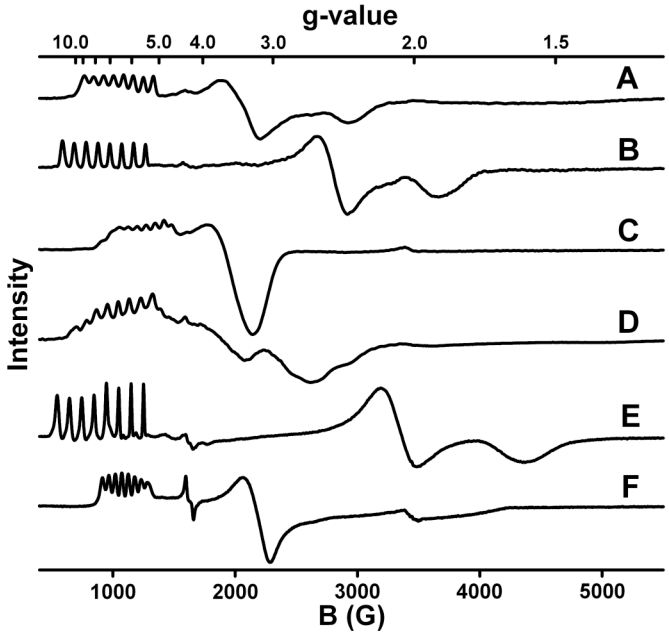

Figure S3. EPR spectra obtained at 9.64 GHz, 20 dB and 20 K in 50 mM MOPS pH 7.5. (A) wt-Co-HPCD[5] (B) anaerobic [wt-Co-HPCD(HPCA)][5], (C) anaerobic [wt-Co-HPCD(4NC)], (D) H200N-Co-HPCD, (E) anaerobic [H200N-Co-HPCD(HPCA)],and (F) anaerobic [H200N-Co-HPCD(4NC)].

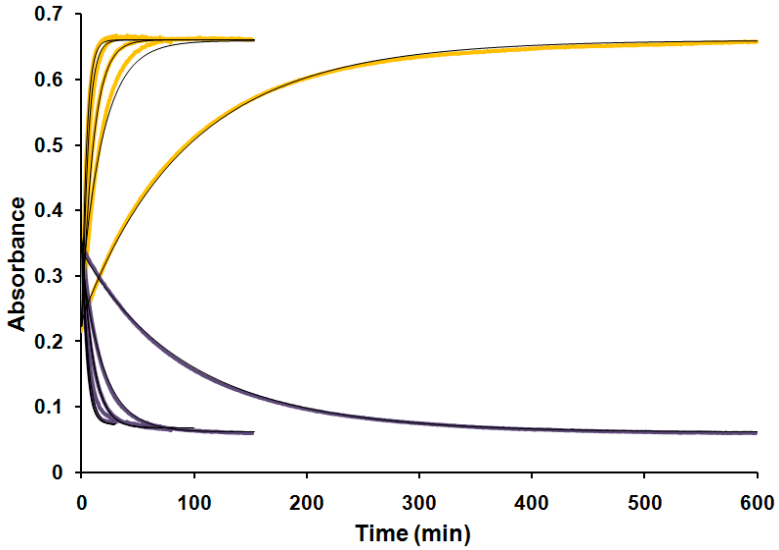

Figure S4. Global analysis fit of single-turnover reactions monitoring the decay of [Co-HPCD(4NC)] enzyme-substrate complex at 516 nm band (purple) and the growth of the 4NC extradiol ring cleaved product at 390 nm band (orange). UV-Vis data was simultaneously fit with EPR data in Figure 3 to rate equations based on the Scheme S1. Reaction conditions: [Co-HPCD(4NC)] (30 μM 4NC and 150 μM Co-HPCD) in the presences of 0.29, 1.37, 2.74, 4.12 and 6.86 mM O_2 (0.21–5 atm O_2, Figure S2), 50 mM MES, pH 6.0 and 22 °C. Thin lines represent fits of data to the O_2 binding and activation mechanism illustrated in Scheme S1 with rate constants k_1 = 40 ± 5 M^{-1} min^{-1}, k_{-1} = 0.05 ± 0.01 min^{-1}, and k_2 = 0.72 ± 0.06 min^{-1}.

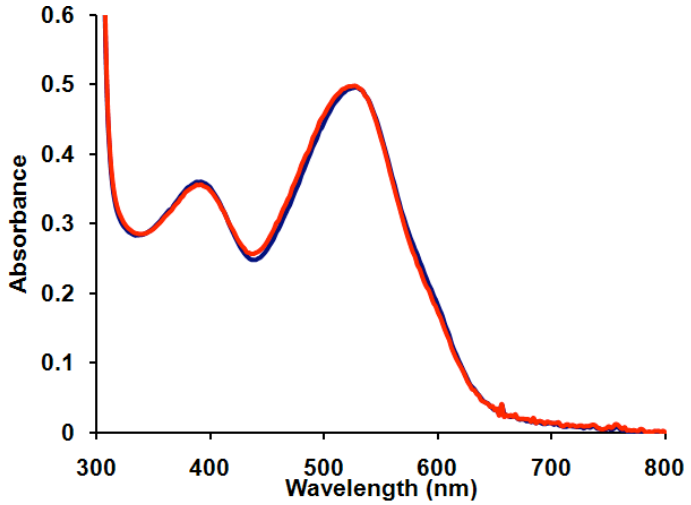

Figure S5. UV-Vis spectra of the anaerobic [H200N-Co-HPCD(4NC)] enzyme-substrate complex (blue), 40 μM 4NC and 150 μM H200N-Co-HPCD, and [H200N-Co-HPCD(4NC)O_2] (red), 40 μM 4NC, 150 μM H200N-Co-HPCD reacted under 2 atm O_2 for 2 hrs at 22 °C in 50 mM MOPS pH 7.5. A 50% yield of the O_2 adduct was measured by EPR for samples prepared under identical conditions (Figure S7).

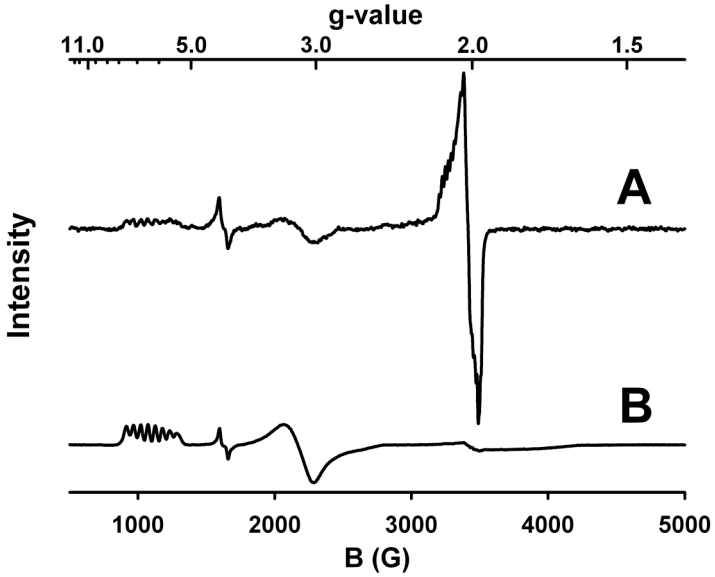

Figure S6. EPR spectra showing reversible binding of O_2 to [H200N-Co-HPCD(4NC)] in 50 mM MOPS pH 7.5. (A) Formation of the [H200N-Co-HPCD(4NC)] complex after exposure to 1 atm O_2 at 4 °C for 10 minutes to afford a ~40% yield of the $S = 1/2$ Co(III)-superoxide species, as measured by EPR spin quantification.[9] (B) Disappearance of the [H200N-Co-HPCD(4NC)O_2] complex after being purged with argon for 1 hr. Both spectra were obtained at 9.64 GHz, 20 dB and 20 K. The signal at $g = 4.3$ derives from contaminating high-spin Fe(III).

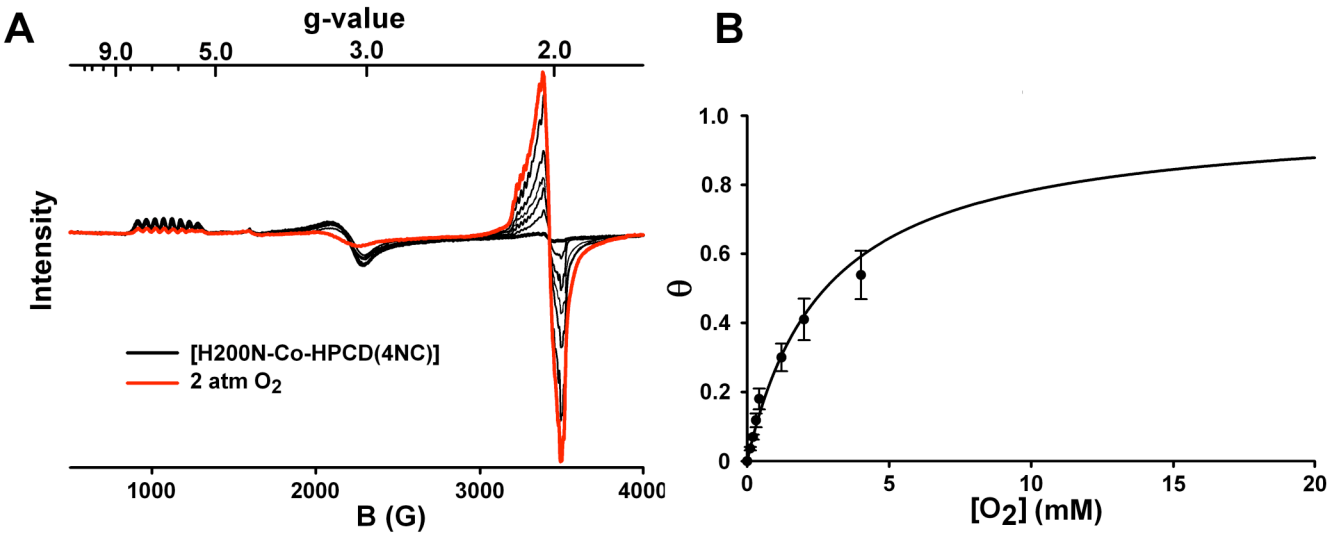

Figure S7. [H200N-Co-HPCD(4NC)] O_2 titration experiment monitored by EPR: **Panel A** EPR spectra of anaerobic [H200N-Co-HPCD(4NC)] incubated under increasing pressures of O_2 and then frozen in EPR tubes. Reaction conditions: 50 mM MOPS buffer pH 7.5 4 °C. **Panel B.** O_2-binding curve from EPR experiments K_D = 2.8 ± 0.2 mM O_2 (P_{50} = 1.4 ± 0.1 atm). The y-axis (θ) is the fraction O_2 bound.

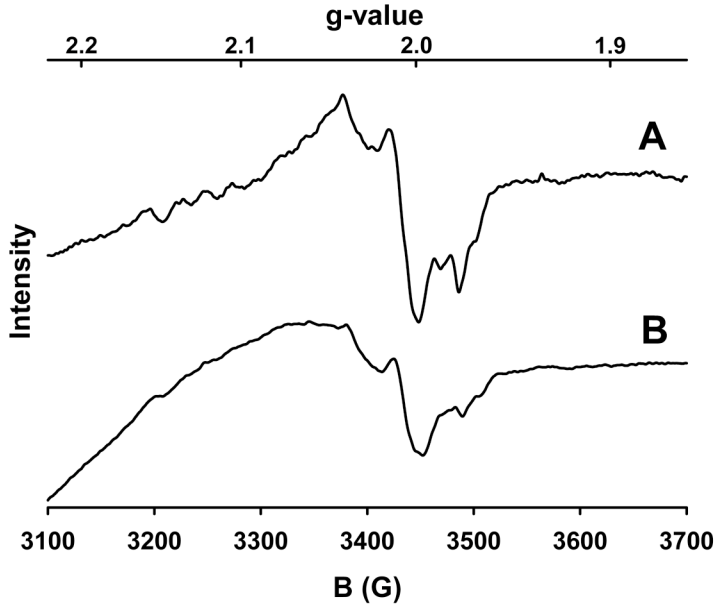

Figure S8. EPR spectra of (A) [Co-HPCD(4NC)$^{16}O_2$], and (B) [Co-HPCD(4NC)$^{17}O_2$] enriched with 45% $^{17}O_2$. Samples were prepared by mixing anaerobic [Co-HPCD(4NC)] in 50 mM MES at pH 5.5 with oxygenated buffer in EPR tubes and reacting the sample for ~5 min at 30 °C before freezing it in liquid nitrogen. Only the g = 2 regions are shown; spectra were obtained at 9.64 GHz, 20 dB microwave power at 20 K.

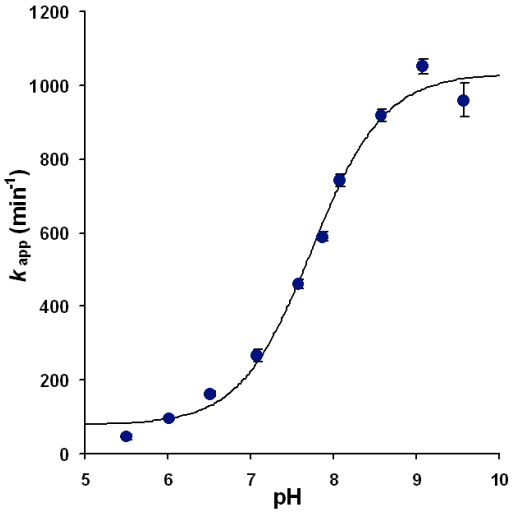

Figure S9. pH-Activity profile (blue circles) of extradiol ring cleavage of HPCA by Co-HPCD. Reaction conditions: 22 °C, 1 atm O_2 (1.37 mM O_2), 180 nM Co-HPCD, and 2 mM HPCA. Product formation was monitored at the pH-independent isosbestic point for the colored product (ε_{350} = 13,900 M^{-1} cm^{-1}). The background rate of the base catalyzed substrate auto-oxidation reaction monitored at 350 nm was subtracted for pH > 8.[7] The thin black line represents fit of data to equation 1 with an apparent pK_a of 7.74 ± 0.06.[8] Buffer mixtures consisting of MES, bis-Tris, MOPS, Tris, CHES, and CAPS, all at 25 mM concentrations were prepared at pH 5.5, 6.0 6.5, 7.0, 7.5, 8.0 and 8.5. The final conductivity of each buffer was then adjusted to 6.4 mS using 2 M NaCl.

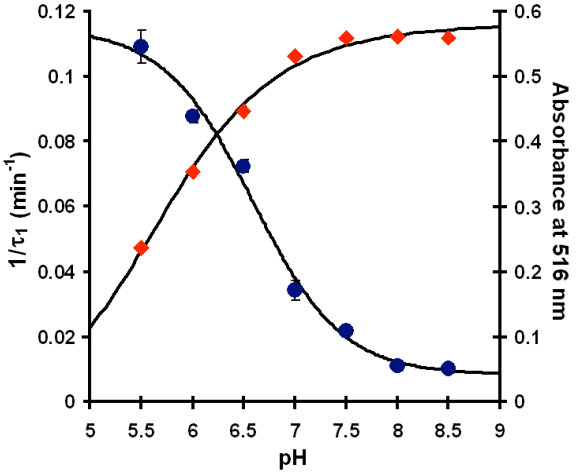

Figure S10. pH-activity profile (blue circles and left axis) of extradiol ring cleavage of 4NC by Co-HPCD. Reactions conditions: 2 atm O_2 (2.75 mM O_2), 40 μM 4NC, 120 μM Co-HPCD and 22 °C. UV-Vis spectrophotometric pH titration curve (red diamonds and right axis) of anaerobic [Co-HPCD(4NC)] enzyme-substrate complex (40 μM 4NC 150 μM Co-HPCD) shown in Figure S11A by monitoring dianionically bound form at 530 nm. Thin black lines represent fit of data to equation 1 and 2 with apparent pK_a's of 6.59 ± 0.09 and pK_a = 5.72 ± 0.05 for the pH-activity profile and spectrophotometric pH titration respectively.[8] Co-HPCD rapidly precipitates at pH ≤ 5.0. Buffer mixtures consisting of MES, bis-Tris, MOPS, Tris, CHES, and CAPS, all at 25 mM concentrations were prepared at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. The final conductivity of each buffer was then adjusted to 6.4 mS using 2 M NaCl.

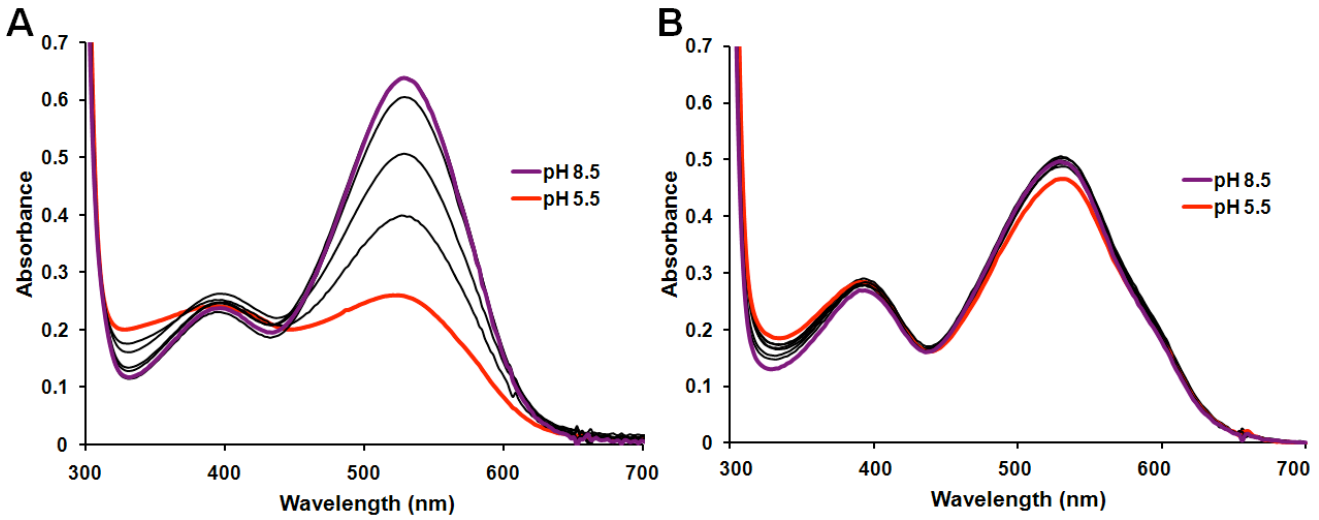

Figure S11. UV-Vis spectrophotometric pH titration of **(A)** anaerobic [Co-HPCD(4NC)] enzyme-substrate complex (40 μM 4NC and 150 μM Co-HPCD) and **(B)** [H200N-Co-HPCD(4NC)] (40 μM 4NC and 150 μM H200N-Co-HPCD). Buffer mixtures consisting of MES, bis-Tris, MOPS, Tris, CHES, and CAPS, all at 25 mM concentrations, were prepared at pH 5.5, 6.0 6.5, 7.0, 7.5, 8.0 and 8.5. The final conductivity of each buffer was then adjusted to 6.4 mS using 2 M NaCl.

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