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A Hyperactive Cobalt-Substituted Extradiol-Cleaving Catechol Dioxygenase

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Abstract

Homoprotocatechuate (HPCA) 2,3-dioxygenase from *Brevibacterium fuscum* (Fe-HPCD) has an Fe(II) center in its active site that can be replaced with Mn(II) or Co(II). While Mn-HPCD exhibits steady state kinetic parameters comparable to those of Fe-HPCD, Co-HPCD behaves somewhat differently exhibiting a significantly higher $K_M^{O_2}$ and k_{cat} . The high activity of Co-HPCD is surprising, given that cobalt has the highest standard M(III/II) redox potential of the three metals. Comparison of the X-ray crystal structures of the resting and substrate-bound forms of Fe-, Mn-, and Co-HPCD shows that metal-substitution has no effect on the local ligand environment, the conformational integrity of the active site, or the overall protein structure, suggesting that the protein structure does not differentially tune the potential of the metal center. Analysis of the steady state kinetics of Co-HPCD suggests that the Co(II) center alters the relative rate constants for the interconversion of intermediates in the catalytic cycle but still allows the dioxygenase reaction to proceed efficiently. When compared with the kinetic data for Fe- and Mn-HPCD, these results show that dioxygenase catalysis can proceed at high rates over a wide range of metal redox potentials. This is consistent with the proposed mechanism in which the metal mediates electron transfer between the catechol substrate and O_2 to form the postulated [M(II) (semiquinone)superoxo] reactive species. These kinetic differences and the spectroscopic properties of Co-HPCD provide new tools with which to explore the unique O_2 activation mechanism associated with the extradiol dioxygenase family.

Keywords

Bioinorganic chemistry; extradiol-cleaving catechol dioxygenase; Co(II)

Introduction

Catechol dioxygenases are essential microbial enzymes for the biodegradation of aromatic compounds [1, 2]. Homoprotocatechuate 2,3-dioxygenase (HPCD) isolated from *Brevibacterium fuscum* is an extradiol-cleaving catechol dioxygenase that catalyzes the cleavage of the C2-C3 bond of homoprotocatechuate (HPCA) with the incorporation of both oxygen atoms of O₂ into the product 5-carboxymethyl-2-hydroxymuconic semialdehyde (5-CHMSA) (Scheme 1) [3]. HPCD contains an active site Fe(II) center bound to a 2-His-1-carboxylate facial triad motif with solvent waters occupying the remaining three coordination sites [4-6]. Closely related is the homoprotocatechuate 2,3-dioxygenase from *Arthrobacter globiformis* (MndD), which is Mn(II)-dependent [7]. HPCD and MndD are very similar structurally, sharing 82% amino acid sequence identity, but they employ metals with standard aqueous redox potentials for the M(III/II) couples that differ by 0.79 V ($E^\circ = 0.77$ V vs SHE for the Fe(III/II) couple and 1.56 V for the Mn(III/II) couple) [8]. Previously, we have described metal-substitution experiments in which manganese was swapped for iron in HPCD and *vice versa* for MndD, producing Fe-MndD and Mn-HPCD [9]. Remarkably, all four enzymes (Mn-MndD, Mn-HPCD, Fe-MndD and Fe-HPCD) exhibit nearly the same activity of *ca.* 400 turnovers/minute at pH 7.8 (22 °C) when the metals are in their active reduced state. The electronic structures of the metal centers in each pair of enzymes are nearly indistinguishable, as demonstrated by the very similar EPR spectra for Mn-MndD vs Mn-HPCD, (Mn-MndD vs Mn-HPCD)/HPCA, and (Fe-MndD vs Fe-HPCD)/HPCA/NO complexes [9]. Moreover, high-resolution X-ray crystal structures of Fe-HPCD and Mn-HPCD are indistinguishable [4, 9], suggesting that the protein does not provide a means to tune the potentials of the Fe(II) and Mn(II) centers differentially, in contrast to the paradigm established by Fe- and Mn-superoxide dismutases [10-12]. For the latter enzymes, redox tuning is achieved by use of different second sphere residues that interact with the metal-bound solvent. Evidence that the Fe and Mn centers in HPCD and MndD maintain their redox potential differences also derives from the observation that only Fe-HPCD and Fe-MndD are inactivated by oxidants like hydrogen peroxide and ferricyanide [9], consistent with the lower potentials expected for the iron centers. This ability to maintain high rates of reductive oxygen activation over a large range of metal redox potentials is unique to the extradiol dioxygenases and mechanistically related enzymes [13-16]. In particular, it suggests that a mechanism is required that differs substantially from the classic heme oxygenase paradigm in which the metal center traverses through several redox states in the course of O₂ activation, implying a strong dependence on the metal redox potential [17, 18].

To harness the oxidizing power of triplet dioxygen, oxygenases generally activate O₂ by first reducing it to a more reactive superoxide or peroxide species [19, 20]. For many metalloenzymes, this entails the initial binding of O₂ to a divalent redox active metal center to yield a M(III)-superoxo species. Upon transfer of an electron from a redox partner, the superoxo species is converted to a M(III)-peroxo species [21, 22] that may itself be reactive or undergo further activation to generate an even more reactive high-valent metal-oxo species. Based on our initial studies [9], this paradigm does not appear to be followed by Fe-HPCD, Mn-MndD and their metal-exchanged homologs. While our recent spectroscopic evidence does show that short-lived M(III)-superoxo species can form in the reactions of O₂ with the ES complexes of Mn-HPCD and active site variants of Fe-HPCD [23, 24], we postulate that formation of the reactive species requires subsequent electron transfer from the catechol substrate to the metal center leading to a M(II)(semiquinone)(superoxo) species

[3, 9, 25]. Recombination of the radicals would then result in a spin-allowed formation of an alkylperoxo intermediate that would rearrange to the observed ring-cleaved product (Scheme 2) [9, 26-29]. This type of mechanism does not require a net change in metal oxidation state between the M(II)-catecholate ES complex and the reactive M(II) (semiquinone)(superoxo) intermediate, so the redox potential of the metal would not be critical so long as it is low enough for the initial O₂ complex to form.

As a further test of the hypothesis that the extradiol cleavage mechanism can operate with metal centers with significantly different redox properties, we have extended our investigation of metal-substituted HPCDs to other first-row transition metal ions. Remarkably, Co-HPCD is found to exhibit catalytic activity much higher than those found for Fe-HPCD and Mn-HPCD under O₂ saturating conditions. These results support the electron conduit hypothesis for the metal center in HPCD and provide new spectroscopic tools with which to further explore the unique mechanism of oxygen activation in the extradiol dioxygenase family.

Materials and methods

Reagents and general procedures

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

Preparation of M(II)-HPCD

Metal-substituted HPCDs were prepared by growing *E. coli* BL21(DE3) carrying plasmid pWZW204 containing the HPCD gene from *Brevibacterium fuscum* as previously described [9, 27, 30]. Upon inducing protein expression with isopropyl- β -D-thiogalactopyranoside (IPTG), the cultures were supplemented with 30 mg/L of an appropriate M(II)Cl₂ salt. In the procedure to obtain Cu-HPCD, CuCl₂ was added slowly, 10 mg/L at a time over 4 h, to avoid killing the cultures. The cells were then harvested and protein purified as previously described [30].

Molar absorptivity of Co-HPCD

The molar absorptivity of Co-HPCD was determined by the Bradford assay using bovine serum albumin (BSA) as a standard and found to be 45,000 M⁻¹ cm⁻¹ per 42 kDa subunit at 280 nm.

ICP-AES metal analysis

Metal incorporation was measured by ICP-AES metal analysis at the Soil Research Analytical Laboratory (College of Food, Agricultural and Natural Resource Sciences, University of Minnesota). Samples were prepared for ICP-AES analysis by digesting 2 ppm protein in 5% HNO₃ overnight and then removing precipitated protein by centrifugation.

Steady state kinetics

Enzyme activity was measured in air-saturated or oxygen-saturated 50 mM MOPS buffer pH 7.8 at 22 °C using a Beckman DU 640 spectrophotometer. Reactions were followed by monitoring formation of the extradiol-cleavage product 5-carboxymethyl-2-hydroxymuconic semialdehyde at 380 nm (5-CHMSA, $\epsilon_{380} = 38,000 \text{ M}^{-1} \text{ cm}^{-1}$) [9]. HPCA concentrations ranging from 1 μM to 4 mM were used to determine the K_M^{HPCA} for Co-HPCD. All other assays used 2 mM HPCA. Metal-substituted HPCDs (Mn, Co, Ni, Cu and Zn) were assayed after first incubating with 1 mM H₂O₂ for 5 min to inactivate any residual Fe-HPCD present

in the preparation. Apparent $K_M^{O_2}$ values were determined using a Clark-type oxygen electrode (Oxytherm from Hansatech Instruments) at 22 °C with 2 mM HPCA in 50 mM MOPS buffer pH 7.8. The apparent kinetic parameters k_{cat} and K_M were determined using nonlinear regression fits of initial velocities to the Michaelis-Menten equation from initial velocities. All k_{cat} values were normalized by dividing the average velocity from multiple assays by the average concentration from ICP-AES metal analysis for the metal of interest. Because of the limited sensitivity of the oxygen electrode at high oxygen concentrations (above 550 μ M O_2), oxygen kinetic experiments were also performed by mixing different ratios of anaerobic, air-saturated, and oxygen-saturated buffers. Reactions were then performed by monitoring the formation of product at 380 nm. Some reactions were performed under 2.0 atm of oxygen in a sealed cuvette (30 psi or 2.75 mM dissolved oxygen at 22 °C) to obtain a higher concentration of dissolved oxygen.

Activation and inhibition studies

The effects of L-ascorbate, H_2O_2 , potassium persulfate, and potassium ferricyanide were studied by incubating Fe-, Mn-, or Co-HPCD with 1 mM reagent for 5 min before assaying for enzyme activity.

Transient kinetic experiments

Anaerobic [Mn- or Co-HPCD(HPCA)] was rapidly mixed with oxygenated 50 mM MOPS buffer pH 7.8 using an Applied Photophysics model SX.18MV stopped flow device at 22 °C. The concentrations of reagents after mixing were 20 μ M HPCA and 50 μ M M-HPCD. The formation of the extradiol-cleavage product was monitored at 380 nm. O_2 concentrations after mixing ranged from 36 to 685 μ M O_2 [31].

X-ray crystallography

Crystals of Co-HPCD were grown by the hanging-drop method at 20 °C in 13% PEG6000, 0.05 M calcium chloride, 0.1 M Tris-HCl pH 6.5, and then were cryo-cooled in liquid nitrogen following a brief transfer into cryoprotectant solution containing 20–25% PEG400 in the mother liquor solution. For anaerobic [Co-HPCD(4-NC)] complex preparation, crystals of Co-HPCD were transferred into mother liquor solution at higher pH (13% PEG6000, 0.1 M calcium chloride, 0.1 M Tris-HCl pH 7.0) to increase the occupancy of the bound 4-NC in the active sites. All mother liquor solutions, crystals of Co-HPCD and 4-NC stocks were equilibrated in the anaerobic glove box atmosphere (Belle Technology) for at least 18 hours prior to mixing. The soaking reaction was initiated by anaerobic addition of 20 mM 4-NC to crystals of Co-HPCD. After 1-hour incubation, crystal complexes were rapidly transferred into mother liquor solution containing 25% PEG400 prior to cryo-cooling in liquid nitrogen inside the anaerobic glove box. Crystals of Fe- and Mn-HPCD were grown in 13% PEG 6000, 0.125 M calcium chloride, 0.1 M Tris-HCl, pH 6.5 and cryo-cooled in liquid nitrogen using 25% PEG400 as cryoprotectant. Buffers with superior buffering capacity to Tris-HCl at pH 6.5 were tested but were found to result in lower quality diffraction data. Adequate buffering was provided by the combination of Tris-HCl, PEG6000 ($pK_a \sim 6$), and the high concentration of the enzyme itself. X-ray diffraction data were collected at 100 K under the stream of liquid nitrogen. All diffraction data were processed using the XDS package [32], with Friedel pairs kept separate for anomalous difference datasets. For high-resolution datasets, the coordinates of the Fe-HPCD (PDB 2IG9) were used as an initial model in rigid body refinement followed by cycles of restrained refinement with Refmac5 [33] as part of the CCP4 program suite [34] and model building using Coot [35]. TLS was used in the final round of restrained refinement, with a single monomer defined as a TLS group. Link restraints to the metal (Mn, Fe or Co) were removed from the refinement to avoid bias in the refined metal-ligand distances. NCS restraints were not used during refinement, and the 4 subunits of the single enzyme molecule

present in the asymmetric unit were refined independently. Anomalous difference maps were calculated in CCP4 [35] using the calculated phases from PDB 3OJJ (Co-HPCD), PDB 3OJT (Fe-HPCD) or PDB 3OJN (Mn-HPCD) and the relevant anomalous difference amplitudes. X-ray data processing and refinement statistics are summarized in Tables S1, S2 and S3. All structure figures were produced using PyMOL Molecular Graphics System, Version 0.99, Schrödinger, LLC

UV-Vis absorption spectroscopy

Concentrated solutions of Co-HPCD exhibit a salmon pink color arising from the weak d-d transitions of Co(II). To accurately determine the extinction coefficients arising from these formally forbidden transitions, the UV-Vis spectrum of a 60 μ M Mn-HPCD, which has no chromophore in the visible region, was used as reference and subtracted from the spectra of 60 μ M Co-HPCD (40 μ M Co) and its complexes to obtain the difference spectra shown in the Results section.

EPR sample preparation and spectroscopic methods

EPR samples were made to a volume of 300 μ L, with 300 μ M Co-HPCD in 50 mM pH 7.8 MOPS buffer. Samples were frozen by slow immersion in liquid nitrogen. Anaerobic samples were prepared by purging Co-HPCD and HPCA separately by repeated cycling under argon and vacuum before mixing, followed by anaerobic sample transfers into EPR tubes using cannula. 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 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.

Results

Metal-substituted enzymes

Metal-substituted HPCDs were expressed in *E. coli* cells grown in M9 minimal media in the presence of added Co(II), Ni(II), Cu(II), or Zn(II) ions. Enzyme preparations were analyzed by ICP-AES for their iron, manganese, cobalt, nickel, copper and zinc metal contents. The average metal occupancies (Table 1) show the highest incorporation for Co(II) and Cu(II) with little incorporation of Ni(II) and Zn(II). Contaminating amounts of iron and manganese were found in all preparations, but were typically less than or equal to 0.13 and 0.04 equivalents, respectively.

Activation and inhibition studies

The activities of metal-substituted HPCD enzymes in the presence of reducing or oxidizing agents were examined to correlate the specific metal present with dioxygenase activity. The residual activity due to contaminating Fe-HPCD could be removed by incubating protein with 1 mM H₂O₂ or another oxidant for 5 minutes before the assay. The activity due to contaminating manganese was calculated by assuming that all Mn detected by ICP-AES gave rise to active Mn-HPCD. Of the various metal-substituted enzymes studied, only Co-HPCD showed significant HPCA cleavage activity (Figure 1, top) that could not be accounted for by contaminating iron or manganese. The Co-HPCD preparations also showed a good correlation between metal content and specific activity (Figure 1, bottom), while preparations with other metals showed no such correlation. Consequently, the Cu(II)-, Ni(II)-, and Zn(II)-substituted enzymes are not considered further here.

As previously reported, Fe-HPCD was found to be susceptible to inactivation by oxidants such as H₂O₂, [Fe(CN)₆]³⁻ and even O₂, but regained full activity after incubation with ascorbate (Table 2) [9]. In contrast, Co-HPCD, like Mn-HPCD, was essentially unaffected

by treatment with the strong oxidizing agents H_2O_2 and potassium persulfate, as only a small decrease in activity was observed for both the Mn-HPCD and Co-HPCD samples assayed. This decrease can presumably be attributed to inactivation of contaminating Fe(II)-HPCD in the samples. Indeed, a small increase in activity was also observed for both Mn-HPCD and Co-HPCD upon incubation with L-ascorbate, reflecting activation of air-oxidized Fe(III)-HPCD contaminant. Figure 2 illustrates the sensitivity of Fe-, Mn- and Co-HPCDs to O_2 . Fe-HPCD afforded only 1,000–2,500 turnovers in air-saturated buffer, while both Mn- and Co-HPCD consumed all the HPCA (33,000 turnovers) under these conditions. The latter observations clearly show that the activity of these metal-substituted enzymes is not due to contamination by Fe-HPCD. The resistance of Co-HPCD to H_2O_2 -induced inactivation suggests that the cobalt center retains a high redox potential relative to iron when it is bound in the protein.

Steady state kinetics

The apparent Michaelis-Menten kinetic parameters for reactions of Fe-, Mn-, and Co-HPCD enzymes with HPCA are reported in Table 1. The apparent k_{cat} for Co-HPCD ($215 \pm 8 \text{ min}^{-1}$), is approximately half the values reported for Fe-HPCD ($470 \pm 20 \text{ min}^{-1}$) and Mn-HPCD ($370 \pm 20 \text{ min}^{-1}$) in air-saturated ($260 \mu\text{M O}_2$) 50 mM MOPS buffer at pH 7.8, 22 °C. However, during the course of efforts to measure the $K_{\text{M}}^{\text{O}_2}$ for Co-HPCD, we found that the k_{cat} continued to increase even as O_2 approached saturation (1.37 mM at 22 °C) (Figure 3). Mn-HPCD showed zero-order kinetics at these O_2 concentrations, and the Fe-HPCD activity decreased slightly ($280 \pm 10 \text{ min}^{-1}$), presumably due to oxidation of the Fe(II) cofactor (Figure 1 top). Co-HPCD was found to be the most active ($590 \pm 20 \text{ min}^{-1}$) of the three enzymes under these high O_2 conditions (Figure 1).

From fitting the Michaelis-Menten plot for oxygen dependence (Figure 3) a maximum rate of $1120 \pm 70 \text{ min}^{-1}$ for Co-HPCD under truly oxygen saturating conditions is possible. The apparent $K_{\text{M}}^{\text{O}_2}$ for Co-HPCD is $1200 \pm 100 \mu\text{M}$ (Figure 3, Table 1), twenty-fold higher than those for either Fe-HPCD ($50 \mu\text{M}$) or Mn-HPCD ($60 \mu\text{M}$). The catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$) of $0.9 \pm 0.1 \mu\text{M}^{-1} \text{ min}^{-1}$ for Co-HPCD is an order of magnitude smaller than the corresponding values for Fe- and Mn-HPCD (7.8 ± 0.3 and $7.4 \pm 0.6 \mu\text{M}^{-1} \text{ min}^{-1}$, respectively). This suggests that [Co-HPCD(HPCA)] has a lower effective O_2 affinity, but the overall rate limiting step for Co-HPCD must be more efficient than those of Mn- and Fe-HPCDs.

X-ray crystal structure comparisons

One possible explanation for the high activity of Co-HPCD is that the structure of the active site may differ from that of Fe-HPCD such that the redox potential of the Co(II) ion becomes more similar to that of Fe(II) in Fe-HPCD. Recently, we reported the full length structures of Fe-HPCD (PDB 2IG9) and for Mn-HPCD (PDB 3BZA) [9, 26]. Higher resolution structures of Fe-HPCD (1.70 Å, PDB 3OJT) and of Mn-HPCD (1.65 Å, PDB 3OJN) are also reported here (Tables S2 and S3) and used for all comparisons. To evaluate the structural consequences of the cobalt substitution, the X-ray crystal structure of Co-HPCD was solved to 1.72 Å resolution (Table S1). The presence of cobalt in the active site of HPCD was confirmed by anomalous difference X-ray diffraction data collected at the cobalt K-absorption edge and pre-edge energies (Figure 4 and S1, Table S1). Four strong anomalous difference peaks corresponding to the positions of the four active site metals in the Co-HPCD tetramer are observed in X-ray diffraction data collected at the cobalt K-edge X-ray absorption energy. Data collected for Fe-HPCD and Mn-HPCD at their respective metal K-edge energies show the metal binding in the same position as in Co-HPCD (Figure S1). The comparison of the resting state structures shows that the identity of the metal (Fe, Mn, Co) has no effect on the overall protein structure as indicated by the RMSD values of

0.18–0.20 Å for superposition of all atoms of the Fe-HPCD (PDB 3OJT), Mn-HPCD (PDB 3OJN) and Co-HPCD (PDB 3OJJ) structures. Moreover, no significant structural differences are observed locally in the active site environment as indicated by the RMSD values of 0.10–0.14 Å for superposition of all atoms within 15 Å of the metal center in the Fe-HPCD and Co-HPCD structures. As illustrated in Figure 5A, cobalt substitution also preserves the distorted octahedral coordination geometry of the metal center in which solvent molecules occupy three coordination positions on one face of the metal as observed in the new, high-resolution structures of Fe-HPCD and Mn-HPCD. In addition, the presence of Co in the active site does not elicit any changes in the mode of substrate binding or in the conformational geometry of the active site residues in the enzyme-substrate complex (Figure 5B). These results suggest that the protein structure does not tune the redox potential of Co(II) into the range of Fe(II), and the observed activity is thus due to Co(II) with a much higher redox potential than that of Fe(II) in the active site. Therefore, any observed differences in activity must arise from the inherent electronic properties of the metal rather than from structural properties of the protein *per se*.

Spectroscopic characterization

It is also possible that the activity of the Co-HPCD stems from a perturbation of the protein structure in solution that is not revealed in the crystal structure, leading to a distinct redox potential tuning. The rich optical and EPR properties of Co-HPCD can be used to examine the electronic and geometric structure of the Co(II) center. There is a well-established correlation between the coordination number and the extinction coefficients of the d-d transitions normally observed in the visible region from 450–750 nm of high-spin Co(II) model complexes [36–38]. As the coordination number and symmetry at the metal center decrease, the intensities of the forbidden d-d transitions increase due to greater p-d orbital mixing. Consequently, typical six-coordinate Co(II) complexes have molar absorptivities less than 50 M⁻¹ cm⁻¹, while five-coordinate complexes have values between 50–250 M⁻¹ cm⁻¹ and four-coordinate complexes, greater than 300 M⁻¹ cm⁻¹. Co-HPCD solutions exhibit a pale salmon pink color that is due to a broad visible absorption at 500 nm with a molar extinction coefficient of 60 M⁻¹ cm⁻¹, suggesting either a five-coordinate or highly distorted six-coordinate high-spin Co(II) center (Figure 6) [36–38]. The anaerobic addition of HPCA to Co-HPCD does not elicit a significant change in the visible spectrum, suggesting no change in the coordination number upon substrate binding. The fairly unstructured broad nature of the absorption bands in both complexes favors a six-coordinate over a five-coordinate center [37]. Therefore, our findings from the UV-Vis spectroscopy (Figure 6) are in agreement with the conclusions drawn from the X-ray crystal structure analysis (Figure 5 and Table S4), which revealed a distortion of the cobalt ligand environment away from an idealized six-coordinate geometry.

X-band EPR spectra of Co-HPCD and its complex with HPCA are shown in Figure 7. These spectra were collected under non-saturating conditions at 20 K, because at lower temperatures (*i.e.* 5 K) the spectra exhibited an absorption-like appearance due to rapid-passage effects commonly observed for cobalt complexes at 4 K due to slow relaxation [39, 40]. Co-HPCD exhibits a dominant rhombic signal with *g* values at 6.7, 3.4 and 2.4 (Figure 7), arising from the ground Kramers doublet of a high-spin d⁷ Co(II) center (*S* = 3/2) [37, 40, 41]. The low field signal exhibits well-resolved 8-fold hyperfine splitting with *A* = 80 G, due to the nuclear spin of ⁵⁹Co (*I* = 7/2). Anaerobic addition of HPCA drastically perturbs the spectrum (Figure 7), leading to an increase in both the rhombicity of the EPR signal (*g* = 7.6, 2.5, 1.9) and the magnitude of the ⁵⁹Co hyperfine splitting of the low-field signal (*A* = 98 G) (Figure 7) [7, 42].

Table 3 compares the EPR parameters of Co-HPCD complexes with those found for other cobalt containing enzymes as well as several model complexes. This collection of data

reveals that $S = 3/2$ Co(II) centers can exhibit a large range of rhombicities that give rise to g values as high as 9 and as low as 1. The spectra of Co-HPCD and [Co-HPCD(HPCA)] have rhombicities comparable to those observed for Co-glyoxalase I, [CoZnAAP(LPA)], and [ZnCoAAP(LPA)] [38, 42, 43]. Co-HPCD and [Co-HPCD(HPCA)] both exhibit ^{59}Co hyperfine splitting with large A values of 80 and 98 G, respectively. In general, octahedral and square pyramidal complexes exhibit well resolved hyperfine features with large A values, while trigonal bipyramidal and tetrahedral complexes have smaller values [37, 44]. Thus, the EPR data support a coordination number of six for the metal centers in Co-HPCD and [Co-HPCD(HPCA)], consistent with visible absorption spectral data as well as the results obtained from X-ray crystallography.

Transient kinetic experiments

The lack of visible absorption features exhibited by the metal center and the native substrate (HPCA) of Fe-HPCD has complicated transient kinetic experiments in the past. One approach has been to monitor the time course of formation of the yellow ring-cleaved product after mixing anaerobic [Fe-HPCD(HPCA)] with oxygenated buffer [31]. In these experiments, a short lag phase prior to product formation was observed and the time course could be fit with two summed exponential phases, neither showing O_2 concentration dependence. Preliminary stopped flow experiments for the reactions of the [Co-HPCD(HPCA)] and [Mn-HPCD(HPCA)] enzyme substrate complexes with oxygenated buffer are shown in Figure 8. Like Fe-HPCD, both Co- and Mn-HPCDs exhibit a lag phase in product formation. While the Mn-HPCD time course shows only a small O_2 concentration dependence, that of Co-HPCD is much more significant and exhibits multiple phases, the detailed analysis of which will be reported in a subsequent publication.

Discussion

Co(II) has been successfully incorporated into the active site of HPCD, producing the first example of an active cobalt-containing, extradiol ring-cleaving, catechol dioxygenase. In combination with our previous study of Mn-HPCD [9], the results reported here show that the native Fe(II) center of HPCD can be replaced by the metal ions to both its left and right in the first-row transition metal series. Co(II)-HPCD is in fact highly active, having the largest k_{cat} yet found for any metal-HPCD preparation. Nevertheless, it is significant that in contrast to Mn-HPCD, Co-HPCD exhibits a $K_{\text{M}}^{\text{O}_2}$ value quite different from that observed for the native Fe-HPCD. This suggests that new insights into the chemical steps of oxygen activation and insertion by extradiol dioxygenases may be revealed by the study of this new metal-substituted enzyme form. This is discussed in the sections that follow in the context of the current proposal for the enzyme mechanism.

Mechanism proposed for extradiol dioxygenases

The mechanism proposed for the extradiol-cleaving catechol dioxygenases is depicted in Scheme 2 [9, 26-29]. In the resting, as isolated state, the high-resolution structure of the enzyme reported here shows that it has a six-coordinate Fe(II) center coordinated by three protein amino acid residues in a 2-His-1-carboxylate facial triad metal binding motif and three *cis*-labile solvent-derived ligands occupying the remaining three facial coordination sites (Scheme 2A) [4, 26, 53, 54]. Catecholic substrate binds to the iron as a monoanionic bidentate ligand, displacing at least two of the bound waters (Scheme 2B) [55, 56]. The third solvent is observed with variable occupancy in some crystal structures but is not observed in others [4, 54, 57], suggesting that it becomes more labile upon substrate binding. The coordination of the anionic substrate serves to lower the redox potential of the Fe(II) center and promote the dissociation of the final solvent and the binding of O_2 to form a transient Fe(III)-superoxo intermediate (Scheme 2C), following the heme paradigm [17, 18].

Although the putative Fe(III)-superoxo intermediate has not to date been directly detected in the wild type enzyme, it has recently been trapped in the case of the H200N mutant with 4-nitrocatechol as substrate [24]. This supports the hypothesis that the Fe(III)-superoxo species is formed, but it has a very short lifetime. At this point in heme enzyme mechanisms, an electron from an external reductant is introduced to form an Fe(III)-peroxo intermediate. Instead for HPCD, an electron is postulated to be transferred from the catecholate substrate to the iron center, generating a substrate radical-Fe(II)-superoxide species (Scheme 2D). Subsequent C–O bond formation between the semiquinone radical and superoxide leads to an alkylperoxo-Fe(II) species (Scheme 2E) that breaks down via a lactone intermediate (Scheme 2F) to afford the ring-opened product (Scheme 2G).

Strong support for this scheme derived from recent crystallographic studies where exposure of [Fe-HPCD(4-NC)] to trace O₂ resulted in intermediates D, E, and G being trapped in different active sites present in the asymmetric unit [26]. The apparent stability of these intermediates *in crystallo* allowed them to be structurally characterized revealing geometries very close to those predicted from biochemical, chemical, and computational studies [26, 29, 58].

Subsequent spectroscopic studies on the reaction of [Mn-HPCD(HPCA)] with O₂ [23] also provided support for this mechanistic scheme. These studies applied rapid freeze-quench techniques and took advantage of the EPR properties of the $S = 5/2$ Mn(II) center of Mn-HPCD to monitor intermediates formed during the reaction. At the shortest observable time after mixing (approximately 15 ms), an $S = 5/2$ species comprised of Mn(III) spin coupled to a radical was observed and proposed to originate from either a Mn(III)-superoxide (Scheme 2C) or a Mn(III)-peroxide-HPCA radical moiety. Within 30 ms this species converted to a second $S = 5/2$ species attributed to a Mn(II) species, which was tentatively assigned to the proposed alkylperoxo-Mn(II) intermediate (Scheme 2E).

Basis for the high turnover rate of Co-HPCD

The substitution of the native Fe(II) in HPCD with Mn(II) or Co(II) to produce comparably active enzymes is quite remarkable, given the redox process implied by intermediate C in the proposed extradiol dioxygenase mechanism (Scheme 2). The three metal ions that afford active HPCD have standard aqueous M(III/II) potentials that span a range of nearly 1.2 V ($E^\circ = +1.92$ V vs SHE for Co(III/II), $+1.51$ V for Mn(III/II), and $+0.77$ V for Fe(III/II), so much slower rates would normally be expected for the Mn(II) and Co(II) containing enzymes relative to the native Fe-containing enzyme for the steps involving electron transfer. In contrast, we show in the current and previous studies that Fe-HPCD and Mn-HPCD exhibit similar rates [9], while Co-HPCD shows a higher turnover rate. One possibility explored here is that the protein tunes the redox potentials to similar values, as seen in the case of the Fe- and Mn-superoxide dismutases where differences in second sphere residues affect the ionization state of a water-derived ligand, and in turn, the redox potential of the metal center [10-12]. However, based on the X-ray crystallographic analysis described above, the structure of resting Co-HPCD is indistinguishable from those of its iron and manganese counterparts (Figure 5A). More relevantly, the second sphere amino acid residues of the enzyme-substrate complex [Co-HPCD(4-NC)] and the [Fe-HPCD(4-NSQ)superoxo] intermediate [26] show no significant structural differences as seen in Figure 5B. Thus, differential redox potential tuning of the metal centers in Fe-, Mn-, and Co-HPCDs is unlikely, and we expect the relative order in redox potential, i.e. Fe < Mn < Co, to be maintained. This notion is supported by the susceptibility of Fe-HPCD to inactivation by O₂ and H₂O₂ and the insensitivity of Mn- and Co-HPCD to these oxidizing agents (Table 2). The absence of differential redox potential tuning of the metal centers by second sphere residues is not unreasonable, considering that the Mn(II) and Co(II) forms of HPCD are created by altering growth conditions and have not evolved naturally.

It is notable that Co-HPCD, with a relatively inaccessible Co(III) state, is active, indeed hyperactive when compared to the native Fe(II)-containing enzyme. To rationalize these observations, one must either consider a non-redox-active role for the metal center (i.e. no intermediate C) or postulate that the metal center in HPCD acts primarily as a conduit for electron transfer between the catechol substrate and O₂ (i.e. short lived intermediate C) in these dioxygenases. In the latter scenario, initial electron transfer from the divalent metal center to O₂ would elicit an immediate subsequent electron transfer from substrate to the nascent trivalent center to form the reactive [M(II)(semiquinone)superoxo] species (Intermediate D). The redox potential of the metal employed would then affect these two steps in a self-compensating manner. The expected higher potentials of the metal centers of the Mn(II)- and Co(II)-substituted enzymes would make them poorer reducing agents in the reductive oxygen binding step than the Fe(II) center in the wild-type enzyme, but the resulting M(III)-superoxo intermediates would be stronger oxidizing agents, thereby facilitating the subsequent oxidation of the bound substrate. The net result would be that the rate of conversion from the M(II)-catecholate to the reactive [M(II)(semiquinone)superoxo] species would be largely unaffected by the specific metal present so long as the O₂ adduct with the metal complex could form. From the point of view of thermodynamics, the electron donor in this oxygen activation reaction is the substrate and the electron acceptor is the O₂, so the potential of the metal catalyst that connects these two redox active species has no effect on the position of equilibrium, provided that the M(III)-superoxo state is accessible.

Co(II) substitution in other dioxygenases can be used to illustrate the significance of the current proposal. Co(II) incorporated into quercetin 2,3-dioxygenase (QueD) from *Bacillus subtilis* (Co-QueD) also results in an active enzyme [14]. This enzyme utilizes a mechanism developed from that proposed in Scheme 2 for the extradiol dioxygenases in which substrate and oxygen bind to the active site metal and are simultaneously activated [14]. Thus, it is reasonable that the enzyme should use metals over a range of potentials. In contrast, taurine/ α -ketoglutarate dioxygenase (TauD) generates an Fe(IV)=O intermediate from the reaction of oxygen with α -ketoglutarate bound to its Fe(II) center [59]. The required change in metal oxidation state from +2 to +4 in this case suggests that the mechanism would be very sensitive to the redox potential of the metal center, and indeed, Co-TauD is inactive [45].

Effect of Co(II) substitution on reaction cycle rate constants

Co-HPCD exhibits a 20-fold larger $K_M^{O_2}$ value (1200 μ M) than do Mn- and Fe-HPCD. To the extent that this value reflects affinity, it suggests that O₂ binds less tightly to Co-HPCD. A similarly high $K_M^{O_2}$ of 1230 μ M is reported for the cobalt-substituted form of QueD [13]. In its simplest form, the ordered bi uni mechanism of HPCD gives $K_M^{O_2} = (k_{-2} + k_3)/k_2$, where the rate constants shown represent collections of rate constants for individual steps involved in O₂ binding (k_2), O₂ release (k_{-2}), and product release (k_3) (Scheme 3) [60]. Thus, the higher $K_M^{O_2}$ of Co-HPCD reflects a lower rate constant for O₂ binding and/or a higher rate constant for the release of O₂ from the enzyme. Given the decrease in the electron sharing capability of the higher potential cobalt center, a lower affinity for O₂ would likely contribute significantly to the high $K_M^{O_2}$ values of Co-HPCD (and Co-QueD).

Transient kinetic studies of the reaction of [Fe-HPCD(HPCA)] with O₂ and more detailed studies of [Fe-HPCD(4-NC)] with O₂ showed that none of the rate constants for the individual steps observed in the product formation and release portion of the cycle were dependent on O₂ concentration, indicating either effectively irreversible O₂ binding or an irreversible step between O₂ binding and the first observable step [31, 61]. A similar detailed analysis of the kinetics of Co-HPCD is complicated by the slow turnover of 4-NC and the multiple, low-amplitude phases of the HPCA reaction, but it is clear that the overall product formation reaction exhibits a dependence on O₂ concentration (Figure 8). Additionally, the true k_{cat} of Co-HPCD, nominally $1120 \pm 70 \text{ min}^{-1}$ under fully saturating

O₂ conditions, is much higher than that of Fe-HPCD ($470 \pm 20 \text{ min}^{-1}$), showing that the rate of at least one previously rate limiting step has more than doubled upon incorporation of cobalt into the active site. For the reaction of [Fe-HPCD(HPCA)] with O₂, the rate limiting step is believed to be part of the product release sequence, perhaps the step immediately before the actual release of product from the active site [31, 61]. The increase in k_{cat} in the case of Co-HPCD shows that this step is at least 2.5 times faster and may be even faster if it is no longer rate limiting as discussed below.

The catalytic efficiency for the use of O₂ by Co-HPCD ($0.9 \pm 0.1 \mu\text{M}^{-1} \text{ min}^{-1}$) is an order of magnitude smaller than the $k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$ values for the Fe(II)- and Mn(II)-containing enzymes (7.8 ± 0.3 , and $7.4 \pm 0.6 \mu\text{M}^{-1} \text{ min}^{-1}$, respectively). The $k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$ value is comprised of all of the rate constants for steps between O₂ association through the first irreversible step, presumably either formation of the alkylperoxo-Fe(II) intermediate or the O–O bond cleavage step that follows [62]. Thus, the low value of $k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$ suggests that a step early in the O₂ activation process is significantly slowed in the Co-HPCD reaction. This is consistent with the expected higher potential of Co(II), which should decrease its affinity for O₂.

The $k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$ value can also be viewed as reflective of the second-order rate constant for the reaction of the enzyme-substrate complex with O₂. The first-order rate constant calculated by multiplying the observed $k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$ value by the O₂ concentration at which it was observed is very close to the turnover number under those conditions. For example, an apparent first-order rate constant of $670 \pm 70 \text{ min}^{-1}$ is calculated in oxygen-saturated buffer ($1370 \mu\text{M O}_2$; 22 °C; pH 7.8) compared to the observed k_{cat} of $590 \pm 20 \text{ min}^{-1}$. This suggests that the rate-determining step has moved from a step in the product release sequence to an earlier step in the oxygen binding and activation sequence. This possibility was examined further in stopped flow transient kinetic experiments in which [Co-HPCD(HPCA)] was rapidly mixed with oxygenated buffer over a range of O₂ concentrations (Figure 8A). Each kinetic time course shows a long lag phase when monitored at 380 nm as expected, because the chromophore results from formation of product in the active site. The fact that a lag phase is easily observed suggests that the steps before product formation are not exceptionally fast compared with the product-forming step itself. However, unlike Fe- and Mn-HPCDs (Figure 8B), the formation of product by Co-HPCD shows significant O₂ concentration dependence [31]. This observation rules out the possibility that the increases in $K_{\text{M}}^{\text{O}_2}$ and k_{cat} are due solely to an increase in product dissociation rate. The O₂ concentration dependence seen for Co-HPCD could be caused by either very weak (i.e. fully reversible) O₂ binding and/or a slow step early in the overall process of O₂ binding and product formation [31]. We argue here that both may be true for the Co-HPCD enzyme. In contrast, for Fe- and Mn-HPCD, the rate determining step occurs late in the process and transient kinetic experiments with Fe-HPCD and its His200 variants indicate that O₂ binding is fast and effectively irreversible [31, 61].

Effects of Co(II) substitution on HPCD catalysis

It seems likely that the presence of cobalt in the active site of HPCD has two effects. First, its high potential slows one or more steps in the oxygen binding process so that it (they) becomes rate limiting and the $k_{\text{cat}}/K_{\text{M}}^{\text{O}_2}$ value falls. Second, it accelerates one or more steps in the product release sequence so that this portion of the reaction cycle is no longer rate limiting. The net result of this is that the overall reaction is faster because the decrease in the O₂ binding sequence rate is not as large as the increase in the rate of the product release sequence.

The studies reported here do not suggest that Co-HPCD utilizes a mechanism different from that shown in Scheme 2 for Fe- and Mn-HPCD, but merely that the relative rates of

intermediate steps have changed. This raises the prospect of trapping early reaction cycle intermediates for detailed spectroscopic characterization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

5-CHMSA	5-Carboxymethyl-2-hydroxymuconic semialdehyde
Co-HPCD	Cobalt-substituted homoprotocatechuate 2,3-dioxygenase from <i>Brevibacterium fuscum</i>
EPR	Electron paramagnetic resonance
ES	Enzyme substrate complex
Fe-HPCD	Iron-dependent homoprotocatechuate 2,3-dioxygenase from <i>Brevibacterium fuscum</i>
Fe-MndD	Iron-substituted homoprotocatechuate 2,3-dioxygenase from <i>Arthrobacter Globiformis</i>
HPCA	Homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid)
ICP-AES	Inductively coupled plasma spectrometry atomic emission spectroscopy
Mn-HPCD	Manganese-substituted homoprotocatechuate 2,3-dioxygenase from <i>Brevibacterium fuscum</i>
Mn-MndD	Manganese-dependent homoprotocatechuate 2,3-dioxygenase from <i>Arthrobacter Globiformis</i>
MOPS	3-Morpholinopropanesulfonic acid
4-NC	4-Nitrocatechol
NCS	Non-crystallographic symmetry restraints
4-NSQ	4-Nitro- <i>o</i> -semiquinone
PEG	Poly(ethyleneglycol)
RMSD	Root-mean-square deviation
TLS	Parameterization for describing the <u>T</u> ranslation, <u>L</u> ibration and <u>S</u> crew-rotation displacements of a pseudo-rigid body.
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride

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Scheme 1.

Extradiol ring cleavage of HPCA to product 5-CHMSA catalyzed by HPCD and MndD.

Scheme 2.

Proposed reaction mechanism for the estradiol cleaving catechol dioxygenases [9].

Figure 1.

(Top) Comparison of activity of metal-substituted HPCDs measured in air-saturated (260 $\mu\text{M O}_2$, light blue bars) and oxygen-saturated buffer (1.37 mM O_2 , dark red bars) in 50 mM MOPS pH 7.8 at 22 $^\circ\text{C}$ determined by monitoring formation of the 5-CHMSA product ($\sim 38,000 \text{ M}^{-1} \text{ cm}^{-1}$). k_{cat} values have been normalized by metal content from ICP-AES results. Error bars were calculated from both the standard deviation in metal concentration measured from multiple samples submitted for ICP-AES metal analysis and from observed rates from multiple assays. (Bottom) Correlation between cobalt concentration determined from ICP-AES analysis and specific activity of Co-HPCD assayed in the presence of 1 mM H_2O_2 . The black line was obtained from a linear regression fit of the data ($R^2 = 0.87$). Error bars represent ± 1 standard deviation in average cobalt concentration determined from multiple samples for ICP-AES analysis.

Figure 2.

Representative progress curves for HPCA turnover by metal-substituted HPCDs carried out in air-saturated (thin lines) and O₂-saturated (thick lines) buffer. Mn-HPCD (red, dotted line), Fe-HPCD (black, solid line), Co-HPCD (blue, dashed line). Reactions contained 6 nM M-HPCD and 2 mM HPCA in 50 mM MOPS pH 7.8 at 22 °C.

Figure 3.

Michaelis-Menten plot of varying O_2 concentration for Co-HPCD (blue circles) and Mn-HPCD (red squares) in the presence of 2 mM HPCA measured at 22 °C. k_{cat} was calculated from initial velocities at different oxygen concentrations. Data points with closed symbols were measured using a Clark-type oxygen electrode, while points with open symbols were obtained by following product formation by UV-Vis spectroscopy. O_2 dependence data were fit to the Michaelis-Menten equation (solid lines).

Figure 4.

A) Model of Co-HPCD tetramer and anomalous difference map calculated from the X-ray diffraction dataset collected at the cobalt K-edge (1.6050 Å, 7.725 keV). The gray, gold, pink and blue ribbons depict backbone representations for the four subunits of Co-HPCD (PDB 3OJJ). The anomalous difference Fourier map (green mesh) is contoured at 8 . B) Active site of Co-HPCD (PDB 3OJJ). Anomalous difference maps were calculated from a dataset collected at the Co K-edge (1.6050 Å, 7.725 keV, green mesh, 8 contour) and pre-edge (1.6077 Å, 7.712 keV, red mesh, 5 contour). Model depicts the metal-(2-His-1-carboxylate) center in subunit C of Co-HPCD structure. Atom color-code: gray, carbon; blue, nitrogen; red, oxygen; magenta, cobalt.

Figure 5. Structural comparison of the active site environments in HPCD enzymes with Fe(II), Mn(II) and Co(II) centers. A) Structure superposition of the metal centers in the resting state of Fe-HPCD (PDB 3OJT; bronze), Mn-HPCD (PDB 3OJN; gray) and Co-HPCD (PDB 3OJJ; color-coded). The bronze, gray and blue cartoons depict the secondary structure elements for Fe-HPCD, Mn-HPCD and Co-HPCD, respectively. B) Structural overlay of active sites in the [Co-HPCD(4-NC)] complex (PDB 3OJK, color-coded) and [Fe-HPCD(4-NSQ)superoxo] complex (PDB 2IGA, bronze). An RMSD value of 0.15 Å is found for the superposition of all protein-derived atoms within 15 Å from the metal center in the substrate-bound complex structures (PDB 2IGA and 3OJK). Atom colors: yellow, carbon; blue, nitrogen; red, oxygen; magenta, cobalt; green, chlorine.

Figure 6.

Visible absorption spectra of Co-HPCD (dark blue) and [Co-HPCD(HPCA)] (red). To accurately determine the extinction coefficients of the weak d-d transitions of Co(II), a solution of Mn-HPCD, which has no chromophore in the visible region, was used as a reference to correct the baseline of spectra in the visible region.

Figure 7.

(Left panel) EPR spectra obtained at 9.64 GHz and 20 K of Co-HPCD at pH 7.8 (top), and [Co-HPCD(HPCA)] (bottom). The small signal at $g = 4.3$ derives from contaminating Fe(III). (Right panel) Magnification of the low field regions of Co-HPCD (top) and [Co-HPCD(HPCA)] (bottom).

Figure 8. Single wavelength (380 nm) stopped flow kinetic data for [Co-HPCD(HPCA)] (panel A) and [Mn-HPCD(HPCA)] (panel B) rapidly mixed with oxygenated buffer. O_2 concentrations upon mixing ranged from 36 to 685 μM O_2 . Final concentrations of reagents after mixing: 20 μM HPCA and 50 μM M-HPCD. Reaction conditions: 50 mM MOPS pH 7.8, 22 $^{\circ}C$. Each inset highlights the observed lag phase that precedes product formation.

Scheme 3.

Simplest form of the ordered bi uni mechanism for HPCD. Rate constants shown represent collections of rate constants involved in substrate and O₂ binding and product formation and release.

Table 1

Apparent steady-state kinetic parameters^a and metal composition analysis of HPCDs.

Metal = (E°, V) ^b	[Metal]/[subunit]		K _M ^{HPCA} (μM)	K _M ^{O₂} (μM)	k _{cat} /[Metal] (min ⁻¹)	k _{cat} /K _M ^{HPCA} (μM ⁻¹ min ⁻¹)	k _{cat} /K _M ^{O₂} (μM ⁻¹ min ⁻¹)
	Target Metal	Fe	Mn				
Fe ^c (0)	0.7	0.7	<0.1	60	470 ± 20	15 ± 3	7.8 ± 0.3
Mn ^c (0.79)	0.8	<0.1	0.8	50 ± 4	370 ± 10	11 ± 2	7.4 ± 0.6
Co (1.15)	0.6(2)	<0.02	<0.01	1200 ± 100	215 ± 8 ^a 590 ± 20 ^d 1120 ± 70 ^e	43 ± 9 120 ± 20 220 ± 50	0.18 ± 0.02 0.49 ± 0.05 0.9 ± 0.1
Ni (1.5)	0.1(2)	0.06	0.02	—	10 ± 9 ^f	—	—
Cu (1.6)	0.5(1)	0.13	0.02	—	12 ± 10 ^f	—	—
Zn (—)	0.1(6)	0.08	0.04	—	4 ± 1 ^f	—	—

^a measured under ambient O₂ at 22 °C in 50 mM MOPS pH 7.8; unless otherwise noted, apparent k_{cat} has been normalized by metal content from ICP-AES results.

^b difference in standard electrode potentials (in water at 298.15 K, pH = 0.0) from E° for Fe(III/II) (+0.77 V vs SHE) [8].

^c data from reference [9].

^d measured in O₂-saturated buffer at 22 °C.

^e V_{max} extrapolated from Figure 3.

^f protein incubated in 1 mM H₂O₂ prior to being assayed; activity due to contaminating manganese detected by ICP-AES has also been subtracted.

Table 2

Effect of various reagents on the activity of M-HPCDs compared to full activity in the presence of L-ascorbate. Standard deviations in percent activities ranged from 2 to 5%.

Reagent	Percent activity		
	Fe-HPCD	Mn-HPCD	Co-HPCD
As isolated	83	93	99
L-ascorbate	100	100	100
Hydrogen peroxide	7	93	93
Potassium persulfate	11	92	91
Potassium ferricyanide	9	100	100

Table 3
EPR and visible absorption data for Co(II)-substituted enzymes and Co(II) complexes.

Sample ^a	Coord #	g values	A (G) ^b	λ_{max} (nm) (e, M ⁻¹ cm ⁻¹)	Ref
Co-HPCD	6	6.7, 3.4, 2.4	80	500 (60)	this work
[Co-HPCD(HPCA)]	6	7.6, 2.5, 1.9	98	500 (60)	this work
Co-TauD	6			530 (70)	[45]
[Co-TauD(taurine)(α -KG)]	5			552 (200), 565 (204)	[45]
Co-QueD'		6.5, 4.3, 3	not reported		[14]
Co-QueD	5 (TBP)	6.16, 3.8, 2.3 7.16	76 95		[13]
[Co-QueD(quercetin)]	5/4	6.94, <1.5 6.23	39 95		[13]
Co-glyoxalase I	6	6.6, 3, 2.5	81	493 (35), 515 (33)	[38]
[CoZnAAP(LPA)]	6/5	6.66, 3.05, 1.90	70	525 (68)	[42] [43]
[ZnCoAAP(LPA)]	6	6.23, 3.40, 1.95	49.5	500 (63)	[42] [43]
Co-MBP-VanX	5	5.92, 2.57, 1.69		520, 550, 580 (~100)	[46]
CoZn-HLADH	4 (Td)	6.5, 2.2, 1.6	37	520 (200), 655 (600)	[47] [48] [49]
Co(Tp) ₂	6	8.48, 1.02	93	460(13.4), 515(1.3)	[40] [50] [51]
[Co(Tp ^{Ph} ,Me)(3,4-HOPO)]	5 (SP)	8.18, 1.42, 0.98	102	445 (379), 555 (89)	[44]
[Co(imidazole) ₂ (acetate) ₂]	4 (Td)	4.49, 4.23, 2.16*	15/38*	550(155)	[36, 52]
[Co(imidazole) ₂ (C ₂ H ₅ CO ₂) ₂]	4 (Td)	5.37, 3.43, 2.06*	14/38*	550(155)	[36]

^a Abbreviations used: TauD taurine/ α -ketoglutarate dioxygenase from *Escherichia coli*; QueD', Quercetin 2,3-dioxygenase from *Bacillus subtilis*; QueD, 2,3-dioxygenase from *Streptomyces sp.*; AAP, aminopeptidase from *Aeromonas proteolytica*; LPA, L-leucine phosphonic acid; MBP-VanX, fusion protein of gene product of vancomycin-resistance gene X from *Enterococcus* and maltose-binding protein; HLADH, horse liver alcohol dehydrogenase; Tp, tris(pyrazolyl)borate; Tp^{Ph},Me, hydrotis(3,5-phenylmethylpyrazolyl)borate; 3,4-HOPO, 3-hydroxy-1,2-dimethyl-4-pyridinone; Td, tetrahedral; SP, square pyramidal; TBP, trigonal bipyramidal.

^b A values listed are for the lowest-field signal, except for the last two entries where hyperfine splitting was also observed for the highest-field signal.