Role of a Tyrosine Radical in Human Ceruloplasmin Catalysis

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ABSTRACT: Multicopper oxidases (MCOs) are a large family of diverse enzymes found in both eukaryotes and prokaryotes that couple one-electron oxidations of various substrates to the four-electron reduction of O₂ to H₂O, functioning through a set of metallocofactors consisting of one type 1 copper (T1 Cu) and one trinuclear copper cluster (TNC). Human serum ceruloplasmin (Cp) is a unique member of MCOs composed of six cupredoxin domains and harbors six Cu ions arranged as three T1 Cu and one TNC. The native substrate of Cp is Fe²⁺. It is an essential ferroxidase critical for iron homeostasis and is closely associated with metal-mediated diseases and metal neurotoxicity. In human serum, Cp operates under substrate-limiting low [Fe³⁺] but high [O₂] conditions, implying the possible involvement of partially reduced intermediates in Cp catalysis. In this work, we studied for the first time Cp reactivities at defined partially reduced states and discovered a tyrosine radical weakly magnetically coupled to the native intermediate (NI) of the TNC via a hydrogen bond. Our results lead to a new hypothesis that human iron transport is regulated as the paired transfer of iron from ferroportin to Cp to transferrin, and the tyrosine residue in Cp acts as a gate to avoid reactive oxygen species (ROS) formation when Fe³⁺ delivery is dysregulated.

INTRODUCTION

Human serum ceruloplasmin (Cp) is a glycoprotein that binds up to 95% of plasma copper.¹ Cp plays important roles in physiological processes, including copper transport and biogenic amine oxidation, and is an essential ferroxidase critical for iron homeostasis.²³ Cp is closely associated with metal-mediated diseases and metal neurotoxicity.⁴⁻⁷ A neurodegenerative disorder resembling Parkinson’s disease has been related to very low levels of Cp circulating in plasma.⁵ Studies of the mutational disease aceruloplasminemia reveal that individuals lacking Cp suffer inefficient Fe eﬄux and consequently develop oxidative injury secondary to Fe accumulation and signiﬁcant neuronal damage.⁹,¹⁰ Defining the mechanism of Cp is fundamentally important in understanding the pathogenesis of metal-mediated diseases and metal neurotoxicity.

Structurally, Cp belongs to a large class of copper-containing enzymes, multicopper oxidases (MCOs), which couple one-electron oxidations of various substrates with the four-electron reduction of O₂ to H₂O.¹¹,¹² MCOs are found in both eukaryotes and prokaryotes and are important for lignin formation in plants; pigment formation, lignin degradation, and detoxification processes in fungi; iron metabolism in yeast and mammals; copper homeostasis in bacteria; and manganese oxidation by bacterial spores.¹² The catalytic motif shared among all MCOs includes a minimum of four copper ions, arranged as a mononuclear type 1 copper (T1 Cu) site and a trinuclear copper cluster (TNC) composed of a type 2 copper (T2 Cu) and a pair of type 3 coppers (T3 Cu) (Figure S1).¹¹,¹² When a fully reduced MCO reacts with O₂, two key intermediates are formed in the reductive cleavage of the O−O bond by the TNC: the two-electron-reduced peroxo intermediate (PI) and the four-electron-reduced native intermediate (NI) (Scheme 1).¹¹−¹³ Spectroscopic studies deﬁned PI as a species with two Cu atoms oxidized and antiferromagnetically coupled, one Cu atom-reduced, and peroxide bridging all three Cu of the TNC.¹⁴−¹⁶ The NI has all Cu oxidized with a μ₁-oxo in the center of the TNC and a μ₂-hydroxo ligand between the two T3 Cu.¹⁷ In the presence of excess amounts of a reductant, NI is rapidly reduced to the fully reduced (FR) form.¹⁸ In the absence of a reductant, NI slowly decays to the resting oxidized (RO) form.¹⁷ Cp, the most complex MCO, contains six Cu ions arranged as three T1 Cu sites and a TNC (Figure 1).¹¹ Notably, only five of the Cu ions are redox-active and one T1 Cu site is permanently reduced.²⁰ Both redox-active T1 Cu sites have nearby Fe³⁺-binding sites. It is intriguing that nature designed such a ﬁve-electron metalloprotein to mediate a four-electron O₂ reduction process, and the role of the additional redox-
active T1 Cu site has been a long-standing question. Recently we reported that chloride, the most abundant anion present in plasma (with a $[\text{Cl}^-] \approx 0.1 \text{ M}$), binds to the partially reduced TNC and shifts the electron equilibrium distribution between T1 Cu sites and the TNC in the Cp reduction process.\(^{22}\) The Cl$^-$-binding-induced reduction potential separation enables controlled formation of a series of partially reduced forms of Cp with well-defined electron distributions, as shown in Scheme 2. The fully oxidized Cp is reduced stepwise following the order of decreasing reduction potentials. The T3 Cu pair with the highest reduction potentials is reduced first (generating the two-electron-reduced, 2e$^-$ species), followed by successive reductions of the two T1 Cu sites (3e$^-$ and 4e$^-$), and the T2 Cu is reduced last (5e$^-$). Note that there is no observable 1e$^-$ form. The addition of one equivalent of reductant results in 50% formation of 2e$^-$).\(^{22}\) The definition of these reduced states serves as an ideal entry point to dissect the reaction pathway of O$_2$ reduction by Cp. In this study we explored the O$_2$ reactivity of these systematically reduced Cp forms. The results define the roles of the two redox-active T1 Cu sites and reveal a newly identified tyrosine radical that plays a key protective role to avoid reactive oxygen species (ROS) formation in Cp catalysis.

### RESULTS AND ANALYSIS

**O$_2$ Reduction by Partially Reduced Cp.** In the iron efflux process, the physiological substrate of Cp is ferrous ion, the concentration of which is over 3 orders of magnitude lower than Cp.\(^{23}\) Therefore, one or more partially reduced Cp forms are likely to be the major species that potentially react with O$_2$. To explore the reactivity of partially reduced Cp, 1e$^-$, 2e$^-$, 3e$^-$, and 4e$^-$-reduced Cp forms (Scheme 2) were mixed with

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**Figure 1.** Crystal structure of Cp, showing the six domains, the locations of the Cu-binding sites, the axial ligand of the T1 Cu sites, and the substrate Fe$^{2+}$-binding sites. The TNC is located at the interface of domain 1 and 6, and each of the T1 Cu is coordinated to a cysteine and two histidine residues from domains 2, 4, and 6. The T1 Cu in domain 6 is coupled to the TNC via a Cys–His pathway similar to other MCOs. The second T1 Cu in domain 4 is 17 Å away but can rapidly transfer its electron to the first T1 Cu.\(^{19}\) The T1 Cu in domain 2, in which the axial methionine is replaced by a leucine residue, has a high potential (>1 V), and has been determined to remain reduced during turnover.\(^{20}\) There are two ferrous-binding sites near the two redox-active T1 Cu sites. Each Fe$^{2+}$ is coordinated by two carboxylate ligands and one histidine ligand (PDB: 2J5W).
air-saturated buffer, and the reactions were monitored by stopped-flow absorption spectroscopy. For the 1e\(^{-}\) and 2e\(^{-}\)-reduced Cp, only the T3 Cu pair is reduced, and minimal spectral change was observed upon exposure to O\(_2\) (Figure S2), indicating neither form was reactive. However, when the 3e\(^{-}\)-reduced Cp (one T1 and two T3 Cu sites are reduced, Scheme 2) was reacted with O\(_2\), two ligand-to-metal charge-transfer (LMCT) bands centered at 330 nm (OH\(^{-}\) → T3 Cu\(_{2}\)(II)) and 610 nm (S(Cys) → T1 Cu(II))\(^{11}\) increased rapidly and plateaued in 3 s (Figure 2a), indicating the oxidation of both the T1 and T3 Cu sites. Moreover, two new peaks at 390 and 410 nm simultaneously appeared with comparable rate constants, followed by a slow decay at 0.1 s\(^{-1}\) (Figure 2a). The 390 and 410 nm peaks with ∼2 M\(^{-1}\) cm\(^{-1}\) extinction coefficients are characteristic of a tyrosine radical,\(^{24,25}\) which provides the fourth electron for the 3e\(^{-}\)-reduced Cp to accomplish the four-electron reduction of O\(_2\). The slow decay of the tyrosine radical reflects its reduction likely by an exogenous electron, either from the protein in vitro or from the serum in vivo (Figure S3).

Scheme 2. The Electron Distribution among Partially Reduced Human Cp in the Presence of 0.1 M Cl\(^{-}\) at pH 7\(^{14}\)

| Fully oxidized | Two-electron reduced (2e\(^{-}\)) | Three-electron reduced (3e\(^{-}\)) | Four-electron reduced (4e\(^{-}\)) | Fully reduced (5e\(^{-}\)) |
|---------------|---------------------------------|---------------------------------|---------------------------------|------------------------|
| T2            | Cu\(^{2+}\)                      | Cu\(^{2+}\)                      | Cu\(^{2+}\)                      | Cu\(^{2+}\)            |
| T3            | Cu\(^{2+}\) Cu\(^{2+}\)         | Cu\(^{+}\) Cu\(^{+}\)           | Cu\(^{+}\) Cu\(^{+}\)           | Cu\(^{+}\)             |
| T1            | Cu\(^{2+}\)                      | Cu\(^{2+}\)                      | Cu\(^{2+}\)                      | Cu\(^{+}\)             |

“Cu\(^{2+}\) and Cu\(^{+}\) ions are shown in blue and green, respectively.

Figure 2. Stopped-flow absorption of partially reduced Cp reacting with O\(_2\) in 25 mM MOPS pH 7.0 buffer with 100 mM NaCl at 4 °C. (a) Three-electron reduced Cp reacted with O\(_2\). (b) Four-electron-reduced Cp reacted with O\(_2\). Black trace: the initial absorption spectrum. Red trace: the spectrum with the 390 and 410 nm peaks reaching the highest absorption. Blue trace: the final spectrum. The reaction models of both three- and four-electron-reduced Cp reacting with O\(_2\) are shown above the figures (the involvement of the NI is demonstrated later). NI, native intermediate; RO, resting oxidized. Singular value decomposition (SVD) was first performed in MATLAB, and these SVD traces were simultaneously fit to extract rate constants and projected spectra of each species in the reaction using Copasi software.
The initial stage of the reaction of the 4e$^{−}$-reduced Cp with O$_2$ paralleled that of the 3e$^{−}$-reduced form, with the four absorptions at 330, 390, 410, and 610 nm increasing in the initial 1 s (Figure 2b, black arrows producing the red spectrum). However, the tyrosine radical features at 390 and 410 nm decayed with a rate constant of 1.3 s$^{-1}$ (red arrow to blue spectrum), 1 order of magnitude faster than that in the 3e$^{−}$-reduced Cp reaction (0.1 s$^{-1}$). This was accompanied by a concomitant increase of the T1 Cu(II) band at 610 nm at the same rate constant (1.3 s$^{-1}$, red arrow to blue spectrum), while the T3 Cu(II) CT band at 330 nm remained unchanged. The spectroscopic changes reveal that, even for the 4e$^{−}$-reduced Cp, a tyrosine radical participated in the four-electron reduction of O$_2$ together with one reduced T1 Cu and two reduced T3 Cu’s. The additional reduced T1 site engaged in intramolecular electron transfer (IET) to quench the tyrosine radical, resulting in its fast decay (1.3 s$^{-1}$). Therefore, in both the 3e$^{−}$- and 4e$^{−}$-reduced Cp, immediately after the O$_2$ reaction, the TNC contains 3 × Cu(II) and a nearby tyrosine radical, where the 3Cu(II) TNC could be in either the RO or NI form (Scheme 1). To distinguish between these two possibilities, rapid-freeze quench techniques were employed to
characterize the initial form of the TNC generated upon the reaction with O₂.

Rapid-Freeze Quench—Electron Paramagnetic Resonance Correlation to O₂ Reaction Kinetics of Partially Reduced Cp. To explore whether the RO or NI TNC was formed in the reaction of 3e⁻ and 4e⁻-reduced Cp with O₂, electron paramagnetic resonance (EPR) spectroscopy was employed. Both forms of the TNC as well as a tyrosine radical should have characteristic EPR signals that can be used to define the electronic and geometric structures of the 3Cu(II) TNC and its possible magnetic coupling with the tyrosine radical.

In the fully oxidized form of Cp, the three EPR-active Cu sites are clearly distinguishable: the T2 Cu of the resting oxidized TNC has a well-separated (mₛ = −3/2) hyperfine peak at 2750 G, and the two T1 Cu sites have distinguishable low-field (mₛ = −3/2) hyperfine peaks at 2880 and 2930 G (Figure S4). The NI form contains a μ₃-oxo ligand bridging all three copper ions of the fully oxidized TNC, which leads to a spin-frustrated $S_{\text{tot}} = 1/2$ ground state. NI exhibits a fast-relaxing, broad EPR signal (observable only at low temperatures and high powers) with g < 2 due to the field-induced mixing with a doublet excited state.26 Following its rapid formation, NI decays due to a loss of the μ₃-oxo bridge to the RO form, resulting in the return of the isolated T2 Cu EPR signal.

The reaction of fully reduced Cp with O₂ followed the same reaction pathway as other MCOs. The EPR spectrum quenched at 35 ms showed a fast-relaxing, broad signal at g ≈ 1.9, indicating the presence of NI (Figure 3a, red). By 1 min, the NI signal has disappeared, and the T2 Cu signal is recovered, indicating that NI had decayed to the RO form (Figure 3a, blue). Alternatively, when the 3e⁻-reduced Cp was reacted with O₂ (Figure 3b and S5), no NI signal was observed in the EPR spectra quenched from 35 ms to 3 s. The T2 Cu signal in these spectra was observed to decrease significantly upon the reaction with O₂ (compared to the starting 3e⁻-reduced Cp) and recovered in the sample collected 1 min after mixing. Given the slow decay of the tyrosine radical in this reaction (0.1 s⁻¹, vide supra), it is notable that no tyrosine radical signal was observed in EPR at any time point. The very small signal at g = 2.0 in Figure 3b reflects <5% radical. This suggests that at 1 s, the tyrosine radical is magnetically coupled to the TNC (either in the NI or RO form), eliminating both the radical and any Cu(II) signals. For a definitive structural assignment of the 3 × Cu(II) TNC, we next turned to magnetic circular dichroism spectroscopy, which will be evaluated in the next section.

Magnetic Circular Dichroism Study of the Intermediates Generated in the Reaction of Fully Reduced and Three-Electron-Reduced Cp with O₂. Similar to other MCOs, the magnetic circular dichroism (MCD) spectrum of NI generated by the reaction of fully reduced Cp with O₂ exhibits an intense derivative-shaped, pseudo-A term (two overlapping MCD C terms with opposite-signed intensities) centered at ∼30000 cm⁻¹ (Figure 4a, top), which is characteristic of the μ₃-oxo-bridged 3 × Cu(II) TNC in NI.17,27 A similar derivative-shaped pseudo-A term pattern is also present in the spectrum of the 3e⁻-reduced Cp following the reaction with O₂ quenched at 0.3 s (Figure 4b, top). The observation of this pseudo-A term feature confirms the formation of NI in this reaction despite the lack of any NI signal in the EPR spectra (Figure 3b).

To explore whether the lack of NI and tyrosine radical signals in EPR might reflect their exchange coupling, the saturation magnetization behaviors of their pseudo-A term features were probed by variable-temperature variable-field MCD (VTVH-MCD). VTVH-MCD data of the NI generated by reacting the fully reduced Cp with O₂ showed no nesting behavior at temperatures up to 25 K (i.e., the field saturation curves measured at different temperatures superimpose when plotted as a function of H/T, Figure 4a, bottom), consistent with the $S_{\text{tot}} = 1/2$ ground state observed by low-temperature EPR.26 However, distinct nesting behavior was observed in the VTVH-MCD isotherms for the NI generated by 3e⁻-reduced Cp reacted with O₂ (Figure 4b, bottom), supporting the existence of a low-lying excited state that becomes Boltzmann-populated by 25 K. This indicates a magnetic coupling between the tyrosine radical (observed in stopped-flow) and the NI (observed in MCD), which would lead to the lack of EPR signals of both NI and the tyrosine radical.

The MCD behavior of two interacting spin systems can be simulated by calculating the contribution of a specific spin center projected onto the spin sublevels of the exchange-coupled spin system.28 The VTVH-MCD data for NI generated by the reaction of 3e⁻-reduced Cp with O₂ were simulated using two coupled $S = 3/2$ centers with g ~ (1.89, 1.98, 2.08) for NI and an isotropic g ~ 2.0023 for the tyrosine radical, and an exchange coupling constant J of ~0.3 cm⁻¹ (−2J $S_1 S_2$ Hamiltonian) was obtained. Therefore, the tyrosine radical and the NI are weakly antiferromagnetically coupled through a superexchange pathway, which requires a bridging ligand between the two paramagnetic centers.

Inspection of the Cp crystal structure reveals a Tyr residue (Y107) near the TNC center that also forms an H-bond to the OH⁻ ligand of the T2 Cu site in the RO form (Figure S6). This residue, which is conserved among the homologous six-domain MCOs, is the most probable candidate for the tyrosine radical that antiferromagnetically couples with NI, as characterized by the above spectroscopies.

**DISCUSSION**

To date, it has been established that all MCOs require a fully reduced TNC to initiate the O₂ reduction process that further proceeds to PI and NI.11 Fully reduced Cp undergoes a similar pathway (Figure S7, bottom), and our previous studies showed that Cp requires a fully reduced TNC to achieve fast reaction with O₂ ($\sim$10⁶ M⁻¹ s⁻¹).25 However, we have demonstrated in this work that partially reduced forms of Cp (3e⁻ or 4e⁻ reduced) are able to reduce O₂ by recruiting one extra electron from a nearby tyrosine residue. To achieve this rapid reaction, the first step likely involves a proton-coupled electron transfer (PCET) process from Tyr107 to the T2 Cu to generate the fully reduced TNC (Figure S7, top). This is a thermodynamically unfavored process and is probably driven by the reaction with O₂, indicating that the reaction should be O₂-concentration-dependent. Indeed, when the 3e⁻-reduced Cp was reacted with varying amounts of O₂, a linear relationship was observed between the tyrosine radical formation rate and the O₂ concentration (Figure S8). Upon the reaction with O₂, the 4e⁻-reduced Cp exhibits the same initial reaction intermediates as the 3e⁻-reduced Cp, but the additional reduced T1 Cu site present in the 4e⁻ sample quenches the tyrosine radical via an IET process with a rate constant of 1.3 s⁻¹. This is much faster than the self-decay (0.1 s⁻¹) of the tyrosine radical observed in the reaction of 3e⁻-reduced Cp
(Figure S7, middle). It is noteworthy that a 420 nm transient absorption had been reported earlier in the reaction of reduced Cp with O2 that was subsequently reported to not be reproduced.28–31 From the present study, this can now be understood as reflecting the O2 reaction of a partially reduced Cp. Also, a tyrosine radical has been observed in small laccases,32,33 and Tyr108 in small laccases forms an homologous H-bond to the OH– ligand of a T2 Cu center. However, the generation of the tyrosine radical in small laccases starts from the fully reduced TNC, while the generation of the tyrosine radical in Cp commences from a partially reduced TNC. Furthermore, the decay rate of the tyrosine radical in small laccases is too slow to be involved in the catalytic cycle.32,33

Iron metabolism is strictly regulated in humans.34,35 The export of Fe2+ from the cell occurs through the membrane protein ferroportin (Fpn)36 followed by oxidation to Fe3+ by Cp in human serum. Fe3+ then binds to transferrin (Tf) to form holo-Tf, which is transported through the blood barrier and cell membranes via transferrin-receptor-mediated endocytosis (Figure 5).37 In human serum, the concentration of Cp is ~2 μM and that of free O2 is ~130 μM, while the concentration of labile Fe2+ is extremely low (<1 nM), indicating that Cp operates under substrate-limiting [Fe2+] but high [O2] conditions. If Fe2+ is individually delivered to Cp from Fpn, Cp would be gradually reduced by two, three, and four electrons. Because of the high O2 concentration in human serum, the first O2-reactive species formed during the reduction process, the 3e−-reduced Cp, would be the major O2-reactive species (Figure S7 top), and each turnover of Cp would generate a tyrosine radical that needs to be quenched by an exogenous electron. To avoid such a scenario with high oxidative stress, Fpn needs to deliver Fe2+ to Cp in pairs to bypass the 3e−-reduced Cp and directly reach the 4e−-reduced form. Such a two-Fe2+ delivery hypothesis is supported by consideration of the iron homeostasis process. First, endogenous Fpn is a dimer,38 and is unable to efflux iron in the absence of Cp or its homologue hephaestin (Hp).39,40 Second, Cp has two redox-active T1 Cu with two nearby Fe2+.

Figure S7. Iron transport in human serum. Cp mediates transfer of the iron in pairs from the cell export pump ferroportin (Fpn) to the plasma metallo-chaperone transferrin (Tf). Cellular internalization of Tf-bound iron occurs after docking with the membrane-bound transferrin receptor (TfR) via clathrin-mediated endocytosis (Figure 5).
with this nearby tyrosine residue acting as a failsafe to avoid ROS formation when Fe^{2+} delivery is dysregulated.

**METHODS**

**General.** All chemicals were purchased from Sigma-Aldrich and used without further purification. Water was purified on a Nanopure Diamond purifier from Barnstead to a resistivity of >17 MΩ cm. No unexpected or unusually high safety hazards were encountered.

**Ultraviolet–Visible Spectroscopy.** UV–vis spectra were recorded on a Hewlett-Packard HP8452A diode array UV–vis spectrophotometer in a 1 cm path length cuvette.

**Stopped-Flow Kinetics.** Stopped-flow data were obtained using an Applied Photophysics SX.19 stopped-flow absorption spectrophotometer equipped with a Hg/Xe arc lamp and a PDA1 photodiode array detector in a purge box (Cleatech Isolation Glove Box 2100) equipped with an oxygen sensor (Neutronics Model 1100). Stopped-flow experiments were conducted at 4 °C and maintained using a water/ethanol temperature bath (Fisher Scientific Isotemp 3016) with a cell path length of 1 cm. All solutions were freshly prepared in an anaerobic glovebox. Both injector ports of the stopped-flow apparatus were degassed with ~3.0 mM sodium dithionite for ~20 min and then washed with the degassed buffer thoroughly. The protein samples were loaded on the stopped-flow apparatus at concentrations of ~50 μM.

**Electron Paramagnetic Resonance.** X-band EPR spectra were obtained with a Bruker EMX spectrometer, an ER 041 XG microwave bridge, and an ER4119HS or ER4116DM resonator. Sample temperatures from 77 K are maintained using an EPR900 continuous flow cryostat. The X-band EPR parameters were as follows: frequency ≈ 9.6 GHz, power = 0.2–200 mW, receiver gain = 30, modulation frequency = 100 kHz, modulation amplitude = 4.00 G. EPR spin quantitation of the paramagnetic copper content was performed using a 1.0 mM standard solution of CuSO₄·5H₂O, 2 mM HCl, and 2 M NaClO₄. EPR simulations were performed using the EasySpin software package.

**Magnetic Circular Dichroism (MCD).** MCD spectra in the UV–vis region (300–900 nm) were collected on a Jasco J810 with an S20 PMT detector. The magnetic field was supplied by an Oxford SM4000 7 T superconducting magnet. The sample temperature was measured with a calibrated Cernox resistor (Lakeshore Cryogenics) inserted into the MCD cell. The MCD sample cells consisted of two quartz disks with a 3 mm rubber spacer, and protein samples for MCD contained ~60 vol % of glycerol in order to form an optical-quality glass upon freezing. The data were corrected for zero-field baseline effects induced by cracks in the glass by subtracting the corresponding 0 T scan.

**Human Cp Purification, Characterization, and Sample Preparation.** Fresh human plasma was obtained from the Stanford Blood Center, and Cp is purified from this by the previously reported rapid one-step method with a yield of ~20 mg dL⁻¹. The purified Cp was treated with 20 equiv. H₂O₂ to achieve its fully oxidized form, and the excess H₂O₂ was removed by a PD-10 desalting column. The quality and function of the human Cp have been assessed by UV–vis spectroscopy, EPR spectroscopy, biquinoline assays (6.3 ± 0.1 Cu ions per Cp), and ferroxidase activity assays (~1 Fe^{2+} oxidized per second per Cp at 25 °C). The partially reduced Cp was prepared by titrating quantitative amounts of Fe^{2+} into the fully oxidized Cp solution. To overcome the interference of leftover Fe^{3+} in the MCD study, an organic substrate dianisidine was used instead of Fe^{2+} to prepare the partially reduced Cp. The oxidized dianisidine was removed by a PD-10 desalting column.

**Rapid-Freeze Quench.** Rapid-freeze quench (RFQ)–EPR samples were prepared by mixing a 0.4 mM Cp solution with an oxygen-saturated buffer (~2.0 mM O₂) at 4 °C using a Kintek quench-flow apparatus, model RFQ-3. The sample was frozen by spraying into approximately -130 °C spectrophotometric grade isopentane in a Pyrex collection funnel attached to the EPR tube. Precooled packing rods and long needles were used for packing the frozen powder into the base of the EPR tube, while maintaining at -120 °C. After packing, the samples were transferred to a liquid nitrogen dewar and stored until collection of the EPR spectra. RFQ-MCD samples were prepared by spraying the reaction mixture through a needle into the MCD cell precooled with dry ice. The MCD cell was then immediately cooled in liquid nitrogen.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.0c00953.

**Figures for Cp structural analysis, EPR speciation of the fully oxidized Cp, RFQ-EPR spectra and kinetics of the reaction of partially reduced Cp with O₂, and mechanisms of O₂ reduction by partially and fully reduced Cp (PDF)**

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**Author Contributions**

S.T. and E.I.S. conceived and designed research; S.T. collected the data; S.T., S.M.J., and E.I.S. analyzed the data; and S.T., S.M.J., and E.I.S. cowrote the paper.

**Notes**

The authors declare no competing financial interest.

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