Published in final edited form as:

J Am Chem Soc. 2019 July 17; 141(28): 11304–11314. doi:10.1021/jacs.9b05230.

O₂ Reduction to Water by High Potential Multicopper Oxidases: Contributions of the T1 Copper Site Potential and the Local Environment of the Trinuclear Copper Cluster

Alina Sekretaryova, Stephen M. Jones, Edward I. Solomon*

Department of Chemistry, Stanford University, 333 Campus Drive, Stanford, California 94305-5080, United States

Abstract

High potential multicopper oxidases (MCOs) have T1 reduction potentials >600 mV (vs normal hydrogen electrode), making them important catalysts for O₂ reduction in various biotechnological applications. The oxygen reduction mechanism for the low-potential MCOs is well-characterized; however, O₂ reactivity of high potential MCOs is not well understood. In this study, we have shown that in the laccase from *Trametes versicolor*, the T1 redox potential is increased by ~350 mV over that of the low-potential MCOs corresponding to an 8 kcal/mol decrease in the driving force, and thus exhibits a slower intramolecular electron transfer (IET) rate to the trinuclear Cu cluster (TNC) in the native intermediate (NI), relative to the low potential MCO from *Rhus vernicifera* laccase. This IET rate is, however, >10² times faster than the decay rate of the NI, demonstrating that this intermediate form of the enzyme is catalytically relevant enabling fast turnover. However, in contrast to the low potential MCOs where T1 reduction by substrate is rate limiting, the rate limiting step in turnover of high potential MCOs is the first IET to NI. Part of the reduction potential difference of the T1 sites in high vs. low potential MCOs is balanced by an ~100 mV higher reduction potential of NI due to the more positive protein environment in the vicinity of the TNC.

Graphical Abstract

*Corresponding Author: edward.solomon@stanford.edu.

Supporting Information

Kinetic analysis, EPR spectra of the NI and T2D resting *TvL*, VT and VH-MCD spectra of the NI and MCD spectrum of RO, saturation magnetization of the NI MCD spectrum, experimental spectroscopic parameters of the NI. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

INTRODUCTION

Multicopper oxidases (MCOs) form a family of enzymes that catalyze the oxidation of various organic substrates and metal ions coupled to the four-electron reduction of dioxygen (O_2) to water.^{1,2} The active sites of MCOs include at least four copper ions, arranged in a mononuclear type 1 (T1) Cu, and a trinuclear Cu cluster (TNC), that in the resting state is composed of a mononuclear type 2 (T2) Cu and a hydroxo bridged, antiferromagnetically coupled binuclear type 3 (T3) Cu center.³ The T1 site is the primary electron acceptor site which receives electrons from substrates and transfers these over 13 Å via a conserved Cys-His superexchange pathway to the TNC where O_2 binding and reduction occurs.⁴

The ligation of the four Cu centers is conserved among MCOs. The coppers of the TNC in the resting oxidized state are ligated by 8 histidine residues.⁵ Each of the T3 Cu's have three His ligands and a bridging μ_2 -OH ligand. The T2 is ligated by 2 His residues and by an aquo-derived hydroxide oriented away from the cluster. The T1 site is coordinated by two His and one Cys residue. In many MCOs, there is an additional weak axial Met ligand. Three-coordinate T1 sites are present mainly in fungal laccases and Fet3p, while four-coordinate T1 sites are found in plant laccases and ceruloplasmin.^{4,6}

One of the key characteristics of MCOs in biotechnological applications is the standard redox potential of their T1 Cu site. The T1 Cu redox potentials in the MCOs vary widely, from as low as ~300 mV, to as high as ~800 mV (vs normal hydrogen electrode).⁷ Depending on the potential of the T1 site, MCOs can be divided into two groups: low potential MCOs with the T1 potential lower than ~600 mV and high potential MCOs, with the T1 potentials in the range of 650 to 800 mV.⁷ Since the T1 Cu is the primary electron entry center, its potential determines the overpotential for O_2 reduction.⁸ This overpotential is remarkably small in high potential MCOs making them attractive biocatalysts for construction of cathodes in biofuel cells.⁹ The potential of the T1 Cu also determines the efficiency of substrate oxidation, since MCOs can directly oxidize only compounds with reduction potentials not too much higher than the redox potential of the T1 Cu. The high potential of the T1 in the high potential MCOs enables them to oxidize both phenolic and nonphenolic lignin-related compounds as well as environmental pollutants having high

redox potentials.¹⁰ This makes the high potential MCOs important biocatalysts in biotechnological processes, including the detoxification of industrial effluents, polymer synthesis, bioremediation of contaminated soils, and wine and beverage stabilization.¹⁰

The reaction mechanism of O₂ reduction by the MCOs has been well characterized through spectroscopic, kinetic, and computational studies of low potential *Rhus vernicifera* laccase (*RvL*) and Fet3p.^{11–14} Initially, a suitable substrate provides four electrons to reduce all four Cu of the resting oxidized (RO) form of the enzyme. The fully reduced form then reacts with O₂ at the rate of $\sim 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ to form a peroxide intermediate (PI) (Figure 1).^{11,15} In this intermediate, O₂ is reduced by 2 electrons and is coordinated by all three Cu's of the TNC, where the T2 Cu and one of T3 Cu's are oxidized. Due to its fast decay rate in the holo enzymes, the PI has only been trapped in enzymes in which the T1 Cu is replaced with Hg^{II},^{16,17} depleted or selectively oxidized (i.e. only three electrons from the TNC are available for reducing O₂).^{18–20} Upon electron transfer from the T1 site and proton transfer from a carboxylate residue near the TNC, the PI decays to the native intermediate (NI) through O-O bond cleavage.^{17,21,22} The NI was spectroscopically defined as a species with all four coppers oxidized as in the resting enzyme, but with different and rather unique spectral features due to the all Cu^{II} bridged structure of the TNC with a μ_3 -oxo in the center of the cluster and a μ_2 -OH bridging the T3s.^{12,23–25} NI decays slowly in the absence of substrate to the RO form. In the presence of substrate, NI is rapidly reduced to the fully reduced form. It has been shown for the low potential *RvL* that the NI, and not the RO form, is the catalytically relevant fully oxidized form that can be rapidly reduced in the catalytic cycle due to fast intramolecular electron transfer (IET) from the T1 to the TNC.²⁶ The significant rate enhancement ($>10^3$) for IET in the NI compared to IET in the RO form is associated with the high proton affinity of the μ_3 -oxo of NI driving this proton-coupled electron transfer process.²⁷

It has been shown that due to the low potential of the T1 site and fast IET to the TNC, the rate of T1 reduction in *RvL* is the rate limiting step which determines the overall turnover frequency of $> 500 \text{ s}^{-1}$.²⁷ High potential MCOs have high turnover frequencies (of 100 – 200 s^{-1} at 25 °C) and high rates of T1 reduction with high potential reductants (e.g. methoxysubstituted phenols, organometallics ([W(CN)₈]^{4–}, [Fe(EDTA)]^{2–})).^{28,29} For rapid turnover in the high potential MCOs there must be efficient IET to the TNC. The resting TNC in high potential MCOs has the same geometric structure as the TNC in the low potential MCOs suggesting formation of the same intermediates in the catalytic site. The potential of the T1 site, however, is elevated by 350 mV in high potential *Trametes versicolor* laccase (*TvL*) (780 mV)³⁰ compared to low potential *RvL* (430 mV)³⁰, significantly lowering the driving force for electron transfer to the TNC and increasing the rate of T1 reduction by substrate. In this study we elucidate the effect of the T1 reduction potential increase on the catalytic mechanism. In parallel to our past studies on low potential MCOs, we examined the O₂ reactivity of high potential *TvL* using stopped-flow absorption data correlated to rapid-freeze-quench electron paramagnetic resonance (EPR) and magnetic circular dichroism (MCD) measurements to define the kinetics of the intermediate formed in this reaction. These studies provide insight into the effect of tuning the reduction potentials of the T1 site and the TNC on IET in MCOs.

MATERIALS AND METHODS

Chemicals and Protein purification.

All chemicals were reagent grade and used without further purification. Water was purified to a resistivity of $> 17 \text{ M}\Omega \text{ cm}$ using a Barnstead Nanopure deionizing system. *Trametes versicolor* stock cultures on malt extract agar plates were obtained from Northern Spalting, Oregon State University. The protein was expressed and purified according to the published procedure³¹ with modification of the expression media. The concentration of copper ions in the growth media was increased by a factor of 5 that led to a 10-fold increase of the protein yield. An additional purification step was included as described elsewhere to further improve enzyme purity.³² Protein concentration was determined using the extinction coefficient of the absorption band at 280 nm ($74\,000 \text{ M}^{-1} \text{ cm}^{-1}$).³³ Total copper content was determined by the biquinoline method.³⁴ The concentration of paramagnetic coppers was determined from spin quantification of EPR spectra using 0.945 mM CuSO_4 in 40 vol% glycerol as a standard.³⁵

Sample preparation.

All experiments were performed in 0.1 M potassium phosphate buffer (pH 6.0) unless otherwise stated. Solutions of protein and buffers were deoxygenated under a constant flow of N_2 for 1–2 h and transferred to an anaerobic glovebox. All other reactants were transferred in a dry form and solutions were freshly prepared in the glovebox. Solutions of fully reduced protein were prepared by reduction of the anaerobic solution of resting oxidized protein by 1.5–2.0 times excess of sodium dithionite. Remaining reductant was removed by size exclusion chromatography using PD-10 desalting columns (GE Healthcare). For the NI reduction studies, solutions of fully reduced protein were mixed with either ascorbic acid or hydroquinone to give the desired concentration of excess reductant prior to the reaction with O_2 . Dioxygen solutions of desired O_2 concentrations were prepared by diluting either air saturated buffer ($270 \mu\text{M O}_2$ at room temperature) or oxygen saturated buffer (1.2 mM O_2 at room temperature) with degassed buffer. For stopped-flow and rapid freeze quench (RFQ) experiments the reaction solutions were loaded into gas tight syringes (Hamilton) and transferred using three way valves to exclude air. Typical protein concentration for stopped-flow experiments was $100 \mu\text{M}$ before mixing.

For RFQ sample preparation of the NI, two syringes (one with the fully reduced protein solution and one with O_2 containing buffer) were assembled for rapid mixing using a KinTek Chemical-Quench-Flow Model RQF-3 with a $156 \mu\text{L}$ mixing loop. RFQ-EPR samples were frozen by spraying directly into liquid N_2 /isopentane bath at -140°C . The frozen samples were then packed into 4 mm quartz EPR tubes. Protein concentrations were $0.8\text{--}1.0 \text{ mM}$ before mixing. For RFQ MCD samples, the protein and O_2 solutions in syringes were premixed with $\sim 50\%$ glycerol before assembling for rapid mixing. RFQ MCD samples were made by direct spraying of the reaction mixture through a needle into a dry ice chilled MCD cell consisting of two infrasil quartz disks with a 3 mm neoprene spacer. After sample collection the cell was immediately cooled in liquid N_2 and assembled into a copper holder under liquid N_2 .

Type 2 - depleted (T2D) *TvL* samples were prepared by anaerobic dialysis of native protein for 4 h against 0.10 M-imidazolium acetate buffer (pH 7.0), containing 50 mM KCN and 5 mM sodium ascorbate.³⁶ Before analysis, protein samples were dialysed against 0.1 M phosphate buffer, pH 6.0. Cu content was determined by biquinoline method and by EPR spectroscopy (*vide supra*).

Kinetic data acquisition and analysis.

Kinetic data with ~2 ms dead time were collected using an Applied Photophysics SX.19 stopped-flow absorption spectrometer equipped with a Hg/Xe Arc lamp and outfitted with PEEK tubing. The spectrophotometer was placed in an anaerobic glovebox (Cleatech custom glovebox) kept under an Ar atmosphere. All experiments were performed at 4 °C with the temperature maintained using a water temperature bath (Fisher Scientific Isotherm 3016). Global fitting of the experimental data was performed using KinTek software. Single kinetic traces were fit using Origin 2018 (Academic) software package.

EPR Spectroscopy.

All X-band spectra were obtained with a Bruker EMX spectrometer, ER 041 XG microwave bridge, with either an ER4119HS or ER4116DM cavity. Sample temperatures of 77 K were maintained using a liquid N₂ coldfinger dewar, and temperatures below 20 K were obtained using an Oxford Instrument ESR 900 Flowthrough Cryostat and an Oxford ITC 503 temperature controller. EPR spectra simulation was performed using the EasySpin MATLAB toolbox. Power saturation data were fit using Origin 2018 (Academic) software package.

MCD Spectroscopy.

MCD spectra were recorded in the visible region using a Jasco J810 spectropolarimeter equipped with an S-20 photomultiplier tube coupled to an Oxford Instruments SM4-7T superconducting magnet with optical access within its sample compartment. MCD spectra were background corrected for baseline effects and natural CD using data collected at 0 T. VTVH-MCD data were collected using a calibrated Cernox temperature sensor (Lakeshore Cryotronics, calibrated to 1.5–300 K with 0.001 K tolerance) inserted directly into the sample cell. Iterative, simultaneous Gaussian fits to the room temperature absorption (RT Abs) and low temperature MCD (LT MCD) spectra (allowing for small energy shifts and band sharpening in the low temperature MCD) were carried out using the program MagicPlot. The MCD temperature dependence and Brillouin curves were simulated using Origin 2018 (Academic) software package.

Structure modeling.

A structural model of *Rhus vernicifera* (*Toxiconendron vernicifluum*) laccase (*RvL*) was generated using SWISS-MODEL^{37,38} based on the sequence in the Uniprot database³⁹(Q8H979_TOXVR). The template used was from *Cucurbita pepo* ascorbate oxidase (PDB ID: 1ASO) with a global mean quality estimation score of 0.68. Structural overlays with this model and the crystal structure of *Trametes versicolor* laccase (TVL, PDB ID:1GYC) were performed using PyMOL 2.3.

RESULTS AND ANALYSIS

Kinetics.

The reaction of fully reduced *TvL* with air-saturated buffer was monitored by spectral changes in stopped-flow (SF) absorption. (Figure 2). An intermediate species with apparent maxima at 315, 365, 420 and 610 nm is observed. The intermediate resembles the native intermediate (NI) previously reported in *Rhus vernicifera* laccase (*RvL*) possessing a characteristic charge transfer (CT) band with a maximum at 365 nm due to the μ_3 -oxo to Cu^{II}_3 CT at the TNC and a transition at 614 nm due to S_{Cys} to Cu^{II} CT of the oxidized T1 Cu site.¹² The rapid formation of the intermediate is followed by its slow, first-order decay to the RO form and is most clearly monitored by the absorption intensity at 420 nm, where the intensity difference between RO and NI is greatest. The T1 site, with an absorption maximum at 610 nm, remains oxidized, consistent with the results on the formation and decay of NI to the RO in *RvL*. Figure 2A and B show the absorbance spectra and absorbance traces of the apparent pseudo-first order formation and decay of the NI in *TvL*. NI formation and decay kinetics were fit to Scheme 1. Since the formation step is an O_2 -dependent second-order process, global fitting of the experimental data (Figure S2) at various concentrations of oxygen was performed. Global fitting yields the component spectra and speciation curves shown in Figure 2C, D with the rate of the NI formation as $1.2(\pm 0.3) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and the rate of NI decay as $0.07(\pm 0.02) \text{ s}^{-1}$. This is comparable to the NI decay rate of *RvL*, but much slower than the turnover frequency of *TvL* (25 s^{-1}).²⁸

As a complementary approach, the decay kinetics of the intermediate were followed by electron paramagnetic resonance (EPR) spectroscopy of rapid freeze quenched (RFQ) samples at different time points of the O_2 activation reaction. RFQ samples at 35 ms and 1, 5, 15, and 50 s after initiation of the reaction of fully reduced *TvL* with O_2 were trapped and 77 K X-band EPR spectra of the samples were collected (Figure S2). Due to its spin frustrated, all-bridged Cu^{II}_3 TNC, the NI does not exhibit the T2 Cu^{II} EPR signal observed in RO at liquid N_2 temperature.¹² Therefore, the NI decay kinetics could be followed by growth of the T2 Cu^{II} EPR signal relative to the T1 Cu^{II} intensity. The decay rate obtained from RFQ-EPR measurements was $0.11(\pm 0.04) \text{ s}^{-1}$, which matches within error the decay rate of the NI obtained by global fitting of the SF experimental data (Figure S2 F).

The rates of NI formation and decay in *TvL* ($1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and 0.07 s^{-1} , respectively) are similar to the rates reported earlier for *RvL* ($2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and 0.007 s^{-1}).²⁷ It was previously shown that the rate of NI decay in *RvL* is pH-dependent. To determine the effect of proton concentration on the decay rate of the NI in *TvL*, the reaction of fully reduced *TvL* with O_2 at various pH values was monitored by SF absorption spectroscopy. Global fitting of the experimental data (300–700 nm) at various pH values to Scheme 1 yields the pH dependence of NI decay rates presented in Figure 3A. The decay rate is higher at low pH and becomes pH independent at pH above 5. Moreover, no significant spectral differences between NI spectra at low and high pH are observed (Figure 3B). If the observed equilibrium involved protonation of μ_3 -oxo, the μ_3 -oxo to Cu^{II} CT transitions would be significantly affected. Thus, the observed pH behavior is consistent with a model in which a nearby carboxylate residue is implicated as the protonatable species. The rate of NI decay

can be fit to a single protonation equilibrium yielding a pK_a value of 3.7. There are two Asp residues, Asp 77 and Asp 456, near the TNC site in *TvL* that could donate the proton for NI decay. It has been shown previously that the rate of NI decay in *RvL* is pH dependent, yielding a pK_a value of 5.4.⁴⁰ From the crystal structure of *TvL* (PDB ID:1GYC)⁴¹ and sequence alignment between *TvL* and *RvL*, Asp 77 and Asp 456 in *TvL* are replaced by Asp 72 and Glu 499, respectively, in *RvL*. It should be noted that the pK_a of the protonatable group in the decay of the PI of *RvL* is 5.8, similar to the pK_a of the protonatable group in NI decay, and it has been shown computationally that the Glu on the T3 edge is the proton donor group involved in the decay of PI.^{17,21} Additionally, mutation of the corresponding Glu in CueO slows decay of the NI-like intermediate.⁴² These observations suggest that Glu 499 in *RvL* and Asp 456 in *TvL* are the proton donating groups involved in the decay of the NI.

It has been shown that NI, not the RO form, is the catalytically relevant fully oxidized form in *RvL* capable of rapid intramolecular electron transfer (IET) during turnover.²⁶ Fast IET in *RvL* is due to the large driving force for proton-coupled electron transfer caused by the strong basicity of μ_3 -oxo and the high potential of the TNC in the NI state relative to the T1 potential.^{26,27} However, assuming a comparable NI state as in *RvL*, the 350 mV higher T1 potential in *TvL* would result in a lower driving force for IET to the TNC. To understand O_2 reactivity of high potential MCOs it is important to evaluate whether the rate of IET to NI in *TvL* is fast enough to outcompete its decay, implicating the NI in the catalytic cycle, similar to *RvL*. Therefore, we studied the kinetics of NI and RO reduction in *TvL*. Fully reduced *TvL* with an additional 5.0 electron equivalents (relative to enzyme) of ascorbic acid was mixed with equimolar O_2 and monitored by SF absorbance spectroscopy. The reaction led to NI formation and decay monitored at 420 and 610 nm (Figure 4A). The 420 nm band decayed in a pseudo-first order fashion ($k_{obs} = 2.8 \text{ s}^{-1}$) which was accelerated ~30 fold over decay of the NI to RO without the reductant present (Figure 2B). Therefore, NI reduction in *TvL* even with minimal excess of substrate is fast compared to NI decay. Between 0.25 mM and 100 mM ascorbic acid ($[TvL] = [O_2] = 0.05 \text{ mM}$), the 420 nm decay rate (*i.e.* the reduction of NI) increased linearly with the concentration of ascorbic acid (Figure 5B).

Parallel kinetic studies were conducted on the RO to obtain the reduction rates under identical conditions (Figure S3). SF traces for the 330 nm band (μ_2 -OH to T3 Cu^{II} CT) and the 610 nm band (T1 Cu^{II}) obtained under anaerobic reduction of RO *TvL* with 5.0 electron equivalents of ascorbic acid are shown in Figure 4B. The rate of T1 reduction in the RO is fast and comparable to the T1 reduction rate in the NI with a pseudo-first order rate constant of 5.3 s^{-1} . The decay of the 330 nm band in Figure 4B is slow and invariant with respect to the concentration of ascorbic acid (Figure S3 B,C). The loss of the 330 nm intensity for RO reduction represents an IET process from the T1 to the TNC. It fits to a first order rate constant of 0.1 s^{-1} , which is slower than both the T1 reduction rate (5.3 s^{-1} with 5 electron equivalents) and the turnover number (25 s^{-1}).²⁸ These results confirm that the reduction of RO is excluded from being part of the catalytic cycle.

These kinetic studies of high potential *TvL* demonstrate that the mechanism of O_2 reduction in *TvL* is similar to *RvL* (Figure 1).⁴ The fully reduced enzyme rapidly reacts with O_2 to form NI. The catalytic cycle then continues with the re-reduction of the fully oxidized native

intermediate formed upon reduction of dioxygen rather than reduction of the resting enzyme. The rate of IET in the NI is faster than the decay of the NI, so in high potential MCOs NI is the catalytically relevant intermediate in parallel to the mechanism of the low potential *RvL*. Further, the rate of NI reduction is consistent with the turnover frequency (*vide infra*). Importantly, *even though the T1 potential is 350 mV higher than in RvL, the IET to NI is fast enough for rapid turnover.*

First IET to TNC.

Full reduction of MCOs requires four electrons. An outer sphere reductant first reduces the T1 site, with subsequent IET to reduce the TNC. There are two additional IET steps to fully reduce the TNC, with the fourth electron remaining on the thus reduced T1 site. Since the absorption at 420 nm is due to the μ_3 -oxo to Cu^{II}_3 CT of NI,¹² it is eliminated with the first electron transfer into the TNC. Thus, the intensity change of this band provides a direct probe of the rate of the first IET to the TNC in NI. Figure 5 A,B displays absorption changes upon NI reduction with various concentrations of ascorbic acid. The intensity of the 420 nm band can be fit to the kinetic model shown in Scheme 2. The scheme accounts for the formation of the NI, denoted as $\text{T1}_{\text{ox}}\text{TNC}^0$, (where the subscript of T1 indicates the oxidation state of the T1 Cu and the superscript of TNC shows the number of its reduced Cu ions), followed by T1 reduction by ascorbic acid and subsequent IET from the T1 site to the TNC. Global fitting of the experimental data (300–700 nm) for various concentrations of ascorbic acid were performed to obtain the rate of the first IET to the TNC in the NI state. To minimize the number of variables in the model, values for kinetic constants k_1 and k_2 were taken from previous experiments (k_1 from the reaction of fully reduced *TvL* with O_2 (Figures 2, S1) and k_2 , the rate of T1 reduction by ascorbic acid, from the reduction of the RO by ascorbic acid (Figures 4B, S3)) and allowed to deviate no more than 10%. The entire data set at various concentrations of ascorbic acid could be fit with the parameters presented in Table 1. Experimental traces and fits are displayed in Figure 5B for the 420 nm band during NI reduction with 0.25, 5.0 and 100 mM ascorbic acid. Global fitting of the experimental data at 4 °C gives the rate of the first IET to the TNC of $20(\pm 3) \text{ s}^{-1}$. This IET rate is similar to the reported turnover frequency of *TvL* of $\sim 25 \text{ s}^{-1}$.²⁸ Importantly, this value of k_{IET} is significantly lower than the corresponding rate in *RvL* which was found to be $> 700 \text{ s}^{-1}$.²⁶

In the experiments presented in Figure 5, one equivalent of O_2 was used to ensure single turnover conditions. In such conditions, a pseudo-first order rate constant for the reaction of fully reduced *TvL* with O_2 is 60 s^{-1} which is comparable to the obtained k_{IET} rate. Thus, the reaction with O_2 can be a rate limiting factor affecting the value obtained for k_{IET} . To further verify the value of k_{IET} , another kinetic experiment was performed. In this case excess O_2 was used for the fast formation of NI, and again one electron equivalent of hydroquinone was employed to ensure single turnover conditions. Due to its lower reduction potential (of -488 mV vs NHE)⁴³ the hydroquinone reaction is very fast even with one electron equivalent, so it does not limit the observed k_{IET} value. Figure 6 displays absorbance changes monitored by SF spectroscopy in the reduction of NI by one electron equivalent of hydroquinone. It can be seen from the absorption trace at 610 nm corresponding to the oxidized T1 site (Figure 6B), that under these experimental conditions, the formation of NI

leads to an increase in T1 intensity that is rapidly reduced by the hydroquinone (in the first 20 milliseconds). Reoxidation of the T1 site is then observed upon IET to NI. The rate of T1 reoxidation corresponds to the rate of the first IET to the TNC. Fitting the trace at 610 nm according to Scheme 3 yields a first order rate constant of $17(\pm 5) \text{ s}^{-1}$, which matches well with the k_{IET} obtained by the global fitting of the experimental data for NI reduction by ascorbic acid. The first IET rate to NI in *TvL* is significantly slower than the first IET to NI in *RvL*.²⁶ In *RvL*, the IET rates are fast and the reduction of the low potential T1 site is slow ($150 \text{ M}^{-1} \text{ s}^{-1}$ with hydroquinone), thus the latter is the rate-limiting step of the catalytic reaction.²⁷ Alternatively, in *TvL*, the T1 reduction is very fast and the first IET to NI is the rate limiting step determining turnover frequency. However, this is still faster than the decay of NI by a factor of 10^2 . Thus, high potential MCOs function using the same mechanism as low potential MCOs but with a change in the rate determining step (Figure 1).

Spectroscopy.

The X-band EPR spectra of NI were obtained at 77 K and 5 K (Figure 7A,B). The 77 K EPR spectrum of the NI in red shows the typical T1 Cu signal with narrow $A_{\parallel}$ and no contribution from the T2 Cu, which is present in the RO spectrum in blue. At low temperatures (below 20 K), new features appear including a g value lower than 2.0 (Figure 7B). To remove the spectral contribution from the T1 signal, the EPR spectrum of T2D under the same (low temperature and high power) conditions was obtained (Figure S4) and subtracted from the NI spectrum. Figure 7B shows the resulting spectrum (black) with its simulated spectrum (red) overlaid. The EPR simulation of NI in Figure 7B is rhombic with g values of 2.08, 1.98 and 1.89. The g values lower than 2.0 indicate the presence of a spin-frustrated trinuclear Cu^{II} cluster similar to the NI in *RvL*.^{12,25, 44} The increase in the two g values below 2.0 in *TvL* compared to the previously reported values for the NI in *RvL* (2.15, 1.86, 1.65) is consistent with a perturbation of the exchange couplings in *TvL*, in which the three Cu-Cu exchange couplings are more similar, placing it closer to the spin frustrated limit (*vide infra*).⁴⁴

An important characteristic of the NI in *RvL* is that the broad EPR signal at low g values is very difficult to saturate indicating the presence of a low-lying excited state.¹² We thus investigated the power saturation behavior of the NI in *TvL*. Figure 8A shows progressive microwave power saturation data for NI in *TvL* at 5 K. It can be seen that the T1 signal saturates at powers higher than 10 mW while features corresponding to the NI continue to increase with increasing power. The saturation behavior of the NI is analyzed according to Rupp's equation:⁴⁵

$$\frac{I}{\sqrt{P}} = \frac{1}{[1 + (P/P_{1/2})]^{b/2}} \quad (1)$$

where I is the intensity of the signal at 3650 G (arrow in Figure 8A), P is the microwave power, $P_{1/2}$ is the microwave power at half-saturation, and b is the inhomogeneity parameter. For the first derivative EPR spectrum, b is 1 in the inhomogeneous limit and 3 in the homogeneous limit. Most EPR signals in proteins are inhomogeneously broadened, so b

should be close to 1. Rearrangement of Eqn. 1 yields Eqn. 2 and power saturation data at 5, 7, 9, 11 and 15 K were fit to this equation with the resulting $P_{1/2}$ and I_{max} parameters given in the inset of Figure 8B:

$$I = I_{max} \frac{\sqrt{P}}{\sqrt{1 + (P/P_{1/2})}} \quad (2)$$

The half-power saturation values at various temperatures were used to determine the energy of a low lying excited state, using an Orbach analysis:⁴⁵

$$\ln P_{1/2} \propto -\Delta/k_B \cdot T \quad (3)$$

where Δ is the energy gap between the ground and the excited state in cm^{-1} , k_B is the Boltzmann constant ($k_B = 0.695 \text{ cm}^{-1}\text{K}^{-1}$), and T is the temperature in K. The Orbach analysis shown in figure 8C reveals a linear relationship at temperatures lower than 15 K with the slope of the line giving the energy of the low-lying excited state $= 14(\pm 3) \text{ cm}^{-1}$. According to a Boltzmann population analysis at 15 K the low-lying excited state will be populated, which leads to the decrease of the half-power saturation value observed experimentally (at $1/T = 0.067$ in Figure 8C). The energy of the low lying state in *TvL* is significantly lower than in *RvL* (150 cm^{-1})¹² demonstrating that the all-bridged TNC structure in NI in *TvL* is closer to the spin-frustrated limit where all three copper atoms have close to equivalent exchange couplings leading to an orbitally degenerate ground state.⁴⁴

The low temperature magnetic circular dichroism (LT MCD) spectrum of NI in the energy region of $10000 - 33500 \text{ cm}^{-1}$ is shown in Figure S5. Panel A shows temperature dependence, and panel B shows the applied field dependence of the intermediate. The temperature and the field dependence are typical of MCD C-term behavior. Similar to the previously reported LT MCD spectrum of NI in low potential *RvL*, a very intense, derivative shaped pseudo-A term MCD feature is observed in the high-energy TNC charge-transfer region with maxima at 315 nm (31780 cm^{-1}) and 362 nm (27600 cm^{-1}). In addition, LT MCD features associated with the oxidized T1 Cu center are visible in the low-energy region (12500 to 20000 cm^{-1} in Figure S6).

Simultaneous Gaussian fitting of the LT MCD and room temperature absorption (RT Abs) spectra for the NI are included in Figure 9. As summarized in Table S1, a minimum of 5 bands is required to fit the LT MCD and RT Abs features associated with NI (bands numbered 1–5 in the region above 20000 cm^{-1} in Figure 9).

Normalized saturation magnetization curves taken at 31780 and 27620 cm^{-1} are shown in Figure S7. The curves were obtained by increasing the magnetic field at fixed temperature of 1.89 K. The curves are dependent on the S_{tot} of the ground state in the limit of no zero field splitting (ZFS). The normalized saturation magnetization curves for different possible spin states are included in the figure. The experimental saturation curves at 31780 and 27620 cm^{-1}

⁻¹ and fit with an $S = 1/2$ Brillouin curve with $g=2.06$ (close to the expected g_z value of 2.08), indicating an $S=1/2$ ground state of the *TvL* NI (Figure S7).

MCD transition intensity of a magnetically isolated $S=1/2$ system decreases as $1/T$ (with a fixed magnetic field).⁴⁶ This is plotted as the black line in Figure 10B. The intensity of the pseudo-A term feature of NI in *TvL* shows a different behavior, similar to that reported previously for *RvL* indicating thermal population of an excited state.¹² The total intensity of a system with thermally accessible excited states is given by the Boltzmann population of these states each with a C term intensity that decreases as $1/T$ (Equation 4).

$$I_{MCD}(\lambda) = \frac{\sum_i \left(\text{Deg}_i \exp\left(\frac{-E_i hc}{k_B T}\right) C_i \frac{1}{T} \right)}{\sum_j \left(\text{Deg}_j \exp\left(\frac{-E_j hc}{k_B T}\right) \right)} \quad (4)$$

where i and j are different electronic states, Deg_i or j is the degeneracy of the state, E_i or j is the i or j state energy, h is Planck's constant (6.626×10^{-34} J s), and c is the speed of light (2.998×10^{10} cm s⁻¹). The degeneracy is 2 for the ground and the first excited state and 4 for the second excited state (for an all spin coupled Cu^{II}₃ frustrated system).¹² C_i is the MCD intensity of state i at wavelength λ (which can be positive or negative and different from that of the ground state). The best fit to the intensity at 27620 cm⁻¹ and 31780 cm⁻¹ (Figure 10B) was achieved with 15 cm⁻¹ for the first excited state and >1000 cm⁻¹ for the second excited state. The energy separation between the ground and the first excited state of 15 cm⁻¹ agrees well with the value obtained from the Orbach analysis of EPR temperature saturation data.

DISCUSSION

By a combination of kinetic and spectroscopic studies, we elucidated the effect of the 350 mV higher reduction potential of the T1 site in high potential *TvL* relative to *RvL* on the catalytic mechanism (Figure 1). Using SF absorption spectroscopy, we have determined that similar to *RvL*, *TvL* rapidly reacts with O₂ to give the native intermediate. Formation of the NI was confirmed through its spectroscopic characterization. The characteristic spectroscopic features of NI are: (1) the absence of a T2 EPR signal at 77 K and the emergence of a broad signal with g_{eff} of 1.89 in the EPR spectrum at lower temperature and high radiant power, and (2) the appearance of a pair of intense field- and temperature-dependent bands with opposite signs in the MCD spectrum at 315 nm (–) and 362 nm (+) that are associated with the $\mu_3\text{-O} \rightarrow \text{Cu}^{\text{II}}$ CT transitions at the trinuclear site. By kinetic studies, we have shown that the decay of the NI to the RO (0.07 s⁻¹) and the reduction of the RO by the substrate (0.1 s⁻¹) are slow compared to the turnover frequency (25 s⁻¹) so the direct reduction of the NI to the fully reduced state occurs in the catalytic cycle of high potential MCOs, as was demonstrated for low potential MCOs.^{26,27}

The striking difference in the catalytic mechanism of the high potential MCOs in comparison to low potential *RvL* is that the first IET rate for the reduction of NI is

significantly slower (TvL : $\sim 20 \text{ s}^{-1}$ compared to RvL : $> 700 \text{ s}^{-1}$). This is still fast enough to enable catalysis through NI but changes the rate determining step from T1 reduction in RvL to IET in TvL . The difference in driving force on the rate of the first electron transfer to NI in TvL vs RvL can be estimated using the Marcus equation:⁴⁷

$$\frac{k_{TvL}}{k_{RvL}} = \sqrt{\frac{\lambda_{RvL} H_{DA}^2(TvL)}{\lambda_{TvL} H_{DA}^2(RvL)}} \exp \left[\left(-\frac{(\lambda_{TvL} + \Delta G_{TvL}^0)^2}{4\lambda_{TvL} k_B T} \right) - \left(-\frac{(\lambda_{RvL} + \Delta G_{RvL}^0)^2}{4\lambda_{RvL} k_B T} \right) \right] \quad (5)$$

where k_i is the electron transfer rate for the indicated MCO, k_B is the Boltzmann constant ($8.617 \times 10^{-5} \text{ eV K}^{-1}$), λ is the reorganization energy required for the electron transfer, H_{DA} is the electronic coupling matrix element between the donor and the acceptor, and G^0 is the total Gibbs free energy change for the electron transfer reaction. It has been shown previously that H_{DA} values for the superexchange pathway in MCOs do not change significantly between proteins.^{48,49} The reorganization energy for the electron transfer from the T1 to the NI in RvL was estimated to be 1.2 eV.²⁷ The reorganization energy for electron transfer from three coordinated T1 sites as in TvL compared to four coordinated T1 Cu sites as in RvL has been calculated to be lower by 0.056 eV,⁵⁰ giving a reorganization energy for TvL of 1.14 eV. Using the previously calculated G^0 for RvL of -12.5 kcal/mol (-0.54 eV) and the experimental k_i for RvL and TvL in Eqn. 5, the driving force for the electron transfer to the TNC in the NI of TvL is estimated to be -0.25 eV or -6 kcal/mol . The decrease in the driving force of 6.5 kcal/mol corresponds to a potential difference of 260 mV , however the potential difference between T1 sites in TvL and RvL has been measured to be 350 mV . For the 350 mV potential difference in T1 sites between TvL and RvL , using the same NI reduction potential, the first IET in TvL should be $\sim 7 \text{ s}^{-1}$. Thus, despite the very high potential of T1 in TvL , the IET rate (with no change in NI) would be fast enough to outcompete the decay rate of NI (0.07 s^{-1}) enabling fast turnover in the catalytic cycle.

The difference between the calculated IET rate (assuming no change in NI relative to RvL) of $\sim 7 \text{ s}^{-1}$ and the experimentally observed IET rate of 20 s^{-1} is consistent with a structural perturbation of NI in TvL and some variation in its protein environment. From the crystallographic structure of TvL (PDB ID:1GYC) and a structural model of RvL there are two conserved second-sphere carboxylate residues near the TNC active site in both proteins (Asp77 and Asp456 in TvL and Asp72 and Glu499, respectively, in RvL). The observed pK_a values of Asp 456 in TvL and Glu 499 in RvL are 3.7 and 5.8, respectively (*vide supra*). Perturbation of a group's pK_a from its intrinsic value mainly occurs due to short- and long-range electrostatic interactions.⁵¹ The experimentally observed lower proton affinity of the Asp 456 in TvL compared to Glu 499 in RvL , thus, reflects a more positive local charge environment in the vicinity of the TNC in TvL compared to RvL . This is supported by the increase in reduction potentials reported by Reinhammar for the T3 Cu pairs from 434 to 782 mV in resting RvL and TvL , respectively.³⁰ Pinpointing residue differences responsible for the overall charge increase in the vicinity of the TNC in TvL is difficult since there is no crystal structure of RvL available. Moreover, structural alignment of TvL and a structural model of RvL shows that despite the entirely conserved first coordination sphere of the Cu ions there are several dozen residues that differ between these two enzymes. These

substitutions would result in differences in both hydrophobicity and charge in the vicinity of the TNC. A more positive local protein environment in the vicinity of the TNC in *TvL* than in *RvL* is consistent with the higher redox potential of NI in *TvL* compared to *RvL* (–100 mV increase based on the above IET rate calculations).

In addition to a difference in the electrostatic environment there are also limited electronic structure differences in NI between *TvL* and *RvL*. Both have $S=1/2$ ground states with $g_{x,y}$ values below 2.0 and pseudo-A terms in MCD indicating a spin frustrated TNC with a μ_3 -oxo bridging all three Cu^{II} s. There must also be a μ_2 -hydroxo at the T3 as all three Cu^{II} are antiferromagnetically coupled and it has been shown in reference⁵² that loss of the μ_2 -hydroxide leads to a rotation of one of the Cu^{II} magnetic orbitals which becomes orthogonal to, and therefore ferromagnetically coupled to the magnetic orbitals on the other two Cu^{II} s,⁵³ which is not observed experimentally in *TvL*.

However, there are quantitative differences in NI between *TvL* and *RvL*. In particular the low lying first excited state is lower in energy in *TvL* ($\sim 15 \text{ cm}^{-1}$) than in *RvL* ($\sim 150 \text{ cm}^{-1}$) and the lowest g value is shifted up in *TvL* (~ 1.9) compared to *RvL* (~ 1.65). These unique features of NI arise from a spin frustrated antiferromagnetic ^2E ground state, which undergoes a ZFS through a combination of differences in symmetric exchange and the presence of an antisymmetric exchange interaction.²⁵ The ZFS of the ^2E ground state is expressed as follows:⁵⁴

$$\Delta = \sqrt{\delta^2 + 3G^2} \quad (6)$$

where δ is the “symmetry lowering” due to small differences in the antiferromagnetic J values due to differences in bridging, and G is the antisymmetric exchange. The lower energy gap between the ground state and an excited doublet () in the NI in *TvL* and g values closer to 2.0 place it closer to the spin frustrated limit, implying there are smaller differences in isotropic exchange coupling constants ($J_{12} \approx J_{23} \approx J_{31}$, where numbered subscripts correspond to the three Cu sites in the TNC).

CONCLUSIONS

This study has evaluated the effect of the 350 mV increase in reduction potential on the mechanism of high potential relative to low potential MCOs. The increase of the T1 redox potential by $\sim 350 \text{ mV}$ contributes to an 8 kcal/mol decrease in the driving force for IET to the NI in high potential MCOs. This decrease results in a slower rate of the first IET to NI, the fully oxidized intermediate in the O_2 reduction reaction. This IET rate is, however, fast compared to the decay rate of the NI, making it the catalytically relevant fully oxidized form of the MCO and enabling fast turnover. However, in contrast to the low potential MCOs where T1 reduction by substrate is rate limiting, in high potential MCOs the first IET step is the rate limiting step in the catalytic cycle, determining the turnover frequency. Part of the reduction potential difference of the T1 sites of high potential in comparison to low potential MCOs is balanced by an $\sim 100 \text{ mV}$ higher reduction potential of the NI due to the more positive protein environment in the vicinity of the TNC and limited perturbation of the

structure of NI. The former is reflected in the lower proton affinity of the proton donating group involved in the decay of NI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study is supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award number R01DK031450 (to E. I. S.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. AS acknowledges support from the Knut and Alice Wallenberg Foundation (KAW 2015.0418).

REFERENCES

- (1). Solomon EI; Sundaram UM; Machonkin TE Multicopper Oxidases and Oxygenases. *Chem. Rev* 1996, 96 (7), 2563–2606. [PubMed: 11848837]
- (2). Giardina P; Faraco V; Pezzella C; Piscitelli A; Vanhulle S; Sannia G Laccases: A Never-Ending Story. *Cell. Mol. Life Sci* 2010, 67 (3), 369–385. [PubMed: 19844659]
- (3). Gray HB; Malmstrom BG; Williams RJP Copper Coordination in Blue Proteins. *JBICJ. Biol Inorg. Chem* 2000, 5 (5), 551–559.
- (4). Solomon EI; Heppner DE; Johnston EM; Ginsbach JW; Cirera J; Qayyum M; Kieber-Emmons MT; Kjaergaard CH; Hadt RG; Tian L Copper Active Sites in Biology. *Chem. Rev* 2014, 114 (7), 3659–3853. [PubMed: 24588098]
- (5). Messerschmidt A; Ladenstein R; Huber R; Bolognesi M; Avigliano L; Petruzzelli R; Rossi A; Finazzi-Agró A Refined Crystal Structure of Ascorbate Oxidase at 1.9 Å Resolution. *J. Mol. Biol* 1992, 224 (1), 179–205. [PubMed: 1548698]
- (6). Jones SM; Solomon EI Electron Transfer and Reaction Mechanism of Laccases. *Cell. Mol. Life Sci* 2015, 72 (5), 869–883. [PubMed: 25572295]
- (7). Morozova OV; Shumakovich GP; Gorbacheva MA; Shleev SV; Yaropolov AI “Blue” Laccases. *Biochem. Mosc* 2007, 72 (10), 1136–1150.
- (8). Quintanar L; Stoj C; Taylor AB; Hart PJ; Kosman DJ; Solomon EI Shall We Dance? How A Multicopper Oxidase Chooses Its Electron Transfer Partner. *Acc. Chem. Res* 2007, 40 (6), 445–452. [PubMed: 17425282]
- (9). Mano N; de Poulpiquet A O₂ Reduction in Enzymatic Biofuel Cells. *Chem. Rev* 2018, 118 (5), 2392–2468. [PubMed: 28930449]
- (10). Rodríguez Couto S; Toca Herrera JL Industrial and Biotechnological Applications of Laccases: A Review. *Biotechnol. Adv* 2006, 24 (5), 500–513. [PubMed: 16716556]
- (11). Shin W; Sundaram UM; Cole JL; Zhang HH; Hedman B; Hodgson KO; Solomon EI Chemical and Spectroscopic Definition of the Peroxide-Level Intermediate in the Multicopper Oxidases: Relevance to the Catalytic Mechanism of Dioxygen Reduction to Water. *J. Am. Chem. Soc* 1996, 118 (13), 3202–3215.
- (12). Lee S-K; George SD; Antholine WE; Hedman B; Hodgson KO; Solomon EI Nature of the Intermediate Formed in the Reduction of O₂ to H₂O at the Trinuclear Copper Cluster Active Site in Native Laccase. *J. Am. Chem. Soc* 2002, 124 (21), 6180–6193. [PubMed: 12022853]
- (13). Andréasson LE; Reinhammar B The Mechanism of Electron Transfer in Laccase-Catalysed Reactions. *Biochim. Biophys. Acta* 1979, 568 (1), 145–156. [PubMed: 221027]
- (14). Andréasson LE; Brändén R; Reinhammar B Kinetic Studies of *Rhus Vernicifera* Laccase. Evidence for Multi-Electron Transfer and an Oxygen Intermediate in the Reoxidation Reaction. *Biochim. Biophys. Acta* 1976, 438 (2), 370–379. [PubMed: 182231]

- (15). Cole JL; Ballou DP; Solomon EI Spectroscopic Characterization of the Peroxide Intermediate in the Reduction of Dioxygen Catalyzed by the Multicopper Oxidases. *J. Am. Chem. Soc* 1991,113 (22), 8544–8546.
- (16). Morie-Bebel MM; Morris MC; Menzie JL; McMillin DR A Mixed-Metal Derivative of Laccase Containing Mercury(II) in the Type 1 Binding Site. *J. Am. Chem. Soc* 1984,106 (12), 3677–3678.
- (17). Palmer AE; Lee SK; Solomon EI Decay of the Peroxide Intermediate in Laccase: Reductive Cleavage of the O–O Bond. *J. Am. Chem. Soc* 2001,123 (27), 6591–6599. [PubMed: 11439045]
- (18). Blackburn NJ; Ralle M; Hassett R; Kosman DJ Spectroscopic Analysis of the Trinuclear Cluster in the Fet3 Protein from Yeast, a Multinuclear Copper Oxidase. *Biochemistry* 2000, 39 (9), 2316–2324. [PubMed: 10694398]
- (19). Severns JC; McMillin DR Temperature and Anation Studies of the Type 2 Site in *Rhus Vernicifera* Laccase. *Biochemistry* 1990, 29 (37), 8592–8597. [PubMed: 2176828]
- (20). Kjaergaard CH; Jones SM; Gounel S; Mano N; Solomon EI Two-Electron Reduction versus One-Electron Oxidation of the Type 3 Pair in the Multicopper Oxidases. *J. Am. Chem. Soc* 2015,137 (27), 8783–8794. [PubMed: 26075678]
- (21). Yoon J; Solomon EI Electronic Structure of the Peroxy Intermediate and Its Correlation to the Native Intermediate in the Multicopper Oxidases: Insights into the Reductive Cleavage of the O–O Bond. *J. Am. Chem. Soc* 2007,129 (43), 13127–13136. [PubMed: 17918839]
- (22). Augustine AJ; Quintanar L; Stoj CS; Kosman DJ; Solomon EI Spectroscopic and Kinetic Studies of Perturbed Trinuclear Copper Clusters: The Role of Protons in Reductive Cleavage of the O–O Bond in the Multicopper Oxidase Fet3p. *J. Am. Chem. Soc* 2007,129 (43), 13118–13126. [PubMed: 17918838]
- (23). Clark PA; Solomon EI Magnetic Circular Dichroism Spectroscopic Definition of the Intermediate Produced in the Reduction of Dioxygen to Water by Native Laccase. *J. Am. Chem. Soc* 1992,114 (3), 1108–1110.
- (24). Chalupský J; Neese F; Solomon EI; Ryde U; Rulíšek L Multireference Ab Initio Calculations on Reaction Intermediates of the Multicopper Oxidases. *Inorg. Chem* 2006, 45 (26), 11051–11059. [PubMed: 17173465]
- (25). Yoon J; Solomon EI Ground-State Electronic and Magnetic Properties of a μ_3 -oxo-Bridged Trinuclear Cu(II) Complex: Correlation to the Native Intermediate of the Multicopper Oxidases. *Inorg. Chem* 2005, 44 (22), 8076–8086. [PubMed: 16241158]
- (26). Heppner DE; Kjaergaard CH; Solomon EI Molecular Origin of Rapid versus Slow Intramolecular Electron Transfer in the Catalytic Cycle of the Multicopper Oxidases. *J. Am. Chem. Soc* 2013,135 (33), 12212–12215. [PubMed: 23902255]
- (27). Heppner DE; Kjaergaard CH; Solomon EI Mechanism of the Reduction of the Native Intermediate in the Multicopper Oxidases: Insights into Rapid Intramolecular Electron Transfer in Turnover. *J. Am. Chem. Soc* 2014,136 (51), 17788–17801. [PubMed: 25490729]
- (28). Farver O; Wherland S; Koroleva O; Loginov DS; Pecht I Intramolecular Electron Transfer in Laccases. *FEBSJ.* 2011, 278 (18), 3463–3471.
- (29). Frascioni M; Favero G; Boer H; Koivula A; Mazzei F Kinetic and Biochemical Properties of High and Low Redox Potential Laccases from Fungal and Plant Origin. *Biochim. Biophys. Acta BBA - Proteins Proteomics* 2010,1804 (4), 899–908.
- (30). Reinhammar BRM Oxidation-Reduction Potentials of the Electron Acceptors in Laccases and Stellacyanin. *Biochim. Biophys. Acta BBA - Bioenerg.* 1972, 275 (2), 245–259.
- (31). Fahraeus GF; Reinhammar B Large Scale Production and Purification of Laccase from Cultures of the Fungus *Polyporus Versicolor* and Some Properties of Laccase A. *Acta Chem. Scand* 1967, 21 (9), 2367–2378. [PubMed: 5585673]
- (32). Bertrand T; Jolival C; Briozzo P; Caminade E; Joly N; Madzak C; Mougou C Crystal Structure of a Four-Copper Laccase Complexed with an Arylamine: Insights into Substrate Recognition and Correlation with Kinetics. *Biochemistry* 2002, 41 (23), 7325–7333. [PubMed: 12044164]
- (33). Malkin R; Malmström BG; Vänngård T The Reversible Removal of One Specific Copper(II) from Fungal Laccase. *Eur. J. Biochem* 2005, 7 (2), 253–259.

- (34). Felsenfeld G The Determination of Cuprous Ion in Copper Proteins. Arch. Biochem. Biophys 1960, 87 (2), 247–251. [PubMed: 13822131]
- (35). Carithers RP; Palmer G Characterization of the Potentiometric Behavior of Soluble Cytochrome Oxidase by Magnetic Circular Dichroism. Evidence in Support of Heme-Heme Interaction. J. Biol Chem 1981, 256 (15), 7967–7976. [PubMed: 6267032]
- (36). Hanna PM; McMillin DR; Pasenkiewicz-Gierula M; Antholine WE; cReinhammar B Type 2-Depleted Fungal Laccase. Biochem. J 1988, 253 (2), 561–568. [PubMed: 2845923]
- (37). Waterhouse A; Bertoni M; Bienert S; Studer G; Tauriello G; Gumienny R; Heer FT; de Beer TAP; Rempfer C; Bordoli L; Lepore R; Schwede T SWISS-MODEL: Homology Modelling of Protein Structures and Complexes. Nucleic Acids Res. 2018, 46 (W1), W296–W303. [PubMed: 29788355]
- (38). Guex N; Peitsch MC; Schwede T Automated Comparative Protein Structure Modeling with SWISS-MODEL and Swiss-PdbViewer: A Historical Perspective. ELECTROPHORESIS 2009, 30 (S1), S162–S173. [PubMed: 19517507]
- (39). UniProt: A Worldwide Hub of Protein Knowledge. Nucleic Acids Res. 2019, 47 (D1), D506–D515. [PubMed: 30395287]
- (40). Huang H; Zoppellaro G; Sakurai T Spectroscopic and Kinetic Studies on the Oxygen-Centered Radical Formed during the Four-Electron Reduction Process of Dioxygen by Rhus Vernicifera Laccase. J. Biol. Chem 1999, 274 (46), 32718–32724. [PubMed: 10551829]
- (41). Piontek K; Antorini M; Choinowski T Crystal Structure of a Laccase from the Fungus Trametes Versicolor at 1.90-Å Resolution Containing a Full Complement of Coppers. J. Biol. Chem 2002, 277 (40), 37663–37669. [PubMed: 12163489]
- (42). Kataoka K; Sugiyama R; Hirota S; Inoue M; Urata K; Minagawa Y; Seo D; Sakurai T Four-Electron Reduction of Dioxygen by a Multicopper Oxidase, CueO, and Roles of Asp112 and Glu506 Located Adjacent to the Trinuclear Copper Center. J. Biol. Chem 2009, 284 (21), 14405–14413. [PubMed: 19297322]
- (43). Huynh MT; Anson CW; Cavell AC; Stahl SS; Hammes-Schiffer S Quinone 1 e⁻ and 2 e⁻/2 H⁺ Reduction Potentials: Identification and Analysis of Deviations from Systematic Scaling Relationships. J. Am. Chem. Soc 2016, 138 (49), 15903–15910. [PubMed: 27960306]
- (44). Yoon J; Solomon EI Electronic Structures of Exchange Coupled Trigonal Trimeric Cu(II) Complexes: Spin Frustration, Antisymmetric Exchange, Pseudo-A Terms, and Their Relation to O₂ Activation in the Multicopper Oxidases. Coord. Chem. Rev 2007, 251 (3), 379–400.
- (45). Poole CP Electron Spin Resonance: A Comprehensive Treatise on Experimental Techniques; Courier Corporation, 1996.
- (46). Neese F; Solomon EI MCD C-Term Signs, Saturation Behavior, and Determination of Band Polarizations in Randomly Oriented Systems with Spin S = 1/2. Applications to S = 1/2 and S = 5/2. Inorg. Chem 1999, 38 (8), 1847–1865. [PubMed: 11670957]
- (47). Marcus RA Electron Transfer Reactions in Chemistry. Theory and Experiment. Rev. Mod. Phys 1993, 65 (3), 599–610.
- (48). Solomon EI; Szilagyi RK; DeBeer George S; Basumallick L Electronic Structures of Metal Sites in Proteins and Models: Contributions to Function in Blue Copper Proteins. Chem. Rev 2004, 104 (2), 419–158. [PubMed: 14871131]
- (49). Hadt RG; Gorelsky SI; Solomon EI Anisotropic Covalency Contributions to Superexchange Pathways in Type One Copper Active Sites. J. Am. Chem. Soc 2014, 136 (42), 15034–15045. [PubMed: 25310460]
- (50). Hu L; Farrokhnia M; Heimdal J; Shleev S; Rulíšek L; Ryde U Reorganization Energy for Internal Electron Transfer in Multicopper Oxidases. J. Phys. Chem. B 2011, 115 (45), 13111–13126. [PubMed: 21955325]
- (51). Fitch CA; Karp DA; Lee KK; Stites WE; Lattman EE; García-Moreno EB Experimental PKa Values of Buried Residues: Analysis with Continuum Methods and Role of Water Penetration. Biophys. J 2002, 82 (6), 3289–3304. [PubMed: 12023252]
- (52). Yoon J; Mirica LM; Stack TDP; Solomon EI Spectroscopic Demonstration of a Large Antisymmetric Exchange Contribution to the Spin-Frustrated Ground State of a D₃ Symmetric

Hydroxy-Bridged Trinuclear Cu(II) Complex: Ground-to-Excited State Superexchange Pathways. *J. Am. Chem. Soc.* 2004, 126 (39), 12586–12595. [PubMed: 15453791]

- (53). Solomon EI; Augustine AJ; Yoon J O₂ Reduction to H₂O by the Multicopper Oxidases. *Dalton Trans.* 2008, No. 30, 3921–3932. [PubMed: 18648693]
- (54). Tsukerblat BS; Kuyavskaya B. Ya.; Belinskii MI; Ablov AV; Novotortsev VM; Kalinnikov VT Antisymmetric Exchange in the Trinuclear Clusters of Copper (II). *Theor. Chim. Acta* 1975, 38 (2), 131–138.

Figure 1.

Mechanism of the high potential *TvL* in comparison with the low potential *Rhus vernicifera* laccase. *TvL* in dark blue, *Rhus vernicifera* laccase in purple.

Figure. 2.

Reaction of the reduced *TvL* with O_2 monitored by a rapid scan stopped-flow instrument at pH 6.0 and 4 °C; $[Lc] = 50 \mu M$, $[O_2] = 130 \mu M$ (A) Absorption difference spectra from 1 ms to 30 s after subtracting reduced enzyme spectrum. (B) Scaled difference absorption trace of the 420 nm NI-TNC trace and (inset) growth of the T1 (610 nm, blue) and TNC (420 nm, red and 330 nm, black) charge transfer bands. (C) Absorption spectra and (D) Absorption traces obtained by the global fitting of the experimental data to the Scheme 1.

Figure 3.

Effect of pH on the native intermediate decay rates. (A) Rates of decay of NI as a function of pH, experimental data (dots) and the fit (red line) to a single protonation equilibrium. The pK_a of the protonatable group is 3.7. (B) Sample absorption spectra of the NI at 3.0 and 7.0 pH.

Figure 4.

Reduction of the native intermediate and the resting oxidized form in *TvL*. (A) Absorption traces (circles), and fits (lines) of the T1 (610 nm, blue) and TNC (420 nm, red) bands in the reduction of the native intermediate. Conditions: $[Lc] = [O_2] = 0.05$ mM; $[Ascorbic\ acid] = 0.25$ mM. (B) Absorption traces (circles), and fits (lines) of the T1 (614 nm, blue) and TNC (330 nm, black) bands in the reduction of the resting oxidized form. Conditions: $[Lc] = 0.050$ mM; $[Ascorbic\ acid] = 0.25$ mM.

Figure 5.

The native intermediate reduction by ascorbic acid monitored by a rapid scan stopped-flow instrument at pH 6.0 and 4 °C; [Lc] = [O₂] = 0.05 mM (A) Absorption spectra from 10 ms to 2 s; [Ascorbic acid] = 0.25 mM. (B) Kinetic data (squares) of the TNC (420 nm) charge transfer bands in the reduction of the native intermediate with 0.25, 5.0 and 100 mM ascorbic acid and overlay of simulations (lines) according to Scheme 2.

Figure 6.

The native intermediate reduction by hydroquinone monitored by a rapid scan stopped-flow instrument at pH 6.0 and 4 °C; [Lc] = [H₂Q] = 0.05 mM, [O₂] = 1.0 mM. (A) Absorption spectra from 1 ms to 250 ms. (A) Kinetic traces of the T1 (610 nm, solid blue line and fit to the Scheme 2, dashed purple line) and TNC (420 nm, red) charge transfer bands in the reduction of the native intermediate.

Figure 7.

EPR characterization of the native intermediate in *TvL*. (A) EPR spectra of the native intermediate (red) and the resting oxidized enzyme (blue) at 10.02 mW and 77 K. (B) EPR spectra of the native intermediate (black with T1 subtracted see text) at 20 mW and 5K, and its simulation (red).

Figure 8.

Power saturation and Orbach analysis. (A) EPR spectra of the native intermediate taken at 0.1–10 mW top and 10–200 mW at 5 K. (B). Normalized intensity of the native intermediate signal at 3640 G (shown by the arrow) plotted against a microwave power at various temperatures (dots) and fits of the experimental data to the Eqn. 1. (C) Plot of the $\ln P_{1/2}$ against inverse temperature. The linear response indicates a low-lying excited state at $14(\pm 3)$ cm^{-1}

Figure 9. Simultaneous Gaussian fit of the Absorbance and MCD spectra of the native intermediate. (A) LT MCD spectrum and (B) RT absorption spectrum of the native intermediate simultaneously fitted with a minimum number of Gaussian bands. Note that low temperature Gaussian bands in MCD are allowed to have narrower band widths at low temperature and shift by up to 200 cm^{-1} in the fits.

Figure 10.

(A) The temperature dependence of the native intermediate MCD spectra at 7T. (B) Fit of the temperature dependence of the native intermediate MCD spectra (average of 3 measurements with MCD Intensity normalized to sample concentration and field) at 27620 cm^{-1} (•) and at 31780 cm^{-1} (◆) (shown with arrows in panel A) to Eqn. 4 assuming the specified energy parameters for the energy of the first and the second excited states.

Fully reduced $\rightarrow NI \rightarrow RO$

Scheme 1.

Kinetic model for the native intermediate formation and decay.

Scheme 2.

Kinetic model for the native intermediate formation and reduction by ascorbic acid (asc).

Scheme 3.

Kinetic model for NI formation and reduction by hydroquinone.

Table 1.

Kinetic parameters obtained from the global fitting to the Scheme 2 of the experimental stopped-flow data of the native intermediate reduction by various concentrations of ascorbic acid.

Parameters	Value	Standard Deviation
k_1	$1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (fixed)	$\pm 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$
k_2	$2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (fixed)	$\pm 2200 \text{ M}^{-1} \text{ s}^{-1}$
k_{IET}	20 s^{-1}	$\pm 3 \text{ s}^{-1}$