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Electron Transfer and Reaction Mechanism of Laccases

Stephen M. Jones and Edward I. Solomon*

Department of Chemistry, Stanford University

Abstract

Laccases are part of the family of multicopper oxidases (MCOs), which couple the oxidation of substrates to the four electron reduction of O₂ to H₂O. MCOs contain a minimum of four Cu's divided into Type 1 (T1), Type 2 (T2), and binuclear Type 3 (T3) Cu sites that are distinguished based on unique spectroscopic features. Substrate oxidation occurs near the T1, and electrons are transferred approximately 13 Å through the protein via the Cys-His pathway to the T2/T3 trinuclear copper cluster (TNC) where dioxygen reduction occurs. This review outlines the electron transfer (ET) process in laccases, and the mechanism of O₂ reduction as elucidated through spectroscopic, kinetic, and computational data. Marcus theory is used to describe the relevant factors which impact ET rates including the driving force (G°), reorganization energy (λ), and electronic coupling matrix element (H_{DA}). Then the mechanism of O₂ reaction is detailed with particular focus on the intermediates formed during the two $2e^-$ reduction steps. The first $2e^-$ step forms the peroxide intermediate (PI), followed by the second $2e^-$ step to form the native intermediate (NI), which has been shown to be the catalytically relevant fully oxidized form of the enzyme.

Keywords

laccase; multicopper oxidase; type 1 copper; type 2 copper; type 3 copper; Marcus theory

I. Introduction

Laccases belong to a family of enzymes known as multicopper oxidases (MCOs), which oxidize a variety of substrates while performing the four electron reduction of dioxygen to water [1–4]. MCOs can be divided into two broad categories: those with high substrate specificity, and those with low substrate specificity. The first category contains the metalloxidases such as Fet3p [5, 6] and ceruloplasmin (Cp) [6, 7], which selectively oxidize iron in yeast and mammals, respectively. Ascorbate oxidase [8] and laccases [1–3] are examples of the latter category, which oxidize a range of organic molecules to facilitate processes such as lignin formation [9] or degradation [10], and wound healing in plants [11]. Laccases are common in fungi including in the polypore mushroom *Trametes versicolor*, and the white-rot fungus *Pleurotus ostreatus* [12], and well known in a number of plants, in particular the lacquer tree *Rhus vernicifera* [13–16]. The first bacterial laccase was reported in the rhizospheric bacterium *Azospirillum lipoferum* [17], and several others have since

*Corresponding Author 333 Campus Drive Stanford, CA 94305 solomone@stanford.edu Phone: (650) 723-9104 Fax: (650) 725-0259.

been characterized [18, 19]. The most widely studied of these prokaryotic laccases is the CotA laccase from *Bacillus subtilis*, now technically classified as a bilirubin oxidase (BOD) [20].

Structurally, MCOs contain at least four copper atoms, which can be classified into three types based on their unique spectroscopic features. In the oxidized state, the Type 1 copper (T1) exhibits an intense ($\epsilon \sim 5000 \text{ M}^{-1} \text{ cm}^{-1}$) absorption feature at $\sim 600 \text{ nm}$ due to a $\text{S}(\text{Cys})_{\pi} \rightarrow \text{Cu}(\text{II})$ charge transfer (CT) transition, and a small parallel hyperfine coupling constant in EPR ($A_{\parallel} = 40\text{-}90 \times 10^{-4} \text{ cm}^{-1}$), both resulting from the highly covalent nature of the Cu-S(Cys) bond [21]. The Type 2 (T2) Cu(II) shows no significant absorbance feature, but has a parallel hyperfine coupling constant similar to that of a typical tetragonal copper center ($A_{\parallel} = 140\text{-}200 \times 10^{-4} \text{ cm}^{-1}$) [22, 23]. Binuclear Type 3 (T3) copper sites are EPR silent due to antiferromagnetic (AF) coupling resulting from a bridging hydroxo ligand between the coppers in the resting oxidized state. This hydroxo bridge is also responsible for the intense absorbance at 330 nm assigned to $\mu_2\text{-OH} \rightarrow \text{T3Cu}(\text{II})$ CT transitions [24]. Figure 1 shows the UV-Vis absorbance and EPR spectra of the resting oxidized (RO) state in the MCOs as described above.

Based on known crystal structures and sequence alignment, the ligation of the four copper centers is largely conserved among MCOs. The T1 site is coordinated by a minimum of two His and one Cys residues [25, 26]. In many MCOs, a fourth ligand, Met, binds axially, resulting in a four coordinate (4C) trigonally elongated tetrahedral geometry as shown in Figure 2(a). This Cu-S(Met) bond is substantially longer and weaker than the other three ligand bonds to the T1 [27]. This structure appears in the redox active T1 sites in Cp, and in plant laccases. Alternatively, Figure 2(b) shows the three coordinate (3C) trigonal planar structure present in fungal laccases and Fet3p, that results when a noncoordinating, hydrophobic residue, such as Phe or Leu, is present in the protein in place of the axial Met. The effects of these perturbations are discussed in Part II. The T2 and binuclear T3 copper sites are arranged in a trinuclear copper cluster (TNC) approximately $13 \text{ \AA}$ distant from the T1 Cu. Type 1 sites are connected to the two T3 coppers of the TNC via a conserved His-Cys-His triad. Figure 3 shows the crystal structure of the TNC from *T. versicolor* laccase (TvL). Including the two His ligands from the Cys-His bridge, there are a total of eight His residues coordinated to the TNC coppers. The T3 Cu's each have three His ligands, and in the oxidized state, a bridging $\mu_2\text{-OH}$ ligand resulting in an approximately trigonal bipyramidal geometry with an open equatorial coordination position directed into the TNC. The T2 Cu(II) is ligated to two His and one hydroxo ligand (external to the cluster) in a square planar geometry, also with an open coordination position directed into the TNC [28].

Additionally, there are two conserved second sphere carboxylate residues located close to the TNC. One carboxylate is situated on the side of the TNC closest to the T2-T3 β edge. Mutational studies have shown that this moiety is required for catalytic activity [29]. Another conserved carboxylate is situated below the T3 site. This residue has been shown to play a key role in the protonation of oxygen-derived products of catalysis [30], described further in Part III.

The focus of this review is on understanding inter- and intramolecular electron transfer (ET) properties of laccases, and outlining the mechanism of O₂ reaction. Part II deals with electron transfer from the substrate to the T1 Cu, and then from the T1 site to the TNC. Part III describes the mechanism of O₂ reduction through the known spectroscopic intermediates, and describe the role of the native intermediate (NI) in catalysis.

II. Electron transfer properties of the T1 copper

Type 1, or “blue copper,” sites are well known in bioinorganic chemistry for their ET properties [21, 27, 31, 32]. In MCOs, substrate oxidation occurs at the T1, which then rapidly transfers electrons through the Cys-His pathway to the TNC [33, 34]. The first and second sphere residues surrounding the T1 control both the intermolecular ET to the T1 from the substrate, and the intramolecular ET from the T1 to the TNC.

Semi-classical Marcus theory provides the description of ET in metalloenzymes. The electron transfer rate constant, k_{ET} , is given by the following equation [35]

$$k_{ET} = K_A S \sqrt{\frac{4\pi^3}{h^2 \lambda k_B T}} |H_{DA}|^2 \exp\left(\frac{-(\Delta G^\circ + \lambda)^2}{4\lambda k_B T}\right) \quad (1)$$

where K_A is the equilibrium constant for the electron donor-acceptor complex, S is a steric term to account for asymmetry in complex formation conducive to ET, λ is the reorganization energy, H_{DA} is the electronic coupling matrix element between the donor and acceptor, and ΔG° is the free energy difference for electron transfer. For intermolecular ET from substrate to T1, the most obvious parameters that influence k_{ET} are the reorganization energy, λ , and the electronic driving force, ΔG° .

The reorganization energy is the energy required for ligand and solvent rearrangements between initial and final states during ET. These inner- and outer-sphere contributions combine to give the total reorganization energy ($\lambda_i + \lambda_o = \lambda_{\text{total}}$) [35]. Inner-sphere reorganization energies are associated with changes in the first coordination sphere of the metal atom, and outer-sphere reorganization energies account for changes in the rest of the protein and solvent rearrangements [36]. Crystallography and X-ray absorption fine structure (EXAFS) data show only minimal differences between oxidized and reduced structures of T1 sites in most MCOs. The changes seen upon oxidation are primarily shortening ligand-Cu bonds, with very little angular change. Significantly, the Jahn-Teller tetragonal distortion associated with oxidation of most copper complexes is not observed in blue copper sites. This is a result of the ligands and the geometry at the T1 which split the Cu and $3d_{x^2-y^2}$ and $3d_{xy}$ levels, eliminating the degeneracy required for electron-nuclear coupling that would result in this geometric distortion [27]. These data indicate that λ_i is small for ET in blue copper sites, and indeed, inner sphere reorganization energies are calculated to be on the order of 0.5 eV [37, 38]. For comparison, the inner sphere reorganization energy of a representative copper tetrammine complex is ~ 1.4 eV [37]. There may, however, be considerable contributions to the total reorganization energies from the substrate (when the T1 accepts the electron), or from the TNC (when the T1 donates the electron). These contributions to λ_{total} would then contribute to k_{ET} through Equation 1.

Changes in reorganization energies which arise from the structural differences of reactive intermediates are found to make relatively minor contributions to the changes in ET rates seen during the catalytic cycle of MCOs (see Part III). We turn now to the free energy difference, G° , which derives from the reduction potentials of T1 Cu sites.

Reduction potentials (E°) of blue copper sites in laccases range from ~ 400 to ~ 800 mV versus NHE [13, 39–41]. Although many factors influence reduction potentials of metal sites in proteins [42], one significant contribution in MCOs is the presence or absence of an axial Met ligand. Plant laccases, which possess the Met ligand, often have potentials on the low end of this range, while fungal laccases, which lack an axial ligand, typically have higher reduction potentials [43]. Evidence for the impact of axial ligation on E° can be seen in two mutants of CotA in *B. subtilis*. Wild type (WT) CotA has a reduction potential of 455 mV, but when the T1 axial ligand Met502 is replaced with non-ligating Phe (M502F) or Leu (M502L), the potentials of these mutants increase to 548 and 515 mV, respectively [44]. Conversely, mutation of the axially non-coordinating F463 residue to Met in *T. villosa* laccase (*TviL*) decreases the reduction potential from 790 mV to 680 mV [45]. Clearly, the presence of the additional Cu-S(Met) bond decreases the reduction potential of the T1 Cu, yet E° can differ substantially even among blue copper proteins with identical ligand sets.

Comparisons of T1 centers over a range of copper proteins have revealed that the strength of the Cu-S(Met) bond can profoundly impact the electronic and geometric structures of these sites. Decreasing the Cu-S(Met) bond length results in weakening and lengthening of the Cu-S(Cys) bond, accompanied by a tetragonal (i.e. Jahn-Teller) distortion of the site, in which the Cu-S(Cys)-S(Met) plane rotates into the Cu-N(His)-N(His) plane [46]. This “coupled distortion” describes the continuum of geometries connecting blue copper sites to so called green copper sites that possess the same 1Cys-2His-1Met ligand motif seen in plant laccases, but differ in electronic and geometric structure. The extremes of this coupled distortion coordinate are the green T1 site in *Achromobacter cycloclastes* nitrite reductase (NiR), which has a strong Cu-S(Met) bond, and the blue T1 Cu in plant laccases and plastocyanin, which have weak Cu-S(Met) bonds (and, by extension, T1 sites in fungal laccases and Fet3p, in which no axial ligand is present) [21, 27]. Effects of this perturbation are manifest in spectroscopic changes including the S(Cys)→Cu π and σ CT bands switching from intense π / weak σ in blue sites to intense σ / weak π in green sites. Density functional theory (DFT) calculations calibrated to spectroscopic data and spectroscopy on NiR variants indicate that it is the strength of the Cu-S(Met) bond that determines whether a site is blue (weak) or green (strong Cu-S(Met) bond) [47]. This, in turn, is a result of the protein backbone, which constrains the Cu-S(Met) bond and enforces an active site structure at a given point along the coupled distortion coordinate. A protein constraint on active site ligation to enhance function is often referred to as the entatic, or rack state [48]. The axial Met constraint contributes to tuning the reduction potential of the T1 as described below.

A demonstration of the importance of the protein backbone comes from the T1 site in *Rhodobacter sphaeroides* NiR, in which the flexible nature of the methionine-containing loop allows the axial Met bond to the T1 to break at high temperatures. Breaking the Cu-S(Met) bond is entropically favored, and this results in a thermodynamic equilibrium between green sites (bound Met) that predominate at low temperature and blue sites

(unbound Met) that are present at higher temperatures. Temperature dependent spectroscopic data in conjunction with DFT calculations yielded Cu-S(Met) bond enthalpies (ΔH) of 4.6 and ~ 1 kcal/mol for the oxidized and reduced sites, respectively. Thus, the presence of the Cu-S(Met) bond stabilizes the oxidized state relative to the reduced state [49]. Most T1 sites do not have flexibility in the protein backbone and do not readily interconvert from blue to green sites [50]. Keeping the ligand bound (i.e. opposing entropy) provides a mechanism (i.e. an “entatic state”) by which the Cu-S(Met) distance enforced by the protein structure stabilizes the oxidized relative to the reduced state to tune down the reduction potential of the T1 by several hundred millivolts.

It is important to emphasize here that the effect of the axial ligand alone is not sufficient to explain the range of reduction potentials exhibited across T1 Cu sites. Other factors that contribute to E° differences among T1 Cu centers include the hydrophobicity of nearby residues, hydrogen bonding to the S(Cys), and electrostatic interactions in the protein backbone [51, 52]. The effects on E° due to hydrogen bonds and protein dipole interactions have been investigated [53, 54]. Mutations of second-sphere residues surrounding the T1 center in *Pseudomonas aeruginosa* azurin resulted in significant changes in reduction potential. These variants included removal of the hydrogen bond to the S(Cys), as well as deletion or insertion of carbonyl dipoles near the Cu-S(Cys) bond. Two types of contributions to tuning E° were identified: covalent contributions, which change the covalency of the Cu-S(Cys) bond, and nonlocal electrostatic contributions, which serve only to change the relative energies of the electrons in the redox active molecular orbital (RAMO). Various spectroscopies and *in silico* experiments were used to quantify each contribution to the potential. Overall, covalent contributions were found to account for a ~ 10 mV decrease in E° for each percent increase in sulfur character of the RAMO. These covalent effects either added to or opposed electrostatic contributions, which consisted mainly of electron-dipole interactions [54]. This study provides insight into how second sphere residues near the T1 Cu also play an important role in tuning the reduction potential over several hundred millivolts.

We now consider the first two terms in the Marcus equation, K_A and S , which describe the interactions between substrate and the protein in the vicinity of the T1 Cu. Increasing K_A or S through favorable binding between substrate and enzyme, and at the proper position for ET, increases the electron transfer rate. This requires an understanding of substrate binding in MCOs.

Phenols and amines are the natural substrates of laccases [55], although some also oxidize inorganic ions [56–58]. The broad range of organic substrates capable of being oxidized by laccases is a result of non-covalent binding near the T1 His ligands for outer-sphere ET. A solved crystal structure by Bertrand and coworkers [59] included the substrate 2,5-xylydine hydrogen-bonded to TvL through the carboxylate D206 and the N ϵ 2 of H458, a first sphere ligand to the T1 Cu. Although the orientation of the substrate can differ, comparisons to other amino acid sequences and crystal structures show that this solvent exposed His binding site is common in laccases [60–63]. Kinetic studies on several laccases yielded Michaelis constants (K_m) which varied by substrate, but were similar across the enzymes studied, suggesting similar substrate binding sites for these enzymes. Data also show that in laccases

an outer-sphere ET to the T1 is the rate determining step in turnover [40, 64, 65]. This is in contrast to metalloxidases, such as Fet3p, which have smaller K_m values due to favorable Fe(II) binding sites at the carboxylate residues E185 and D409 [66, 67]. These carboxylates are then hydrogen-bonded to the His residues of the T1 site, providing an efficient ET pathway in these metalloxidases. Single and double mutations of each of these residues to Ala change the coordination environment at the substrate, decreasing the effectiveness of Fet3p as a metalloxidase and turning on laccase-like activity [68, 69]. Another interesting result of these mutational studies involves the E185D mutant of Fet3p. This mutant exhibits the same T1 reduction potential as the WT enzyme and the same K_m , yet the electron transfer rate is an order of magnitude slower than the WT. This is attributed to changes in the hydrogen-bonding network that disrupt the ET pathway [68]. This leads us to the next term in the Marcus equation: H_{DA} .

The electronic coupling matrix element, H_{DA} , is primarily dependent on three factors: (i) the covalency of the ligand-metal bond, (ii) the anisotropy of the redox active molecular orbital, and (iii) the orbital overlap of the superexchange pathway between donor and acceptor through the protein [27]. For ET from the substrate to the T1 in MCOs, the short distance between donor and acceptor leads to minimal attenuation of the electronic coupling and therefore a relatively large H_{DA} . As noted above, the E185D mutant of Fet3p shows that disrupting the superexchange pathway from the substrate to the T1 greatly decreases H_{DA} [68]. In laccases, the through bond pathway from substrate to the T1 is shorter since the binding occurs near a nitrogen of the histidine ligated directly to the T1 suggesting a reasonable electronic coupling constant despite the limited covalency of the Cu-N(His) bond. The T1 site is, however, well-positioned to rapidly transfer electrons to the TNC due to the highly covalent Cu-S(Cys) bond.

In laccases, the covalency and anisotropy in the ground state, as well as the Cys-His superexchange pathway contribute to a large H_{DA} for intramolecular ET from the T1 to the TNC. The RAMO, a Cu $d_{x^2-y^2}$ orbital, contains a substantial contribution from the S(Cys) p_π orbital [70–72]. UV-visible absorption (UV-Vis), X-ray absorption (XAS), circular dichroism (CD), and magnetic CD (MCD) spectroscopies explicitly quantify the ground state wavefunction of the oxidized T1 site. This lowest unoccupied beta molecular orbital (β -LUMO) is shown in Figure 4(a). Through Cu L-edge and S K-edge XAS it has been determined that the RAMO of blue copper sites contains ~ 42% Cu character, and ~ 38% S character [73]. Compared to CuCl_4^{2-} , a “normal” Cu complex with 61% Cu character in the ground state [74], this represents a substantial increase in covalency of the T1 Cu. Simultaneous fitting of the UV-Vis, and MCD spectra reveal a strong S(Cys) $p_\pi \rightarrow \text{Cu(II)}$ ligand to metal charge transfer (LMCT) transition and weak S(Cys) $p_\sigma \rightarrow \text{Cu(II)}$ LMCT [71]. This relationship is the inverse of normal copper and green copper sites which have a strong p_σ LMCT and a weak p_π LMCT (*vide supra*). These data show that in blue copper sites, the Cu-S(Cys) bond bisects the Cu $d_{x^2-y^2}$ orbital of the copper, resulting in considerable overlap with the S p_π orbital. This highly covalent π -anisotropy of the Cu-S(Cys) bond in the ground state of the oxidized Cu activates the site for rapid intramolecular ET through the Cys-His pathway to the TNC.

The Cys-His pathway provides a link through the protein backbone between the Cu-S(Cys) π bond at the T1 to the Cu-N(His) σ bond at the T3 site [21, 27, 75, 76]. There is an additional hydrogen bond overlap, shown in Figure 4(b,c), which serves as an additional superexchange pathway that also contributes to H_{DA} in MCOs [75]. Numerous studies have shown a strong interaction between the T1 and TNC. Augustine and coworkers, working with Fet3p, studied the effects of mutating the His ligands at the T3 Cu sites to Cys and Gln. Resonance Raman (rR) spectroscopy revealed that this weakened the bonds at the T3 Cu and strengthened the T1 Cu-S(Cys) bond. A decreased reduction potential of the T1 sites in these mutants was attributed to this increased donation of the S p_π orbital to the Cu [77]. Changes in Cu-S(Cys) bonding were proposed to arise due to a conformational change of the pathway, which would result in changes to H_{DA} .

A recent computational study by Hadt, *et al.* on the Cys-His pathway in NiRs and MCOs quantifies how the anisotropy (π versus σ overlap) and covalency (% S contribution) of the T1 Cu-S(Cys) bond significantly changes H_{DA} [75]. Models of the Cys-His pathway in NiRs and MCOs were used to probe the differences between blue and green T1 sites. This study showed a direct correlation between the covalency of the Cu-S(Cys) bond (% S character) and H_{DA} and that k_{ET} goes as $(H_{DA})^2$ in non-adiabatic ET (Equation 1). Blue and green sites were also found to activate different superexchange pathways in the same bridge, which led to marked changes in H_{DA} . In MCOs, the hydrogen-bond pathway in Figure 4(b), in addition to the Cys-His π to σ pathway, results in large H_{DA} 's from the blue T1 sites. If a green site existed in MCOs, H_{DA} 's would be small. Conversely, in NiRs, structural differences in this same H-bond pathway result in larger H_{DA} 's for green relative to blue T1 sites [75]. It is worth noting that additional, unstudied pathways exist in MCOs between the T1 and the other Cu's in the TNC that may lead to additional constructive or destructive interferences for ET. However, it is clear that the highly covalent, π -donor bonding, blue copper site in laccases promotes rapid intramolecular ET during the catalytic cycle.

The above discussion of electron transfer processes described by Marcus theory as applied to blue copper sites illustrates how rates of electron transfer depend sensitively on a several terms, and changes in the first and second-sphere ligand environment can result in significant changes to the relevant terms in the ET rate expression. Laccases are highly tuned by nature to accept electrons from substrates, and shuttle them to the TNC, where dioxygen is reduced. In one of the most widely studied MCOs, RvL, intramolecular ET must be fast given a maximal turnover rate of 560 s^{-1} [15], and kinetic data show that during turnover this electron transfer rate has a lower limit of 700 s^{-1} [78]; however pulse radiolysis studies on the resting enzyme give a rate of 1.1 s^{-1} [79]. This indicates that k_{ET} changes during the course of the catalytic cycle, and suggests that it is dependent on the state of the TNC. In fact, the resting site as described by crystallography is not the catalytically relevant fully oxidized form of the TNC. This will be discussed in Part III that explores the mechanism of O_2 reduction through the known spectroscopic intermediates.

III. O_2 reduction mechanism

The mechanism of O_2 reduction by MCOs has been determined through a large body of spectroscopic, kinetic and computational studies of native enzymes, and mutated or

otherwise chemically altered derivatives. Investigations have largely focused on the two most widely studied MCOs, *RvL* and *Fet3p*, where selective perturbations of the different copper sites stabilize certain reactive intermediates which can then be characterized spectroscopically. Figure 5 shows an overview of the mechanism, which begins with the fully reduced enzyme, followed by two $2e^-$ steps that reduce O_2 to H_2O . The catalytic cycle then continues with the re-reduction of the fully oxidized intermediate formed upon reduction of dioxygen rather than reduction of the resting enzyme (*vide infra*).

The resting oxidized (RO) form of the enzyme (Figure 5, bottom left) has four oxidized coppers including a hydroxide-bridged T3 center with the spectroscopic features described above and shown in Figure 1. The mechanism begins when a suitable reductant donates four electrons to reduce all four Cu(II) atoms to Cu(I). Electrons enter through the T1 and are transferred to the TNC. In the absence of a redox active T1 site, no reduction of the TNC is observed upon addition of native substrates. However, in these altered enzymes, the T2 and T3 sites can still be reduced with smaller, inner-sphere reductants, and the reduced TNC, even in the absence of the T1, will react with and reduce O_2 . Elimination of T1 reactivity was accomplished either through site-selective mutation which inhibits Cu loading at the T1, resulting in a T1 depleted enzyme (T1D) [81], or through a derivative of tree laccase in which the T1 Cu has been replaced by the redox innocent Hg^{2+} ion (T1Hg) [82, 83]. These derivatives have been crucial to the elucidation of the mechanism of O_2 reduction at the TNC, as described below.

Anaerobic reduction of resting laccases reveal complex, pH-dependent reduction behavior for the four copper sites. There remains some ambiguity in the exact steps involved in Cu(II) reduction, but it is clear that formation of a fully reduced TNC is required for O_2 reaction (*vide infra*). The Cu(II) sites are essentially fully reduced after addition of ~ 4 electron equivalents, although the order in which each site becomes reduced varies among enzymes and reaction conditions [13, 84–87]. The binuclear T3 Cu site reduces as a two electron acceptor, which is supported by the lack of a half-met (i.e. Cu(II)Cu(I)) T3 EPR signal during reduction of the resting enzyme. The T2 is often the last site to fully reduce during anaerobic titrations [13]; however, where clear data exist, all 4 Cu(II)s reduce at approximately the same potential.

In T1D/T1Hg, the fully reduced TNC reacts with dioxygen to form the peroxide intermediate (PI) with a bimolecular rate constant of $\sim 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [88, 89]. In the absorbance spectrum of PI there are two CT bands at 340 and 480 nm (Figure 6). These two CT bands decompose into four bands in CD. Also present in CD are several ligand field ($d \rightarrow d$) transitions signaling the presence of oxidized copper [88]; yet PI lacks of any paramagnetic features in either EPR or MCD, indicating an $S=0$ ground state. Furthermore, SQUID magnetic susceptibility studies confirm that it is diamagnetic with strong AF coupling between two Cu(II)s ($-2J > 200 \text{ cm}^{-1}$). Isotope ratio mass spectrometry (IRMS) with $^{18}O_2$ showed that PI retains both oxygen atoms from the reaction with O_2 [90]. It decays slowly to a species with an MCD spectrum identical to the features of the native intermediate (NI, *vide infra*), and subsequently to a species with an EPR spectrum of an oxidized T2 Cu as in the resting form [19]. PI is therefore a catalytically competent precursor to the NI, which is discussed below. These findings lead to the assignment of PI as

a peroxide-level species, with peroxide bridging two oxidized coppers of the TNC, leaving one reduced.

Although they were originally considered the same, it is important to note the distinction between the binuclear T3 center in the TNC and the binuclear Cu sites found in the coupled binuclear Cu proteins hemocyanin (Hc) or tyrosinase (Tyr). In Hc and Tyr, reduced copper sites reversibly bind O₂ to form a planar $\mu\text{-}\eta^2\text{:}\eta^2$ peroxo-bridged structure (oxy Hc) [91], similar to the hypothetical T3 site shown in Figure 7(a). Although both PI and oxy Hc exhibit antiferromagnetic coupling of two Cu(II)s, their distinct CT spectra reveal a large geometric difference between these two peroxide-bound species [92]. Moreover, when the T2 Cu of RvL is selectively eliminated (T2D), the reduced T3 site is unreactive toward O₂ [92]. This reduced T3 site is still capable of binding small molecules, but in contrast to Hc and Tyr, exogenous ligands do not bridge the T3 Cu centers. The origin of these differences lies in the protein architecture around the active sites. In reduced Hc (deoxy Hc), the Cu(I)-Cu(I) distance is tightly constrained by the protein to ~ 4.2 Å, which destabilizes deoxy Hc and activates it for O₂ binding. Reduced T3 sites in laccases are more flexible with equilibrium Cu-Cu distances of ~ 6.5 Å, leading to less electrostatic repulsion and a more stable reduced site, which explains its lack of O₂ reactivity [93]. These data show that not only is the T3 site in laccases different from the binuclear site in Hc, but also that the presence of the T2 Cu is required for O₂ reaction in laccases leading to PI formation.

Mutational studies provide further insight into the structure of the TNC of PI. T1D mutants of Fet3p, expressed with either the T3 α or T3 β Cu sites (see Figure 3 for labels) selectively deactivated due to a His to Gln T3 ligand mutation, showed that the T2 and T3 β coppers are electron donors in the first 2e⁻ step of O₂ reduction [80]. Other mutational studies involved transformation of the conserved carboxylate D94 (D77 in Figure 3) in Fet3p. Enzymes with D94 transformed to Ala or Asn were unreactive toward O₂, yet the D94E mutant still formed PI upon reaction with O₂, showing that an anionic residue at this position was required for reactivity [29]. Computational models that include the negative charge arising from this residue yielded the $\mu_3\text{-}1,1,2$ peroxo bridging structure shown in Figure 7(b) [94]. Electronic structure calculations of PI indicate that the negative charge of D94 lowers the reduction potential of the T2 and T3 β sites, allowing them to donate the two electrons to O₂. This results in a peroxide bridged site that has good overlap of the O₂²⁻ π_{σ}^* HOMO with the $d_{x^2-y^2}$ LUMOs on the T2 and T3 β Cu(II)s, consistent with the strong AF coupling observed in PI [94]. Thus, in contrast to the coupled binuclear Cu sites in Hc and Tyr, the TNC is set up to bind O₂ as peroxide bridging all three Cu centers. This geometry is essential for the reductive cleavage of the O-O bond.

The next step in the mechanism is the decay of PI through O-O bond cleavage. In the absence of T1 Cu, PI decay exhibits an ¹⁸O₂ kinetic isotope effect (KIE) of 1.11 indicating that O-O cleavage is part of the rate-limiting step [95]. Furthermore, at low pH, the rate is modestly enhanced, and exhibits an inverse solvent kinetic isotope effect (SKIE) of 0.89 in Fet3p. Increasing the pH slows the decay rate and eliminates the SKIE. In Fet3p, when E487, the carboxylate nearer the T3 Cu site (D456 in Figure 3), is mutated to Ala the pH effect is eliminated. The E487D mutant still exhibits a rate increase at low pH, but shows a “normal” SKIE of 2.0. Meanwhile, mutation of D94, the carboxylate at the T2/T3 β edge, to

Glu changes the SKIE to 2.3 [30]. All three mutations result in slower PI decay rates, indicating that these carboxylates are critical for proton transfers during the reductive cleavage of peroxide.

Similar findings were later reported in T1D CotA, in which mutations of E498 (equivalent to D456 in Figure 3) to Leu, and D116 (D77 in Figure 3) to Ala each slowed PI formation and essentially halted PI decay. Similarly, the T1D-E498D and T1D-D116E CotA mutants formed PI at similar rates to WT, but exhibited significantly slower PI decay rates [96, 97]. The model which explains these observations involves two O-O cleavage reaction pathways: a proton-unassisted pathway at high pH and a proton-assisted pathway at low pH as shown in Figure 8 [30, 94]. Both pathways invoke proton donation to peroxide from E487 through the H-bond network to an oxygen atom bridging the T3 Cu's. This model also includes the effect of the D94 residue, which is responsible for deprotonating the water bound to the reduced T2 upon its oxidation. The resulting hydroxide lowers the reduction potential of the T2, facilitating electron transfer to the peroxide. Computation of the potential energy surface for PI decay in the holo enzyme begins with an additional electron, which would be donated from the T1 Cu(I), to form a $PI+e^-$ species. At low pH, the proton is transferred first from E487 to the oxygen bridging the T3 (O^2 in Figure 7(b)), followed by reductive cleavage of the O-O bond. In accordance with the Westheimer model, the inverse SKIE is attributed to a product-like transition state in which the stronger O-H bond of the μ_2 -OH has formed and the weaker carboxylate O-H proton donor bond is broken. At high pH, peroxide O-O bond elongation occurs first, followed by proton transfer after the transition state [30]. The activation energy for the former, proton-assisted pathway is slightly lower compared to the proton-unassisted pathway, which is consistent with the limited rate increase at low pH. Both activation energies are also in line with the experimental activation energy of ~ 3 -5 kcal/mol [94].

Figure 9 shows the 2-D potential energy surface calculated for this conversion of PI to NI. Examining the orbital contributions to the RAMOs during the reaction pathway shows that the TNC is set up for this reaction with good orbital overlap between the e^- donating HOMOs on the T2 and T3 α Cu's (both reduced) and the LUMO, the $O_2^{2-} \sigma^*$ orbital. The T3 β is already oxidized in forming PI, but in the triangular topology of the TNC contributes to the reaction by acting as a Lewis acid in lowering the energy of the peroxide σ^* electron acceptor orbital [94]. A schematic of this interaction is shown in Figure 10. This overlap allows for cleavage of the O-O bond with the concerted, two-electron reduction of peroxide, a highly exothermic process.

The formation of NI from PI (Figure 5, bottom right) must be rapid in holo laccases with reported second order NI formation rate constants of 0.7 to 5 $\mu M^{-1}s^{-1}$ [88, 98]. From Cu K-edge XAS, all four coppers are oxidized in NI, with characteristic absorbance features at 318, 330, 365, and 614 nm. NI exhibits an $S=1/2$ ground state determined through MCD with EPR g-values of 2.15, 1.86, and 1.65 in low temperature (< 20 K) EPR [99]. Importantly, it has a low lying excited state at ~ 150 cm^{-1} observed in temperature dependent MCD and in the temperature dependent saturation behavior of the low g-value EPR signals [100]. NI must therefore have an all bridged trinuclear Cu(II) cluster. EXAFS shows a Cu-Cu distance of 3.3 Å, which, through magneto-structural correlations, gives an

isotropic exchange coupling ($-2J$) of 520 cm^{-1} from one pair of Cu(II)s. Inclusion of two more bridging interactions closing the 3 Cu(II) triangle results in a condition known as spin frustration in which all three spins want to be, but cannot be AF coupled. This results in the low lying excited state at 150 cm^{-1} , which restricts the range of possible values for the three exchange coupling constants. These isotropic coupling constants for the other two bridges were determined by evaluating the contribution from each Cu(II) to the ground and excited state C terms in MCD. The resulting analysis yields exchange coupling constants ($-2J$) of 430 and 470 cm^{-1} for the other two bridges [100]. This spin frustration also gives rise to the g values below 2.0 due to a phenomenon known as antisymmetric exchange [101]. This requires good ground state to ground state exchange coupling between two adjacent Cu(II)s, spin orbit coupling between the ground and excited states on a single Cu(II), and exchange coupling between this excited state and the ground state on the adjacent Cu(II). The result is that in the EPR experiment, for fields perpendicular to the 3 Cu(II) plane, there is a nonlinear, field dependent mixing of the ground and a low-lying excited state that gives rise to $g_{\perp} \ll 2$, as shown in Figure 11 (right). Finally, analysis of the orbital contributions to the pseudo-A terms in MCD (i.e. two C terms with opposite signs) based on studies of structurally defined relevant model complexes identified the structure of NI as a μ_3 -oxo bridged species with a μ_2 -OH bridge between the T3 Cu(II)s as shown in Figure 12 [102]. Further protonation of the μ_2 -hydroxide bridge has not occurred as this would result in a ferromagnetic ground state, which is not observed experimentally [103]. Both oxygen atoms come from O-O cleavage, and the μ_2 -OH remains bound.

Although both NI and RO have fully oxidized TNCs, they exhibit very different spectral properties and reactivities due to their different oxygen bridges (μ_3 -O and μ_2 -OH in NI and μ_2 -OH in RO). In the absence of excess reductant, NI decays slowly to the RO form with an observed rate constant of $\sim 0.05\text{ s}^{-1}$ [78, 105]. IRMS on $^{18}\text{O}_2$ revealed that one of the oxygen atoms from dioxygen remained bound to the TNC in RO [90, 106]. A model linking the NI structure to RO requires the addition of protons and the loss of H_2O . Protonation of the μ_3 -oxo bridge is energetically favorable relative to protonation at the μ_2 -hydroxo ligand, and the protonated μ_3 -oxo rotates through the T2-T3 α edge with a calculated barrier of 8.5 kcal/mol, which is consistent with the experimentally observed barrier of 8.8-13.9 kcal/mol in the decay of NI to the resting enzyme [103]. Importantly, the rate of decay of NI is several orders of magnitude slower than catalytic turnover, which is further evidence that RO is not part of the catalytic cycle. Instead, as summarized below, NI has been shown to be the fully oxidized form of the enzyme that is relevant to catalysis.

It has been observed that the turnover rate of R_vL (560 s^{-1}) was significantly higher than either the T1-T3 electron transfer rates in RO (1.1 s^{-1}), or the decay of NI to RO (0.05 s^{-1}). For this reason, the RO form of the enzyme is not catalytically relevant. Instead, re-reduction of NI has now been shown to be the catalytically relevant reduction step. The kinetics of RO reduction versus re-reduction of NI were studied via stopped flow absorption spectroscopy [78]. The two processes show markedly different behavior for the T1 and T3 electron transfer rates. In RO, the T1 reduces quickly, followed by slow reduction of the T3 at an initial rate of 0.111 s^{-1} at 4°C . In contrast, the rate of T1 to T3 ET in the first e^- reduction of NI was observed to be $>700\text{ s}^{-1}$. Both processes are proton coupled electron

transfers (PCET), and this dramatic change in k_{ET} between the two fully oxidized TNC's is due to the substantially higher basicity of the μ_3 -oxo ligand of NI relative to the μ_2 -OH ligand present in the RO structure [78]. The increased proton affinity of the μ_3 -oxo species drives the PCET enabling fast re-reduction of NI.

DFT calculations were used to evaluate changes in the parameters of the ET rate equation. The differences in driving force between these two PCET steps reveals that NI reduction is 7 kcal/mol more favorable than reduction of RO. In these calculations, the reorganization energy for the first PCET in NI reduction is ~ 0.3 eV lower than for reduction of RO. Using these values in the Marcus equation (with H_{DA} held constant) yields a factor of 10^3 greater ET rate for NI reduction, in agreement with experimental data [78]. The structure of NI is therefore responsible for rapid turnover, since reductive cleavage of the O-O bond forms the μ_3 -oxo ligand and allows for proton coupling leading to fast rereduction and high turnover rates.

The mechanism described here likely applies in general to all MCOs. Turnover rates of laccases vary widely among substrates, but are generally several orders of magnitude greater than rates measured for metalloxidases [6, 15, 98]. Although PI has not been observed in native enzymes, it has been observed in several MCOs lacking a T1 center, including derivatives of RvL [107], Fet3p [89], CueO [108], and CotA [97]. PI forms at a rate consistent with it being in the reduction of O_2 . NI-like features have been observed in RvL, BOD [109], Cp [105, 110], and CueO [108]. Interestingly, in some MCOs including BOD, Cp, and CueO, NI features are not long lived; however, in both CueO and BOD, mutations at sites equivalent to E487 in Fet3p, result in more pronounced NI features [108, 111]. This suggests that although differences in second-sphere residues around the Cu centers may lead to changes in rates for individual mechanistic steps, the intermediates described here are common to all MCOs. Future studies will expand this model to include other relevant features that exert a mechanistic influence on different MCO activities.

IV. Conclusion

In laccases, the enzyme ET properties, largely governed by the T1 Cu, combined with the unique triangular topology of the TNC effectively couple the oxidation of substrates to the reduction of dioxygen. As illustrated above, structural constraints imposed by the protein modulate electron and proton transfer rates both to and from the T1 Cu, and at the site of O_2 reduction. Reductive cleavage of the O-O bond, effectively a $4e^-$ process, leads to the formation of a species, NI, that is then poised for rapid proton coupled reduction to generate H_2O and continue the catalytic cycle.

It is fascinating to consider that while laccases and other MCOs have nearly identical Cu active sites they exhibit substantial diversity in substrate interactions and catalytic rates. As structure-function relationships are established for second-sphere residues that explain differences in reactivity, it will be interesting to correlate these changes to the roles that particular enzymes serve in biological systems. Understanding how enzyme activity is regulated by changes in electron and/or proton transfer rates will both provide fundamental insight into how nature controls the fundamentally important process of O_2 reduction to

H₂O and also be important to the development of industrial processes and biomedical applications.

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Figure 1. UV-Vis absorbance (A) and EPR spectra (B) of resting laccase. Reprinted with permission from [3].

Figure.

2. T1 sites of CotA from *B. subtilis* with axial methionine (a) and of laccase from *T. versicolor* without the axial methionine (b). PDB accession numbers: 2X88 and 1GYC.

Figure 3.

T2 and T3 Cu sites in MCOs. Labeled histidines H454 and H452 connect T3 α and T3 β to the T1 Cu through the Cys-His bridge. Also shown are conserved carboxylates D77 and D456. All residues are numbered according to PDB:1GYC

Figure 4. β -LUMO coupled into Cys-His pathway (a). Calculated spin density of ground state wavefunction with H-bond pathway shown in blue (b). π to σ superexchange pathway from T1 Cu to TNC (c). Reprinted with permission from [21]. Copyright 2006 American Chemical Society.

Figure 5.

Mechanism of O_2 reduction by MCOs. Red arrows show steps in the catalytic cycle. Black arrows show reduction of resting enzyme to enter catalytic cycle and decay of the native intermediate which terminates catalysis. Adapted with permission from [80]. Copyright 2010 American Chemical Society.

Figure 6.

(a) Absorbance spectra of the peroxy intermediate at different times during its decay. Inset shows the decay at 340 nm. (b) Absorbance difference spectra of the spectra in (a) relative to fully oxidized TlHg laccase.

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Figure 7. Optimized structures of the peroxy intermediate without (a) and with (b) the D94 residue. Reprinted with permission from [94]. Copyright 2007 American Chemical Society.

Figure 8. Schematic showing the roles of carboxylates E487 and D94 in O-O bond cleavage during PI decay. Reprinted with permission from [94]. Copyright 2007 American Chemical Society.

Figure 9.
2D potential energy surface of the decay of the peroxy intermediate. Reprinted with permission from [94]. Copyright 2007 American Chemical Society.

Figure 10. Schematic of the overlap between the HOMOs on the T2 and T3_A sites and the $\text{O}_2^{2-} \sigma^*$ orbital. Reprinted with permission from [94]. Copyright 2007 American Chemical Society.

Figure 11.
Origin of the low g-values observed in NI. Reprinted from [104] with permission of The Royal Society of Chemistry.

Figure 12.

Optimized structure of the NI. Reprinted from [103]. Copyright 2007 National Academy of Science, USA.