Published in final edited form as:

J Am Chem Soc. 2013 August 21; 135(33): . doi:10.1021/ja4064525.

Molecular Origin of Rapid vs. Slow Intramolecular Electron Transfer in the Catalytic Cycle of the Multicopper Oxidases

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Abstract

Kinetic measurements on single-turnover processes in Laccase establish fast Type 1 (T1) Cu to trinuclear Cu cluster (TNC) intramolecular electron transfer (IET) in the reduction of the native intermediate (NI), the fully oxidized form of the enzyme formed immediately after O-O bond cleavage in the mechanism of O₂ reduction. Alternatively, slow IET kinetics in the reduction of the resting enzyme is observed, which involves proton coupled electron transfer (PCET) process based on isotope measurements. The $> 10^3$ difference in IET rate between the two processes confirms that the native intermediate, not resting enzyme that has been defined by crystallography, is the fully oxidized form of the TNC in catalytic turnover. Computational modeling shows that reduction of NI is fast due to the larger driving force associated with a more favorable proton affinity of its μ_3 -oxo moiety generated by the reductive cleavage of the O-O bond. This defines a unifying mechanism of coupling the reductive cleavage of the O-O bond to the rapid intramolecular electron transfer in the multicopper oxidases.

Reduction of dioxygen to water is performed in nature by the Multicopper Oxidases (MCOs) to carry out a variety of single-electron oxidations of metal ion or organic substrates.^{1,2} This requires at least four Cu ions: a type 1 (T1)^{3,4} Cu site and a trinuclear Cu (TNC)^{5,6} site composed of mononuclear type 2 (T2) and coupled binuclear type 3 (T3) Cu centers. The T1 site receives electrons from substrate and transfers them through the protein over ~ 13 Å to the TNC where O₂ binds and is reduced. Understanding of the chemistry of these enzymes have been motivated by their relevance to human health (ceruloplasmin)⁷ and application to biofuel cells.^{8,9} The mechanism of O₂ reduction has been well studied.² The fully reduced enzyme (4 Cu^I) reacts with O₂ to form the Peroxy Intermediate (2 Cu^I, 2 Cu^{II}), where dioxygen is bound as peroxide to the TNC. This then undergoes O-O bond cleavage (with protonation) of one oxygen to produce the Native Intermediate (NI, Scheme 1 left), which is fully oxidized (4 Cu^{II}) with all three Cu's of the TNC bridged by a μ_3 -oxo and the T3s are additionally bridged by a μ_2 -OH. Both moieties originate from the 4 electron reduction of O₂.¹⁰ NI is structurally distinct from the fully oxidized resting (R) form of the enzyme, that is observed crystallographically,^{11,12} with respect to bridging ligands in that R only has the T3Cu μ_2 -OH bridge (Scheme 1 right).¹³

While much effort has been made to describe the mechanism of O₂ reduction by the MCOs, the mechanism of re-reduction in catalysis is less explored especially concerning the rates of intramolecular electron transfer (IET) from the T1 to the TNC. The most well-studied enzyme in the MCO family is *Rhus vernicifera* Laccase (Lc) that naturally oxidizes phenolic substrates and for which the maximal turnover rate is 560 s⁻¹ at 25 °C.¹⁴ However, IET

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Supporting Information.

Experimental and computational details and supporting data. This Material is available free of charge via the Internet at <http://pubs.acs.org>.

rates for R have been measured using pulsed-radiolysis and are slow (1.1 s^{-1} , 25°C) compared to this turnover rate, excluding the reduction of R from the catalytic cycle.¹⁵ Upon reaction with O_2 , NI forms rapidly and decays slowly (0.058 s^{-1} , 23°C)¹⁶, which strongly suggests that NI is reduced directly with fast IET rates to be consistent with turnover.¹ For the first time, we report kinetics on the reduction of NI, demonstrating fast IET and thus the relevance of this intermediate in the reduction cycle in turnover. This is important as it demonstrates that NI, not R studied by crystallography (Scheme 1), is the fully oxidized form of the enzyme that is catalytically active. Parallel experiments on the slow IET in R define the geometric and electronic structural contributions of the TNC in NI that determine its fast IET.

Monitoring absorption spectral changes via stopped-flow (SF), in the reaction of $100 \mu\text{M}$ fully reduced Lc with equal molar O_2 shows initial rapid ($\sim 10^6 \text{ M}^{-1}\text{s}^{-1}$) formation of NI based on the appearance of its 365 nm band associated with the $\mu_3\text{-oxo}$ to Cu^{II}_3 CT at the TNC and the 614 nm Cys to Cu^{II} charge transfer (CT) of the oxidized T1 site.^{17,18} This is followed by slow first-order decay (0.0070 s^{-1}) of the 365 nm band while the T1 remains oxidized consistent with previous findings on the formation rate of NI and its decay to R (Figure S1). Carrying out the same SF experiment now with 5.0 electron equivalents (0.250 mM) hydroquinone (H_2Q), a widely used phenolic substrate for kinetic studies of Lc,^{14,19} added to fully reduced Lc leads to analogous NI formation now followed by reduction of both the 365 and 614 nm bands (Figure 1A). The 365 nm band (Figure 1A and 1B red) reduces in a first-order fashion ($k = 0.029 \text{ s}^{-1}$), which is accelerated ~ 4 -fold over the rate of decay of NI (Figure 1B black). Therefore, NI is reduced before it can decay with minimal excess substrate. The 365 nm reduction increases linearly with $[\text{H}_2\text{Q}]$ (Figure 1B red to orange) affording a second-order rate of $168.2 \text{ M}^{-1}\text{s}^{-1}$ (Figure 1B inset).

Full reduction of the MCOs requires 4 electrons where H_2Q first reduces the T1 followed by IET to reduce the TNC. This occurs three times to fully reduce the TNC with the fourth electron reducing the T1. The SF traces in Figure 1A indicate a number of points concerning the reduction of NI. The 614 nm band (Figure 1A blue) reduces in a multiphasic fashion with limited loss of intensity at $< 20 \text{ sec}$ indicating that the T1 effectively remains oxidized at the early stages of the reaction (Figure S3). Since electrons enter at the T1 Cu, while no reduction of the T1 is evident (i.e., loss of 614 nm intensity), the T1 re-oxidation is rapid indicating fast IET. This is further substantiated by the observation of second-order (in $[\text{H}_2\text{Q}]$) reduction of the 365 nm band (insert Figure 1B).

Since the absorption at 365 nm is due to the $\mu_3\text{-oxo}$ to Cu^{II}_3 CT of NI¹⁸, it is eliminated with entry of the first electron into the TNC because this significantly alters the electronic structure of NI. Therefore, the kinetics of this band intensity can provide a direct probe of the rate of the 1st IET in NI reduction, which can be fit to a mechanism (equation 1) accounting for the formation of NI (denoted by $\text{T1}^{\text{ox}}\text{TNC}^0$ indicating the oxidation state of the T1 and the number of reduced Cu's in the TNC, respectively) followed by reduction of the T1 proceeded by IET from the T1 to the TNC. The 365 nm absorbing species in this rate law would be the sum of the two TNC^0 intermediates since the reduction of the T1 does not alter the absorption at the TNC.

In order to obtain an estimate of k_{IET} , SF traces at 365 nm were obtained at higher $[\text{H}_2\text{Q}]$ (Figure 1C) where the T1 reduction rate is increased such that NI intensity is not maximized. Fit to the rate law in equation (1) are obtained with $k_{\text{ox}}[\text{O}_2] = 1.2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$, $k_{\text{red}}[\text{H}_2\text{Q}] = 182.7 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{IET}} > 700 \text{ s}^{-1}$ (Figure S8). k_{IET} is a lower limit as illustrated in Figure

1D where the fit of equation 1 to the high $[H_2Q]$ data converges at k_{IET} rates $> 700\text{ s}^{-1}$. This is significant in that it establishes that the rate of IET in the MCOs for the reduction of NI is faster than turnover, while its decay is much slower (*vide supra*) and therefore requires that *NI reduction is the catalytically relevant process for the re-reduction of the fully oxidized enzyme*.

As slow rates have been reported for the IET process in R with another method,¹⁵ parallel kinetic studies were conducted on R to obtain the rate difference under identical conditions. SF traces were obtained at the 330 nm band (μ_2 -OH to T3 Cu^{II}_2CT) and 614 nm (T1 Cu^{II}) upon anaerobic reaction of R with H_2Q (Figure 2B). The 614 nm band trace is multiphasic and reduces initially at rates proportional to $[H_2Q]$, which is the opposite of the behavior observed in NI reduction, where the T1 is mostly oxidized initially and the TNC reduction is proportional to $[H_2Q]$, implying slow IET in R (blue traces in Figure 2). The 330 nm band reduction in R is first-order, invariant with $[H_2Q]$ and exhibits biphasic behavior with a major component (~75%) having 0.111 s^{-1} and a minor (~25%) component of 1.29 s^{-1} (Figures S10). The slow reduction of the 330 nm band for both components and its substrate independence demonstrate that the IET from the T1 to the TNC in R is slow relative to T1 reduction (and turnover).

Kinetic experiments were performed in the pH range of 6.5–8.5 (Figure S12–14). At high pH, the 614 nm band reduces faster consistent with the first pK_a of $H_2Q \sim 9$ –10.²⁰ Due to its limited contribution the minor component of the 330 nm band reduction is difficult to study while the rate of reduction of the major component clearly increases at low pH. The major component exhibits an inverse solvent kinetic isotope effect (SKIE) of 0.67 (Figure S11). This SKIE on the IET rate can be understood on the basis of the Westheimer model where the bonds of the transition state are stronger than in the reactant complex.²¹ Since the protons are delivered to the TNC via a distal carboxylate residue, these have a weaker O-H bond than that of the resulting $Cu^{II}OH_2$ complex (protonating the T3 μ_2 -OH bridge in Scheme 2B).^{10,22} There could also be a secondary isotope effect from the substitutable proton on the oxygen of the T3 μ_2 -OH bridge (Figure S15).²³ Importantly, the observed SKIE effect on reduction of the 330 nm absorption requires that a proton be involved in the IET in R. Thus this is a proton coupled electron transfer process (PCET). IET in NI is too fast at pH = 7.5 to measure a SKIE. However, since R has an SKIE that involves protonation of the T3 μ_2 -OH and NI has a μ_3 -oxo bridge that is more basic (*vide infra*), the IET in NI reduction must also involve a PCET process.

Observation of this $> 10^3$ difference in IET rates ($> 700\text{ s}^{-1}$ in NI, 0.111 s^{-1} in R) provides an opportunity to determine the molecular factor(s) that lead to fast IET in the MCOs. It can be assumed that electrons and protons come from sites (T1 and carboxylate, respectively) that do not vary much between NI and R. Therefore, the $> 10^3$ difference in rates reflects the structure of the TNC electron and proton acceptor. In the context of Marcus Theory, first order IET rates can be expressed in terms of the driving force (G°), reorganization energy (λ), and the electronic coupling matrix element (H_{DA}), the latter reflecting the electron transfer pathway.²⁴ Since these reactions are PCETs the proton affinity is included in the driving force and the structural changes that contribute to the reorganization energy.²⁵

Density functional theory (DFT) optimizations were performed on models (Scheme 1) of singly reduced and protonated NI and R sites to probe the thermodynamics and structural changes upon PCET (Scheme 2). Models include second sphere carboxylate (D72 in Lc)²⁶ as the equivalent of this residue has been shown to provide a critical negative charge in Fet3p (the MCO in yeast)^{10,27}. The first electron into NI reduces the T3aCu (the T3Cu furthest from D72) and elongates the T3aCu- μ_2 -OH bond. Protonation of this species is more favorable (by 16.8 kcal/mol) at the μ_3 -oxo (relative to the T3 μ_2 -OH) resulting in a μ_2 -

OH bridge between the oxidized T2 and T3b that are antiferromagnetically coupled. Protonation of the μ_2 -OH bridge in this one-electron reduced form uncouples the T3 coppers resulting in the reduction of the T3a, the same copper reduced in NI reduction.

Free energies for electron transfer ($G^\circ(e^-)$) and proton transfer ($G^\circ(H^+)$) were obtained with solvation correction. From previous studies, there are four conserved second sphere carboxylates near the TNC active site that neutralize its large positive charge (Figure S19).¹³ If negative point charges in the carboxylate positions are included, the free energies of electron transfer go up and proton transfer energies go down proportionally but the sum of these energies is not sensitive to the inclusion of these point charges (Table S3). Therefore, the important comparison is the difference in PCET free energies $G^\circ(e^-+H^+)$, which is approximately -7.0 kcal/mol more favorable for NI. Additionally, an estimate for the inner-sphere reorganization energy (λ), the energy of the starting geometry with an H-atom bound to the TNC compared to the optimized geometry for PCET, is computed as 1.2 eV for NI and 1.5 eV for R.

The Marcus equation can be used to obtain an expression for the ratio of the two IET rates (Equation S8). Since the same copper is reduced in both reactions, the H_{DA} are the same. Using the known potentiometric electrochemical potential difference between the T1 and T3 in R ($G^\circ = -2.0$ kcal/mol)²⁸, it is found that with equivalent reorganization energies, the calculated $G^\circ(e^-+H^+) = -7.0$ kcal/mol gives $\sim 10^2$ difference in IET rates (Table S4). The experimentally determined difference ($> 10^3$) is obtained if the free energy difference is ~ 10 – 12 kcal/mol or with if $G^\circ(e^-+H^+) = -7.0$ kcal/mol and the reorganization energy of R is ~ 0.3 eV higher than that of NI, consistent with above estimates (Tables S5–6). Thus, the difference in PCET driving force is the principle factor in determining the large difference in k_{IET} .

The molecular origin for the difference in PCET driving force in NI vs. R is the structural difference of the two fully oxidized forms. In both PCET processes, the electron goes to the T3aCu where the major structural difference between NI and R is the presence of the μ_3 -oxo ligand in NI. The strong donation of the μ_3 -oxo to the T3aCu results in a lower driving force for IET in NI compared to R, which would make IET to NI slower than R. However, since both IET reactions are PCET processes, the proton affinity contributes to the driving force and the μ_3 -oxo in NI is a far stronger base than the T3 μ_2 -OH in R. The free energy difference in proton affinity between the μ_3 -oxo and μ_2 -OH in NI is 20.6 kcal/mol which represents a pK_a of ~ 15 between these basic ligands. Therefore the large difference in G° for IET to NI vs. R is due to the μ_3 -oxo acting as a strong base driving proton transfer.

This establishes that IET in NI reduction is fast, catalytically relevant and due to the basicity of the μ_3 -oxo ligand. The μ_3 -oxo of NI originates from the reduction of O_2 and also provides the driving force for rapid enzyme re-reduction. Therefore, in addition to being the 4-electron oxidant, O_2 plays a critical role in enabling fast, catalytic re-reduction of these oxidoreductases. This work focuses on the first electron in the reduction of NI. Two additional fast, catalytically relevant electron transfers are required to complete the catalytic cycle, which are the focus of present research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research is supported by NIH grant DK-31450 (E.I.S.).

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Figure 1.

(A) Scaled absorption traces of the T1 (614 nm, blue) and TNC (365 nm, red) in the reduction of Ni: $[Lc] = [O_2] = 0.050$ mM; $[H_2Q] = 0.125$ mM, and (inset) time dependent absorption spectra from 1 to 300 secs (B) $[H_2Q]$ dependence of 365 nm trace (black 0.00 mM, Red to Orange 0.125, 0.250, 0.500 and 1.25 mM) with (inset) first-order dependence of 365 nm reduction on $[H_2Q]$. Color of trace matches color of dot in inset (C) Traces of the 365 nm band (identical conditions as 1A but with 42.8(black), 85.5(green), 145(blue), and 271(red) mM $[H_2Q]$). (D) 42.8 mM Data from (C) Illustrative fitting k_{IET} of 42.8 mM 365 nm data with $k_{IET} = 0, 1, 5, 10, 50, 100, 500$, and 1000 s⁻¹.

Figure 2.

(A) Scaled absorption traces of the T1 (614 nm, blue) and TNC (365 nm, red) in the reduction of NI [Lc] = [O_2] = 0.050 mM; [H_2Q] = 0.500 mM (B) Scaled absorption traces of the T1 (614 nm, blue) and TNC (330 nm, red) in the reduction of R [Lc] = 0.050 mM; [H_2Q] = 97.5 mM.

Scheme 1.

Experimentally determined geometric structures of the TNC in NI and Resting states of the TNC.

Scheme 2.

Computationally derived PCET schematic for NI (A) and R (B). Details included in SI.