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Correlating the structures and activities of the resting oxidized and native intermediate states of a small laccase by paramagnetic NMR

Michael C. Machczynski *, Jeffrey T. Babicz Jr. [1]

Department of Chemistry, State University of New York at New Paltz, 1 Hawk Drive, New Paltz, NY, 12561, United States

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ABSTRACT

Multicopper oxidases (MCO) are the fastest and most efficient known catalysts of the oxygen-reduction reaction. When all four copper ions are oxidized during catalysis, the native intermediate state (NI) decays in seconds to the resting oxidized state (RO), which returns to the catalytic cycle via reduction, but at a much slower rate than NI. We report the long-lived (months at 4 °C) NI state of the small laccase (SLAC) MCO and the subsequent characterization of both its RO and NI states by paramagnetic ^{1}H NMR. We find that the RO state of the trinuclear cluster (TNC) is best described as an isolated Type-3 dicopper site, antiferromagnetically coupled by a hydroxo group with $-2J = 500\ cm^{-1}$. The NI state is more complicated; we develop a theoretical treatment for the case in which all three copper ions in the TNC are coupled, and find that the results are consistent with three coupling constants of $-2J = 300$, 240, and 160 cm^{-1}. These couplings result in a ground doublet state, a low-lying excited doublet state at 121 cm^{-1}, and a quartet excited state at 411 cm^{-1}, in good agreement with DFT models in which the Type-2 copper has a terminal hydroxo ligand.

1. Introduction

Multicopper oxidases (MCO) are a family of ubiquitous enzymes that couple one-electron oxidations to the catalytic reduction of dioxygen to water [1,2]. Biological substrates for oxidation among the enzymes vary widely, and include many phenols, ascorbate, iron, copper, and manganese ions [3,4]. Surface features near the initial oxidation site thus vary widely, but the metal sites (vide infra) that perform these reactions are highly conserved [5]. This family attracts attention for industrial application due to the production of water as a waste product [6]. The oxygen-reduction reaction is the limiting factor in the efficiency of fuel cells; MCO are useful candidates for this cathodic reaction, because they are the most active known catalysts of oxygen reduction [7].

MCO perform these reactions at two sites, separated by 10–12 Å. At the enzyme surface, a Type-1 (T1) mononuclear copper site oxidizes the organic or metallic substrate. The copper geometry is planar, with the copper bound by two histidine sidechains and a cysteine. A weak bond is often provided by an axial methionine, although some MCO eschew this bond in favor of a hydrophobic, nonbonding interaction. This geometry and the covalent copper thiolate bond provides the blue color (λ_{max} ~ 600 nm, ε ~ 5000 $M^{-1}\ cm^{-1}$) and small parallel hyperfine constant ($A_{||} = 40–90 \times 10^{-4}\ cm^{-1}$) that are hallmarks of the cupric form of this site [8]. The electrons from the initial oxidation reaction are then transferred to a trinuclear copper cluster (TNC), consisting of a Type-3 (T3) binuclear center and a Type-2 (T2) copper ion, which together turnover O_2. T2 sites do not absorb significantly in the visible or UV. However, their long electronic relaxation times (10^{-8}–10^{-9} s) result in intense electron paramagnetic resonance (EPR) spectra that are marked by parallel hyperfine constants similar to tetragonal model compounds ($A_{||} = 140–200 \times 10^{-4}\ cm^{-1}$). T3 sites contain two copper ions that are each terminally bound by three histidine sidechains and bridged by the oxygen of a hydroxo ion. This coordination in the dicupric state results in a charge-transfer band in the UV (λ_{max} ~ 330 nm) [1]. The site is also EPR-silent, due to a singlet ground state, which is caused by the hydroxo bridge antiferromagnetically coupling the two copper ions.

In the 1970s, the groups of Hiromi, Vänngård, and Malmström detected the presence of a paramagnetic species that was generated by mixing reduced forms of ceruloplasmin or fungal laccase with dioxygen [9–11]. More recently, this species, since dubbed the native intermediate (NI), has also been observed in other MCO, such as CueO [12] and bilirubin oxidase [13]. This species was originally recognized by an increase in UV–Visible absorbance near 365 nm and the absence of the T2 Cu signal in its EPR spectrum, the latter feature owing to the coupling of the T2 ion to the other Cu in the TNC. For the overall reaction cycle, Solomon and coworkers have shown [14] that O_2 binds to the fully reduced TNC and is reduced by two electrons to the peroxide intermediate (PI), from which it is rapidly reduced further by two electrons to water, leaving the enzyme in the fully-oxidized, NI state (Scheme 1). EPR and MCD measurements support the model that, in the NI state, all three copper ions of the TNC are magnetically coupled by a shared oxygen atom in the center of the cluster [15]. In the absence of residual reductant, the NI state further relaxes to a resting oxidized state (RO),

* Corresponding author.
E-mail address: machczym@newpaltz.edu (M.C. Machczynski).
[1] Present address: Department of Chemistry, Stanford University, Stanford, CA, 94305.

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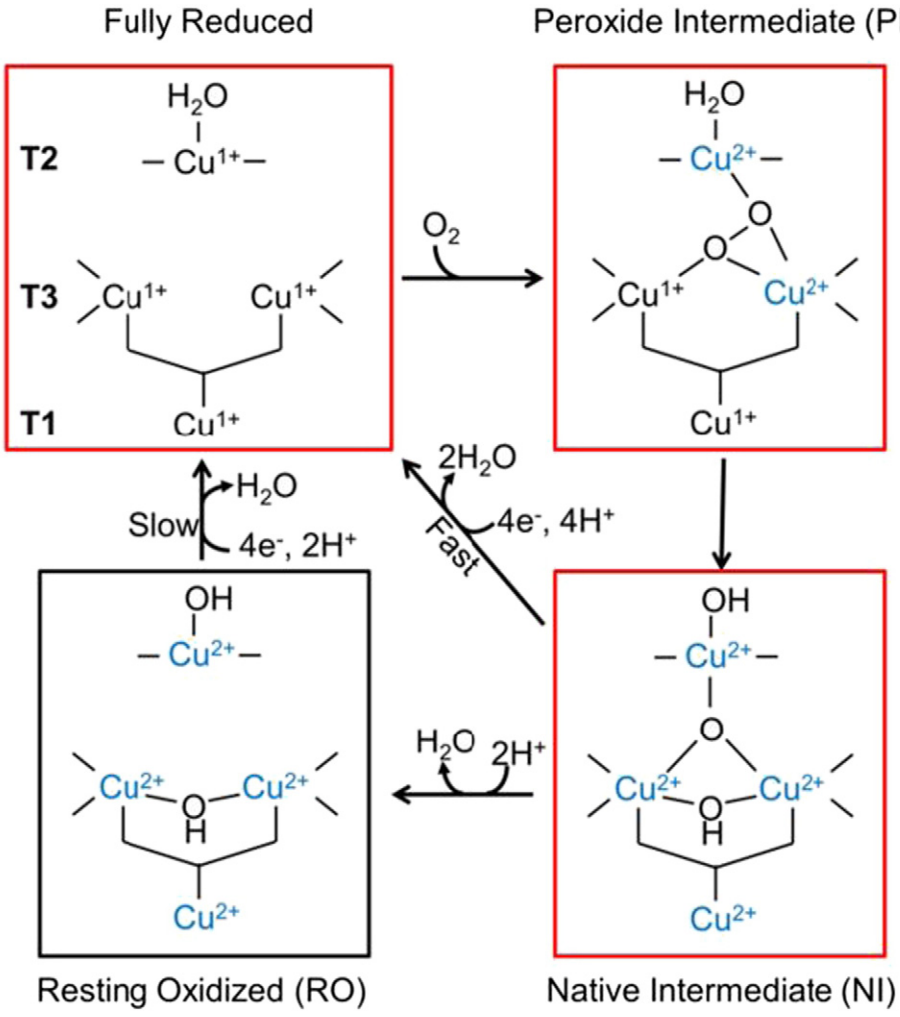

Scheme 1. The Mechanism of O_2 Reduction by MCO [2].

where the coupling between the T3 and T2 sites is broken when the bridging oxygen atom is protonated to become a hydroxo ligand to the T2 ion. These two states differ in their rates of reduction, and thus in their ability to return to the catalytic cycle [16]. For *Rhus vernicifera* laccase (RvL), an MCO, the electron transfer from the T1 to the TNC in the NI is more than 6000 times faster than in the RO.

^{1}H NMR spectroscopy can be an excellent tool for probing both the electronic and physical structures of oxidized copper sites in proteins. This technique has been adapted for the fast-relaxing signals of paramagnetic sites and applied to T1 and T3 sites [17–19]. Recently, Vila and coworkers measured and analyzed ^{1}H NMR spectra of the Fet3p MCO from *Saccharomyces cerevisiae*, assigning signals to the T1 and isolated T3 sites, as well as the T2 site, whose electronic relaxation is enhanced enough by very weak ($|J| \sim 1–4\ cm^{-1}$) coupling to the T3 site that several resonances are observed [20]. In contrast, studies of the RvL reported that all observed signals originated in the T1 site, because removal of the T1 copper ion resulted in loss of all peaks observed in the holo-protein [21,22].

We report here a ^{1}H NMR spectroscopic study of an MCO, the small laccase (SLAC) from *Streptomyces coelicolor* [23]. In contrast to other MCO, the NI of this MCO may be prepared in high yield and is stable for months at 4 °C. We have prepared wild-type SLAC in both the RO and NI states and have monitored their spectra as a function of temperature. These states, and their ^{1}H NMR spectra, have been directly correlated to their enzymatic activity. Additionally, mutants that lack certain copper sites, the Type-1 deleted (T1D) and Type-2 deleted (T2D) forms, have been created to aid in the assignment of NMR resonances. This work shows that the RO, which reacts slowly, is accurately described as a magnetically-isolated T3 site, and, the NI, which reacts quickly, is accurately described as the three copper ions of the T2 and T3 sites all similarly coupled to each other. We also present a theoretical treatment for simulating paramagnetic NMR spectra in the case of three coupled copper ions.

2. Methods and materials

2.1. Mutagenesis and protein preparation and characterization

SLAC protein was expressed and purified in the manner previously reported [23]. SLAC was isolated as a mixture of NI and RO states. The T1D (C288S) and T2D (H102A) mutations were introduced by using the method of Liu and Naismith [24]. All chemicals were of reagent grade and used without further purification.

Flame atomic absorption spectroscopy confirmed the expected number of copper ions in the protein samples. Samples for NMR were prepared with 2–4 mM protein in 100 mM sodium phosphate buffer at pH 7.2, with either 10% or 100% D_2O. Activity assays were performed in 100 mM sodium acetate, pH 4.2, with 1 mM potassium ferrocyanide as the substrate, at 298 K. Absorbance of the product was monitored by a Cary 50 UV–Visible spectrophotometer at 420 nm and converted to concentration using an extinction coefficient of 1100 $M^{-1}\ cm^{-1}$ [25].