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Redox Investigations of the Type 3 Site in Type 2 Copper Depleted *Rhus* Laccase

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We report the results of experiments designed to characterize the Type 1 and Type 3 copper sites in *Rhus* laccase depleted of Type 2 copper (T2D). Use of the Lowry method for determining protein concentration, with native laccase as a standard, established the value 5680 ± 710 M^{-1} cm^{-1} for the extinction at 615 nm for T2D laccase. Anaerobic reductive titrations of T2D laccase have been performed using Cr(II)aq ions, $Ru(NH_3)_6^{2+}$ and hydroquinone as reductants. For five independent titrations on three independent T2D laccase preparations, 3.1 ± 0.25 reduction equivalents were required to reduce the protein completely, as judged by the absorption at 615 nm. Thus the fact that the Type 3 copper ions are in the +2 oxidation state in fresh T2D laccase is unequivocal.

The data from equilibrium reductive titrations with hydroquinone fit a model in which the Type 1 site copper in T2D laccase is 35 mv more oxidizing than those of the Type 3 site, and the Type 3 site coppers are reduced in equipotential single electron steps. Both these findings contrast with the case for the native enzyme. In addition theoretical considerations governing the electron distribution in multinuclear equivalent site species during reduction imply that reduction of the Type 3 site coppers is accompanied by a protonation or conformational transition within the site itself in T2D laccase.

Treatment of T2D laccase with a 70-fold excess of H_2O_2 induced a new shoulder at 330 nm ($\Delta\epsilon$ = 660 M^{-1} cm^{-1}), minor intensity enhancement of the ultraviolet absorption band and a decreased absorption at 615 nm ($\Delta\epsilon$ = −900 M^{-1} cm^{-1}). The following difference spectra were constructed from experimental data: (native laccase minus T2D laccase) and (peroxy native laccase minus peroxy T2D laccase). Both were found to exhibit maxima at 325 nm, ($\Delta\epsilon$ ~1200 M^{-1} cm^{-1}) and shoulders near 370 nm ($\Delta\epsilon$ ~500 M^{-1} cm^{-1}). The similarity of these spectra throughout the near ultraviolet region confirms previous CD results that peroxide is bound to the Type 3 copper site of T2D laccase in a fashion similar to that of the native protein, despite the diminished binding constant (10^4 M^{-1} *vs* > 10^8 M^{-1}, respectively). The lack of any other new near-UV feature on treatment with peroxide corroborates the reductive titrations in demonstrating the oxidized state of the Type 3 copper ions in resting T2D laccase.

Dioxygen reoxidation of ascorbate reduced T2D laccase produced new difference bands at 330 nm ($\Delta\epsilon$ = 770 M^{-1} cm^{-1}) and 270 nm ($\Delta\epsilon$ = 13000 M^{-1} cm^{-1}), the former assigned to a bound peroxide dioxygen reduction intermediate. In the corresponding epr spectrum of this material new low intensity $Cu(II)g_{\|}$ features ($A_{\|}$ ~130 G) indicative of a magnetically isolated copper ion, as well as a new triplet signal near 3400 Gauss were observed. Reoxidation by dioxygen as mediated by iron hexacyanide, or by ferricyanide alone, produced a reoxidized protein having the original spectral determinants. Thus not only does reduced T2D laccase react directly with dioxygen, but the magnetism of the reoxidized Type 3 site may occasionally be perturbed during turnover. Conformational transitions occurring in T2D laccase during turnover may therefore diminish and perhaps obviate the binuclear coupling of the Type 3 copper ions.

In summary, removal of the Type 2 copper from *Rhus* laccase has produced an altered Type 3 site. It appears that T2D laccase is conformationally labile around the Type 3 site, and therefore that direct analogies relating the chemistry or spectroscopy of native and T2D laccase should be drawn with caution.

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Cu(II)-Coordination in the Bimetallic Sites of Laccase and Cytochrome Oxidase

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Molecular oxygen plays a key role in many biological oxidation reactions. Dioxygen is reduced to peroxide by most oxidases but a few enzymes can reduce it all the way to two water molecules. Among