number of water molecules bound to the Eu^{3+} [4], thereby providing a probe to explore the changes at the hemocyanin Ca^{2+} binding site related to deoxygenation [3].

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Selective Chemical and Physical Perturbations for the Different Copper Sites in the Multicopper Oxidase, *Rhus* Laccase

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Laccase [1] contains one blue (T1) and one normal (T2) copper which interact with the single binuclear copper (T3) site to couple one-electron oxidations of substrate to the four-electron reduction of dioxygen to water. We here report reversible chemical simplifications and the application of spectroscopic methods particularly suited for the multicopper oxidases which together enable detailed study of the T3 site in laccase and structure-function correlation to the simpler binuclear copper containing proteins, hemocyanin and tyrosinase.

Derivatives of the simplified type 2 copper-depleted (T2D) [2] protein form have been prepared which allow systematic variation of the coupled binuclear copper site in the presence of an oxidized T1 center. Spectroscopic study of these derivatives (deoxy, [Cu(I)Cu(I)], half met [Cu(I)Cu(II)], met [Cu(II)···Cu(II)], and dimer [Cu(II)Cu(II)]) has allowed determination of the spectral features associated with the binuclear copper centers in laccase. Comparison [3] of deoxy and met T2D identifies the optical features of the binuclear cupric site (ϵ_{330} = 2000 M^{-1} cm^{-1}, ϵ_{420} = 175 M^{-1} cm^{-1}, and ϵ_{745} = 150 M^{-1} cm^{-1}); anion binding studies of the EPR-detectable half met derivatives directly probe geometric structure at the cupric T3 center, demonstrating that exogenous ligands are only weakly bound and do not bridge the binuclear coppers [4].

Spectral comparison of the T2D derivatives and their anated forms has demonstrated the existence of allosteric interactions between the T1 and T3 sites. Laser excitation into the deoxy T2D Blue copper CT band results in a 60% reduction of intensity and increased frequency of the ~380 cm^{-1} vibration compared to native and met T2D protein, whose spectra are similar. In the EPR, $A_{\parallel}$ increases from 37.8 to 42.9 × 10^{-4} cm^{-1} when deoxy is oxidized to met, demonstrating that changes in the T1 copper geometry are linked to oxidation of the T3 copper [3].

Finally, chemical and spectroscopic comparison [5] of anion binding to the oxidized T3 site in met T2D and native laccase indicates a unique T3–T2 intersite interaction which leads to an especially high affinity for exogenous ligands. Ligand competition studies systematically probe the chemical nature of these binding sites and their interdependence, while a combination of variable excitation energy resonance Raman and variable temperature MCD techniques are used to spectroscopically define the role of the T2 copper in greatly increasing ligand binding affinity at the T3 site. L → Cu(II) CT transitions are present for a number of ligated forms of the native enzyme, and resonance Raman enhancement together with mixed isotope studies provide assignment of these features and insight into binding geometry; a comparison to analogous studies of met T2D probes the T2 Cu(II) contribution. Finally, MCD of these features will be presented and their temperature dependence used to distinguish ligand binding to the antiferromagnetically coupled T3 copper centers from binding to the paramagnetic T1 and T2 Cu(II) centers which are predicted to exhibit C terms for which MCD intensity is proportional to 1/T.

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