spectra of the newly formed [4Fe–4S] cluster [6] correspond to those obtained for the subsites of the Fd_R cluster (Fig. 1). The data show that the externally added iron occupies either one subsite or at most two structurally equivalent sites of the [4Fe–4S] cluster [6, 7]. This isotopic labeling of specific cluster subsites allows us to study in considerable detail the hyperfine interactions of the [4Fe-4S] core structures. Moreover, this technique will allow us to correlate spectra of subsites in different oxidation states. Application of this technique of isotopic labeling to individual clusters of enzymes with multiple clusters (*e.g.*, hydrogenase) will greatly simplify the spectral assignments.

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Spectroscopic Probes of Homotropic and Heterotropic Interactions of the Hemocyanin Active Site

YEONG TSYR HWANG, EDWARD I. SOLOMON*

Dept. of Chemistry, Stanford University, Stanford, Calif. 94305, U.S.A.

and LEONARD J. ANDREWS

GTE Laboratories, Waltham, Mass. 02154, U.S.A.

Under physiological conditions, both arthropod and mollusc hemocyanins are assembled into highly aggregated biopolymers with a molecular architecture that differs dramatically between the two phyla. For arthropods, a single subunit has a molecular weight of ~70,000 and contains one binuclear copper active site. At pH below 8 and in the presence of Ca^{2+} these subunits aggregate, and in the case of *Limulus polyphemus* which is the arthropod discussed here, eight hexamers comprise the resultant biopolymer. For molluscs, the smallest subunit contains eight binuclear copper active sites covalently linked in a single polypeptide chain of ~400,000 daltons. At pH less than 8, *Busycon canaliculatum* being representative, twenty chains aggregate to form a whole molecule. When aggregated, all of the hemocyanins are highly cooperative in their oxygen binding, with Hill coefficients dependent on pH and the presence of divalent cations. The cooperative oxygen binding can be interpreted as the equilibrium between a low oxygen affinity tensed quaternary structure (deoxy) and a high oxygen affinity relaxed quaternary structure (oxy), in which the conformational change is induced by the homotropic effector (O_2) and heterotropic effectors (H^+, Ca^{2+}).

We have been able to develop a direct spectroscopic approach to study these allosteric interactions at the active site level through the preparation of Spectral Probe (SP) derivatives of the hemocyanin biopolymer [1]. These SP derivatives contain a small fraction of EPR-detectable half met [Cu(II)-Cu(I)] sites homogenously dispersed among the EPR nondetectable oxy binuclear copper active sites. Our studies demonstrate that deoxygenation of the dominant oxy site (homotropic effect) induces change in the hemocyanin quaternary structure which results in significant spectral and structural changes in the half met probe sites. The extensively defined and well-understood chemistry and spectroscopy of the half met derivatives [2], suggest that the changes in half met SP sites represent elimination of the exogenous ligand (due to the steric competition of endogenous ligand, OR^-) and rearrangement of the geometric structure at the copper site. Furthermore, *Busycon* hemocyanin SP derivatives show the regulatory role of the heterotropic effectors: where Ca^{2+} can induce cooperativity by stabilizing the tensed quaternary structure, proton increases the oxygen affinity and stabilizes the relaxed quaternary structure. Finally, we use the SP derivatives to study the intersubunit and intrasubunit interactions in hemocyanin.

We have also been able to probe the allosteric relationship between changes in the coupled binuclear copper site and the Ca^{2+} binding site [3]. Here, we substitute Eu^{3+} for Ca^{2+} and study its resonance emitted f–f fluorescence. Direct excitation of Eu^{3+} emission is accomplished by a pulsed laser source. Transition between the nondegenerate ground (^7F_o) and excited (^5D_o) states gives a single unsplit line. Since this transition energy is very sensitive to the environment of Eu(III), we are able to differentiate the bound Eu^{3+} peak from the free Eu^{3+} peak. Excited state life times of bound Eu^{3+} measured in D_2O and H_2O permit a direct estimation of the

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

DARLENE J. SPIRA, MARK D. ALLENDORF and EDWARD I. SOLOMON*

Department of Chemistry, Stanford University, Stanford, Calif. 94305, U.S.A.

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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