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Structure–function correlations in copper clusters in proteins

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Abstract- A coupled binuclear copper active site is present in a wide variety of proteins and enzymes which perform different biological functions utilizing O_2. In the multicopper oxidases, the Type 2 and Type 3 centers comprise a trinuclear Cu cluster which represents the active site for the multielectron reduction of O_2.

A coupled binuclear copper active site is present in a wide variety of proteins and enzymes which perform different biological functions utilizing O_2. The hemocyanins (Hc) reversibly bind O_2, the tyrosinases (Ty) are monoxygenases which hydroxylate monophenols to o-diphenols and oxidize these to o-quinones, and the multicopper oxidases (laccase (Lc), ascorbate oxidase and ceruloplasmin), which contain additional copper centers (Type 1 and Type 2) catalyze the four electron reduction of O_2 to water. Our original chemical and spectroscopic studies over a series of protein active site derivatives of Hc and Ty demonstrated (ref. 1,2) that these proteins have very similar active sites. The key features of this spectroscopically effective model for the coupled binuclear copper active site, reproduced in Fig. 1, are that dioxygen binds as peroxide, bridging two tetragonal Cu(II)'s in a cis μ-1,2 fashion, with an additional endogenous bridge between the coppers (RO^-). The endogenous bridge is likely hydroxide based on the crystal structure (ref. 3) of deoxy Hc. The major difference between the Hc and Ty sites is the high accessibility of the Ty active site to exogenous ligands (ref. 4). We found that substrate analogues bind directly to the copper in Ty and compete with peroxide for the same binding site (ref. 5). These studies resulted in the proposed structural mechanism for hydroxylation and oxidation catalysis given in Fig. 2. Alternatively, chemical and spectroscopic studies of a series of protein active site derivatives of Lc (which is the simplest multicopper oxidase, containing one Type 1, one Type 2, and one coupled binuclear Type 3 center) showed the Type 3 site to be strikingly different from the coupled binuclear site in Hc and Ty (ref. 6). Low-temperature magnetic circular dichroism (LTMCD) spectroscopy was found to be a powerful probe of the different copper centers in the multicopper oxidases, allowing a correlation of excited state spectral features with the ground state magnetic properties. From LTMCD studies, the Type 3 site was in fact found to be part of a trinuclear copper cluster with the Type 2 center (ref. 7). This new class of copper site has recently been supported by crystallographic studies on ascorbate oxidase (ref. 8). Our recent studies have focussed on I) Spectroscopy of model complexes to further develop our understanding of the unique spectral features of oxy Hc; II) A detailed comparison of the coupled binuclear site in Hc and the Type 3 site in Lc; III) Evaluation of the metal centers required for the O_2 reactivity of the multicopper oxidases; and IV) Detailed LTMCD spectral studies of the trinuclear copper cluster to establish its geometric and electronic structure and interactions with exogenous ligands as related to the mechanism of multielectron reduction of O_2. These studies are summarized below.

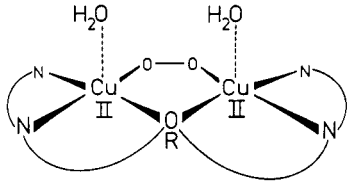

Fig. 1. The Spectroscopically Effective Active Site of Hemocyanin and Tyrosinase.

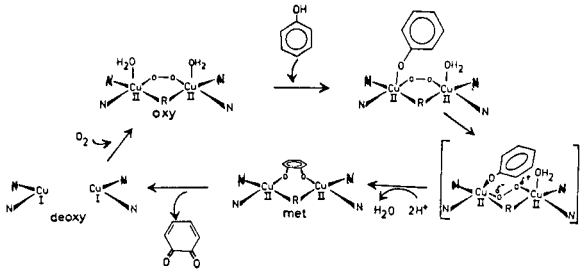

Fig. 2. Active site structural mechanism for hydroxlyation and oxidation of phenols to form o-quinones by tyrosinase.

I. ELECTRONIC STRUCTURE OF OXY HEMOCYANIN

In oxy Hc, the two Cu(II) ions show strong antiferromagnetic coupling in the ground state, and are consequently inaccessible to ground state spectral study. The excited state spectroscopic features are therefore the most useful probe of the electronic structure of

the oxy Hc active site. By comparison of oxy Hc to met aquo Hc, a fully oxidized derivative of Hc in which peroxide has been displaced, the intense oxy Hc absorption (abs.) bands at 345nm (ϵ = 20,000 M^{-1} cm^{-1}) and at 570nm (ϵ = 1000 M^{-1} cm^{-1}), as well as the CD band at 480nm($\Delta\epsilon$ = +2.5 M^{-1} cm^{-1}), have been assigned as $O_2^{2-}\rightarrow$Cu(II) charge transfer (CT) transitions (ref. 1). In order to gain insight into the electronic structure of oxy Hc, a transition dipole vector coupling (TDVC) model has been developed for analysis of CT transitions of exogenous bridging ligands and has been tested on several small molecule model complexes. The highest occupied molecular orbitals (HOMOs) of peroxide are a degenerate π^* set which splits in energy upon coordination to copper, with one σ bonding to the half-filled $d(x^2-y^2)$ acceptor orbital of the copper. The CT transition from the former (π^*_σ) will be higher in energy and more intense, due to greater overlap, than that from the latter (π^*_v). Peroxide has been shown by mixed-isotope resonance Raman studies to bind terminally to one copper in a binuclear copper complex prepared by Karlin et al. (ref. 9). The rR enhancement profiles of the (O-O) and (Cu-O) stretching modes show two $O_2^{2-}\rightarrow$Cu(II) CT transitions, with the more intense feature at higher energy (505nm, ϵ = 2400 M^{-1} cm^{-1}; 610nm, ϵ = 2400 M^{-1} cm^{-1}) as predicted above.

The HOMOs of azide are a π^{nb} degenerate set, similar to the π^* set in peroxide. Copper complexes with azide terminally bound show one $N_3^-\rightarrow$Cu(II) CT transition in the absorption spectrum at 400nm (ϵ = 2000 M^{-1} cm^{-1}), indicating that only the $\pi^{nb}_\sigma \rightarrow$ Cu transition contributes significant intensity. In the μ-1,3 azide bridged copper dimers, two $N_3^-\rightarrow$Cu(II) CT transitions are seen at 365nm (ϵ = 2000 M^{-1} cm^{-1}) and 420nm (ϵ = 1000 M^{-1} cm^{-1}). From the TDVC model, these are the symmetric and antisymmetric combinations of the $\pi^{nb}_\sigma\rightarrow$Cu(II) transition in the monomer, both of which are electric dipole allowed. The energy splitting estimated from the Coulomb interactions between the transition moments is significantly less than the experimental value, which has been accounted for by introduction of an exchange mediated excitation transfer contribution (the L integral) to the excited state energies. The met azide derivative of Hc shows spectroscopic features which are remarkably similar to those of Reed's μ-1,3 azide bridged dimer model, demonstrating the applicability of the TDVC model to the spectra of the protein site (ref. 10).

The abs. and CD spectra of oxy Hc, shown in Fig. 3, are interpreted within the framework of this TDVC model (ref 1.). In the μ-1,2 peroxide bridged structure in Fig. 1, each of the two $O_2^{2-}\rightarrow$Cu(II) CT transitions observed in the monomer model complex split into two transitions in the bridged dimer. In the C_{2v} effective site symmetry, these would be of A_1 and B_1 symmetry for π^*_σ and A_2 and B_2 for π^*_v. All except the A_2 transition should be electric dipole allowed, while the A_2, B_2, and B_1 transitions should be magnetic dipole allowed. Thus, $\Delta A/A$ must be greatest for the A_2 transition, and the 480nm CD band can be assigned to this transition. The 570nm abs. band is assigned as the electric dipole allowed B_2 component of π^*_v. This ordering of the A_2 and B_2 transitions is as calculated using the Coulomb interactions, but as in the azide complexes the splitting is underestimated (840 cm^{-1} compared to the observed value of 3500 cm^{-1}). For the π^*_σ transitions, B_1 is expected to be at higher energy than A_1 ($E(A_1)$-$E(B_1)$ is calculated to be -5000 cm^{-1}), with A_1 more intense based on vector coupling of the CT moments. Thus, the 345nm band is assigned as the A_1 component of π^*_σ. The fact that three $O_2^{2-}\rightarrow$Cu(II) CT transitions are observed for oxy Hc requires that the peroxide bridges the active site, and these CT spectral features are shown to be well interpreted in the context of the TDVC model which has now been calibrated by structurally defined binuclear copper model complexes.

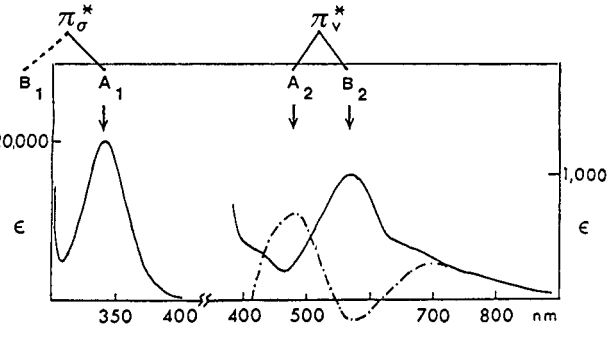

Fig. 3. Assignment of the $O_2^{2-}\rightarrow$Cu(II) charge transfer spectrum of oxy hemocyanin using the TDVC model.

II. COMPARISON OF THE BINUCLEAR SITE IN Hc AND THE TYPE 3 SITE IN T2D Lc

A parallel series of met [Cu(II) Cu(II)], half met [Cu(I) Cu(II)] and deoxy [Cu(I) Cu(I)] derivatives of Hc and of the Type 3 site in a Type 2 depleted (T2D) derivative of Lc (where the Type 2 Cu is reversibly removed and the Type 1 Cu is oxidized) have been prepared. In the met form, both the Hc and Lc binuclear sites contain two tetragonal Cu(II) ions. However neither site gives rise to an EPR signal; therefore, the Cu(II) ions are antiferromagnetically coupled. Preliminary SQUID susceptibility measurements place a lower limit on the singlet-triplet splitting of -2J > 400 cm^{-1} for met Hc and -2J > 200 cm^{-1} for met T2D Lc. Lowering the pH and adding azide produces broad triplet EPR signals in both

systems which account for <10% of the sites. These signals arise from two dipolar interacting Cu(II) ions. These results are interpreted in terms of an endogenous bridge in both met aquo sites which provides the superexchange pathway for strong antiferromagnetic coupling. Protonation of this bridge uncouples the sites, and from the pH dependence of the dipolar EPR signals the endogenous bridge ligands are calculated to have similar intrinsic pK_a's (>6.7).

The spectroscopic and chemical properties of the half met Hc and T2D Lc sites are very different. In Hc, the electronic spectral features of the half met NO_2^- derivative indicate a localized Cu(II) center with ~ tetragonal geometry. However, an additional low energy band at ~1000nm is observed in the Br^- derivative. This band is reasonably intense in abs. but has very little associated LTMCD intensity. This allows assignment as an intervalent transfer (IT) transition, since the selection rules for C term MCD require two perpendicular transition moments and an IT transition is polarized only along the Cu-Cu axis. Detailed analysis of the IT transition over the half met-L series ($L=Cl^-$, Br^-, I^-) allows an estimation of the electron delocalization between the coppers in the ground state wavefunction. The delocalization increases with increasing covalency of the exogenous ligand, indicating that this is the pathway for electron delocalization and thus must bridge the two coppers at the half met Hc site (ref. 11).

Exogenous ligands bind to the half met Hc site with unusually high affinity (at 298K: $K_{N_3^-}$ (half met)=3.2 x 10^4, (met)=80, (deoxy)=150 M^{-1}). Temperature dependent studies indicate that this high affinity results from a very favorable entropy contribution. In contrast to half met Hc, the spectral features of the mixed valent T3 site in half met T2D Lc show no evidence for electron delocalization. Furthermore, exogenous ligands bind to the mixed valent T3 site with affinities similar to aqueous Cu(II). Taken together, these results strongly suggest that exogenous ligands do not bridge two coppers at the half met Type 3 site in T2D Lc (ref. 6).

Deoxy Hc contains a pair of Cu(I) ions and readily reacts with O_2 to form oxy Hc, which contains a pair of Cu(II) ions bridged by peroxide (Fig. 1). Using a quantitative Cu X-ray absorption edge method we developed to assay Cu redox state composition, we have demonstrated that T2D Lc as isolated contains an oxidized Type 1 site and a reduced Type 3 site (deoxy T2D) (ref. 12). In contrast to deoxy Hc the reduced Type 3 site in deoxy T2D Lc is stable to oxidation by O_2. The linear correlation of the formation of a 330nm abs. band with the percent oxidation of the Type 3 site during peroxide titration of T2D Lc indicates that peroxide oxidizes but does not bind to the Type 3 site, thus casting doubt on the existence of peroxide intermediates in Lc. Like Hc, exogenous ligands bind at the deoxy T2D Lc active site, indicating that the lack of O_2 reactivity in deoxy T2D Lc compared to deoxy Hc is not due to inaccessibility of small molecules, but likely relates to terminal compared to bridged exogenous ligand binding modes, respectively.

III. O_2 REACTIVITY OF LACCASE

The reactivity of fully reduced T2D and TlHg Lc (a derivative in which the Type 1 Cu is replaced by Hg(II), containing a Type 2-Type 3 trinuclear Cu cluster) with O_2 has been investigated using Cu X-ray absorption edge spectroscopy. Upon exposure of reduced T2D Lc to air, biphasic reoxidation kinetics are observed. A small amount of Cu reoxidizes quickly, which corresponds to ~10% native Lc regenerated under reducing conditions. As monitored optically, the bulk of the Type 1 centers reoxidize slowly (half-time ~12 h). However, an intense Cu(I) peak at 8984 eV is present in the Cu edge of a sample exposed to air for 3.5 days, indicating that the Type 3 centers remain reduced. In contrast, both the Type 2 and Type 3 centers in fully reduced TlHg Lc completely reoxidize rapidly upon exposure to air. Taken together, these results indicate that the Lc Type 2 Cu is critically required for facile reactivity with O_2, whereas the Type 1 center is unnecessary. Thus, the Type 2-Type 3 trinuclear Cu cluster represents the minimum functional unit required for O_2 reactivity.

IV. THE TRINUCLEAR Cu CLUSTER

Having defined the reactivity of the trinuclear Cu cluster, we have employed a combination of abs., CD and LTMCD spectroscopies to characterize the electronic and geometric structure of the trinuclear Type 2-Type 3 Cu cluster and to probe its small molecule interactions. In order for an electronic transition to be observed in the LTMCD spectrum, it should have significant absorption intensity and be associated with a degenerate ground state (C-term MCD). CT and d→d transitions at the paramagnetic (S=1/2) Type 2 Cu(II) will thus be strong in the LTMCD spectrum. In contrast, the pair of Cu(II) ions in the Type 3 center are antiferromagnetically coupled, giving rise to an S=0 ground state. Thus, transitions of the Type 3 center will only be observed in abs. and CD.

The Type 2 d→d bands are evident in the LTMCD of TlHg Lc (Fig.4), and their energies define a tetragonal effective geometry at this site. Three sharp CT bands are present at 320-370nm, indicating that the Type 2 center exhibits significant contribution in this spectral region. Fluoride is known to bind with very high affinity to the Type 2 Cu(II) ($K>10^4$ M^{-1}).

Addition of F^- causes a dramatic decrease in the amplitude of the d→d and CT MCD features, thus confirming their assignment to the Type 2 site. A band at ~900nm, which is observed in the CD but not LTMCD spectra, is assigned as a d→d band of the diamagnetic Type 3 site. Upon binding F- at the Type 2 Cu, the Type 3 d→d band shifts to lower energy, indicating an additional strong interaction of this exogenous ligand with the Type 3 site.

The mechanism of N_3^- binding to TlHg Lc has been probed at the molecular level via LTMCD and abs. studies of N_3^-→Cu(II) CT features. Upon binding N_3^-, a paramagnetic N_3^-→Type 2 CT band at 485 nm and a perturbation of the Type 3 d→d transition titrate in parallel ($K \sim 700\ M^{-1}$). In addition, this N_3^- uncouples <10% of the diamagnetic Type 3 sites, rendering them paramagnetic, and giving rise to three paramagnetic N_3^-→uncoupled Type 3 CT bands, and a broad triplet EPR signal. Thus, a single azide bridges the Type 2 and Type 3 centers (Fig. 5). The similarity of the N_3^--induced uncoupling process and Type 3 d→d perturbations in T2D and TlHg Lc indicate that the interaction of N_3^- with the Type 3 center is not significantly perturbed by interaction with the Type 2 center.

Thus, we have determined that the Type 2 and Type 3 centers in the multicopper oxidases comprise a trinuclear Cu cluster capable of exogenous ligand bridging, and that this represents the active site for the multielectron reduction of O_2. Detailed studies of the molecular mechanism of this reaction are currently underway.

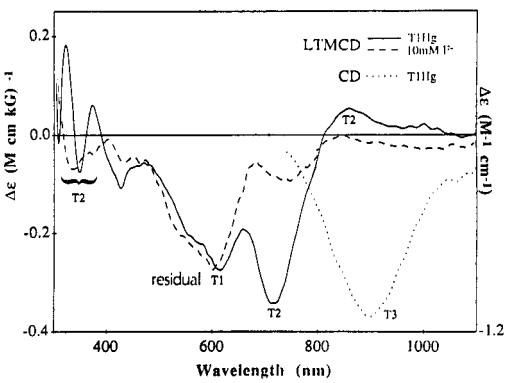

Fig 4. Comparison of the LTMCD and CD spectra of TlHg laccase.

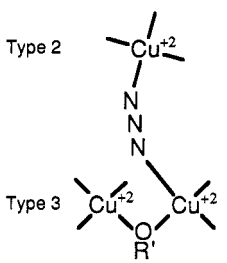

Fig. 5. Model for bridged binding of N_3^- at the trinuclear Cu cluster.

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