Eisenberger *et al.* used extended X-ray absorption fine structure (EXAFS) to measure the Fe–N distance in deoxyhaemoglobin and concluded that the irons lie only $0.2^{+0.1}_{-0.2}$ Å from the plane of the porphyrin nitrogens and that the cooperative mechanism I had proposed is therefore invalid. We have now compared the EXAFS of deoxyhaemoglobin with that of the ferrous 'picket fence' 2-methylimidazole complex in which the displacement of the iron from the plane of the porphyrin nitrogens is known to be 0.399 ± 0.004 and 0.426 ± 0.004 Å from the mean porphyrin plane. The two EXAFS spectra are very similar, consistent with similar displacements of the irons. We find the same Fe–N distance of 2.06 ± 0.01 Å in deoxyhaemoglobin as Eisenberger *et al.*, but show that the displacement of the iron cannot be calculated from that distance.

A4

Electronic and Geometric Structure-Function Correlations of the Coupled Binuclear Copper Active Site

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A coupled binuclear copper active site exists in a variety of proteins and enzymes which perform different biological functions. These metalloproteins are listed in Table I. The hemocyanins are highly aggregated molecules whose sub-units exhibit significant intersite interactions, while the multicopper oxidases contain additional coppers which are involved in interactions with the coupled binuclear copper site. The unique spectral features associated with these binuclear copper active sites and their variation in the proteins of Table I provide an important opportunity for structure-function correlations in Bioinorganic Chemistry.

TABLE I. Coupled Binuclear Copper Proteins.

Hemocyanin	Arthropod	deoxy + $O_2 \rightleftarrows$ oxy
	Mollusc	deoxy + $O_2 \rightleftarrows$ oxy
		$2H_2O_2 \rightleftarrows O_2 + 2H_2O$
Tyrosinase		deoxy + $O_2 \rightleftarrows$ oxy
		$2H_2O_2 \rightleftarrows O_2 + 2H_2O$
		phenol + $2e^- + O_2 + 2H^+ \rightleftarrows$ *o*-diphenol + H_2O
		2*o*-diphenol + $O_2 \rightleftarrows$ 2*o*-quinone + $2H_2O$
Laccase, Ceruloplasmin, Ascorbic acid Oxidase		$4AH + O_2 \rightarrow 4A + 2H_2O$

A variety of different spectroscopic and magnetic techniques have been used to probe the unique electronic structure of the coupled binuclear copper active site. However, in order for results from these studies to be meaningful, the system must be subjected to perturbations and the spectral responses systematically investigated. In studying active sites in bioinorganic chemistry, not only can physical perturbations such as magnetic fields (Zeeman effect) or electric fields (Stark effect) be applied, but one also has the ability to chemically perturb the system. For the coupled binuclear copper active site, a series of protein derivatives has been prepared which allows the site to be systematically varied. These are summarized in Fig. 1.

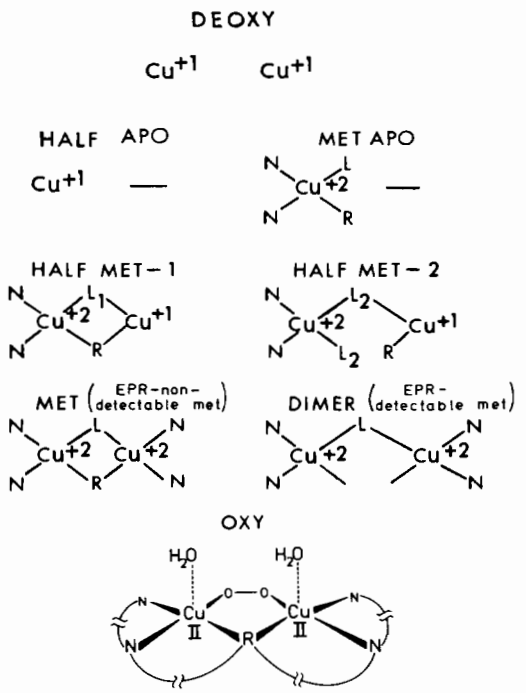

Fig. 1. Coupled binuclear copper active site derivatives.

Detailed chemical and spectroscopic studies of these derivatives of the hemocyanin site have led to the 'spectroscopically effective' model for the coupled binuclear copper active site shown at the bottom of Fig. 1. A comparison of the half met and met apo derivatives indicates the presence of class 2 mixed valence behavior which correlates with the exogenous bridging ligand providing the pathway for electron delocalization. The met and dimer derivatives both contain formally binuclear cupric active sites, yet met (like oxy) exhibits no EPR signal. This has been related to the presence of an endogenous protein bridge (RO^-) which provides a superexchange pathway for antiferromagnetic coupling between the coppers. When the endogenous bridge is broken, either by a group 2 ligand maintaining the coppers

at a distance >5 Å (dimer derivative) or by protonation at low pH, EPR signals are observed which can extend over several thousand gauss and which result from zero field splitting of the spin triplet. A comparison of met and oxy demonstrates that the unique optical spectral features of oxyhemocyanin (bands at 600 nm, $\epsilon \sim 1000\ M^{-1}\ cm^{-1}$ and 350 nm, $\epsilon \sim 20{,}000\ M^{-1}\ cm^{-1}$) are $O_2^{=} \rightarrow Cu(II)$ charge transfer transitions. Finally, a transition dipole vector coupling model has been developed which predicts the charge transfer spectrum of a peroxide bridged to two copper(II)'s and strongly supports the μ-1,2 $O_2^{=}$ bridging geometry in Fig. 1.

Cooperativity among the hemocyanin biopolymer active sites in dioxygen binding has been studied through the preparation of a spectral probe derivative which contains ~10% EPR-detectable half met sites dispersed among the EPR-nondetectable oxy sites. Deoxygenation produces large changes in the mixed valence properties of the EPR signal; these effects appear to correlate to a distortion of the site with changes in the endogenous bridge.

Finally, chemical and spectroscopic studies have been extended to the metalloproteins listed in Table I in order to correlate active site structure and biological function. These studies have indicated that hemocyanin from both phyla, and tyrosinase, all have very similar coupled binuclear copper active sites. The lack of catalase activity in arthropod hemocyanin appears to relate to a geometric structural distortion of the active site. The monooxygenase and oxidase activity of tyrosinase correlates with the high accessibility of its active site to organic substrates, and has led to the structural mechanism for monophenol hydroxylation and oxidation shown in Fig. 2.

Parallel studies on active site derivatives of the coupled binuclear copper (type 3) active site in laccase have demonstrated very different chemical and spectroscopic behavior. This has been interpreted in terms of the preliminary 'spectroscopically effective' active site model shown in Fig. 3 where exogenous ligands do not bridge the two coppers of the type 3 site. In contrast to the μ-1,2 peroxo bridging geometry found for reversible dioxygen binding in hemocyanin, lack of exogenous ligand bridging in the type 3 site of laccase appears to correlate with irreversible binding of the peroxide intermediate produced in the reduction of dioxygen to water.

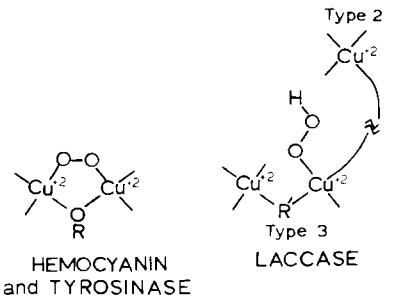

Fig. 3. Comparison of the spectroscopically effective models for peroxide binding to the coupled binuclear copper site in hemocyanin, tyrosinase, and laccase. OR and R' denote the endogenous protein bridge in the hemocyanins and laccase, respectively.

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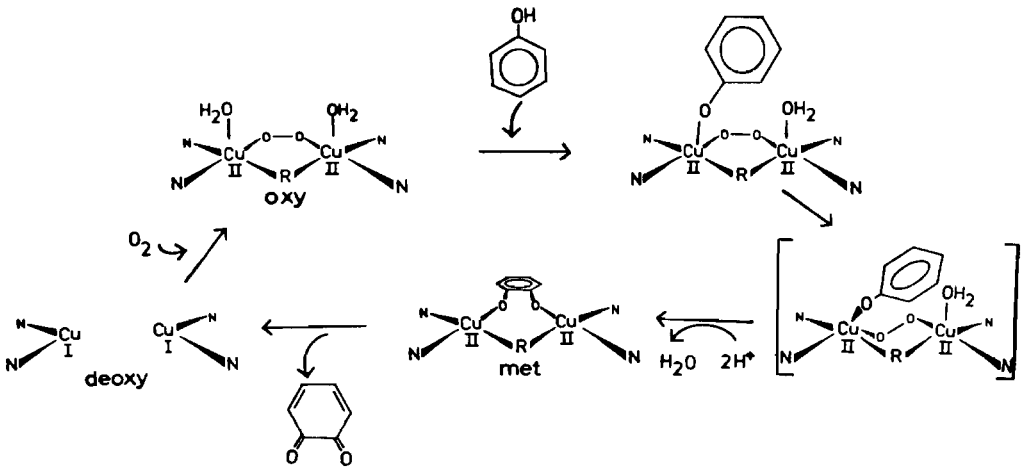

Fig. 2. Proposed mechanism of phenol hydroxylation and oxidation to form *o*-quinones by tyrosinase.