Cooperative Phenomena in Polynuclear Metalloproteins

ANDREA SCOZZAFAVA
Institute of Biological Chemistry, University of Sassari, Italy
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Polynuclear metalloproteins are rather numerous in nature, and these systems still now constitute a formidable puzzle for biochemists and chemists in the comprehension of their spectroscopic and catalytic properties. Probably most of the present interest in the field of coordination chemistry toward the synthesis and theoretical studies of simple inorganic polynuclear complexes arises from the necessity of finding reliable models for explaining the spectroscopic properties of the more complex, naturally occurring, polynuclear systems.

The metals present in a metalloprotein can play different roles, and for this reason they have been generally distinguished as (a) structural, or (b) catalytic or functional. This obviously represents a rather naive classification, because the situation is more complex. For example, as will be shown later, each catalytic ion plays also a structural role and in many cases structural ions can also influence the catalytic efficiency of the enzyme.

It is aim of this paper to illustrate with reference to some selected polynuclear metalloproteins the structure and the location of the metal binding sites, as well as to point out the role of the different metals and the mutual influences between the various centers. I have selected two systems to discuss here: bovine superoxide dismutase, and the blue copper oxidases. The first represents an example in which both functional and structural ions are present, whereas the blue copper oxidases represent systems in which different metal sites all concur in a cooperative fashion to the catalytic reaction.

Bovine Superoxide Dismutase

Bovine superoxide dismutase (BSOD), a dimeric enzyme with a molecular weight of 32,000, contains 2 Cu(II) and 2 Zn(II) ions. Its biological role is to catalyze the dismutation of the superoxide ion into O_2 and H_2O_2 [1].

In the past, spectroscopic studies on various metal substituted BSOD established before the X-ray structure was solved that the copper and the zinc sites in each subunit were in close proximity to each other. For example, Fee and Rotilio's group independently replaced the zinc atom with the cobalt(II) ion obtaining the $Cu_2(Co, Zn)_2$BSOD derivative, and they discovered that this derivative did not show any ESR signal from either the Co(II) or the Cu(II) ions [2–4]. Only upon reduction of the copper(II) to copper(I) did the ESR signal of the cobalt ion become detectable. This was attributed to antiferromagnetic coupling between the cobalt and the copper ions, and it was therefore suggested that a common ligand should be shared by the two ions [4].

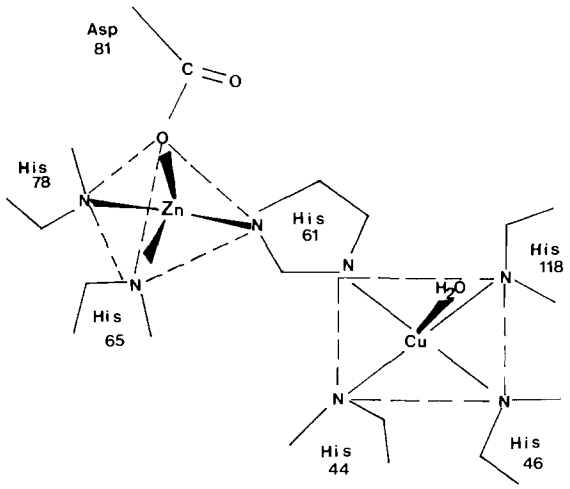

Fig. 1. Schematic drawing of the active site of bovine superoxide dismutase.

Later, Fee prepared the Cu_2Cu_2BSOD derivative and again the proximity of the copper to the zinc site (in the above derivative, occupied by copper(II)) became apparent through ESR spectroscopy since a spectrum typical of a triplet state was detected [5]. From the temperature dependence of the ESR signal Fee estimated an energy separation between the singlet and the triplet state of about 50 cm^{-1}.

The X-ray structure at 3 Å of resolution is now available, showing that the two metals share a

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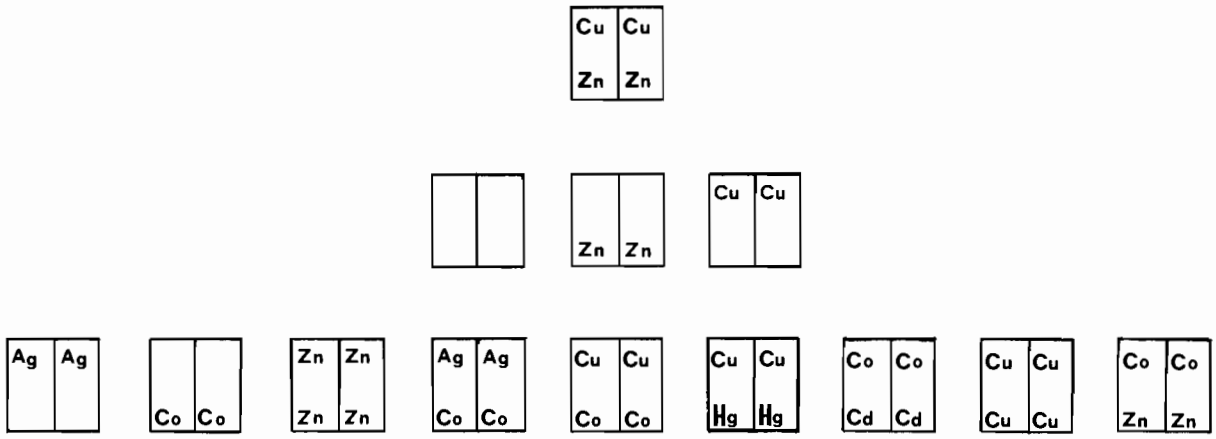


Fig. 2. Metal derivatives of bovine superoxide dismutase. (From Ref. 12 and references therein).

common bridging imidazolate residue from the side chain of histidine 61 [6] (Fig. 1).

The coordination around the copper ion is distorted square pyramidal, the other donors being represented by three histidines and a water molecule, as revealed through ^1H T_1^{-1} measurements [7]. In particular the histidine 61 is somewhat above the plane defined by the copper atom and the remaining three nitrogens, on the same site which is exposed to the solvent. The coordination around the zinc is pseudotetrahedral, the four donors being represented in addition to the bridging imidazolate by two histidines and one aspartyl carboxylate residue [6].

Which are the peculiar chemical properties of this heterodinuclear coordination compound?

First of all the two ions can be removed, leading to a stable apoprotein which in turn can re-accept different ions. In such a way it has been possible to prepare a large variety of metallo substituted superoxidodismutases (Fig. 2). Up to now only Ag(I) and Co(II) have been inserted in the place of the native copper(II), whereas a larger number of metals have been substituted in the place of the native zinc ion [8, 10]. The two sites have unusual properties from the point of view of the coordination chemistry. Starting from the native enzyme, and on lowering the pH below 3, all the metals dissociate into the solution. At pH values between 3 and 4.5 only the site which in the native enzyme contains copper is able to bind metals, its affinity being 10^6 times higher than the zinc site [11]. At higher pH the zinc site can also be populated, and the affinity constant of this site increases with pH in such a way that at alkaline pH this site competes with the first one for metals. For example, starting from the zinc depleted enzyme Cu_2E_2BSOD at pH values above 7.5 there is a preferential migration of part of the copper toward the zinc site of subunits containing copper in the native site; in such a way the apoprotein and the Cu_2Cu_2BSOD derivative are obtained [9, 12].

TABLE I. Relative Activity of Metal-Substituted Bovine Superoxide Dismutases.[a]

Native enzyme	100
Apoprotein	0
Cu_2Co_2BSOD	90
Cu_2Co_2BSOD	90
Cu_2Cd_2BSOD	70
Cu_2Cu_2BSOD	100
E_2Co_2BSOD	0
E_2Zn_2BSOD	0
$Ag(I)_2Cu_2SOD$	5

[a]From Ref. 12.

The copper ion in the native enzyme represents the catalytic center whereas the zinc ion has only an ancillary structural function. As a matter of fact every derivative which maintains copper(II) in the native site retains almost full activity, whereas no derivative in which the native copper is replaced by other metals retains this activity (Table I). The copper atom also has a structural role and stabilizes the apoenzyme quite strongly. For example it has been reported that the T(50%/10 min), the temperature which causes irreversible loss of half of the activity in 10 minutes, increases from 49.4 to 73.2 °C on passing from the apoprotein to the Cu_2E_2BSOD [13].

Addition of the superoxide ion to the fully oxidized enzyme causes a bleaching of the color whereas addition of the same ion to the fully reduced enzyme partially restores the color. This behavior indicates that the copper ion is alternatively reduced and oxidized during the catalytic cycle. A possible mecha-

TABLE II. Nuclear Relaxation Parameters for Anions Bound to Copper(II) in Superoxide Dismutase (SOD) and Cu(Me_4(14)-aneN$_4$)(ClO_4)$_2$ (TMC).[a]

Ligand	SOD		TMC	
	$(fT_{1p})^{-1}$, s^{-1}	$(fT_{2p})^{-1}$, s^{-1}	$(fT_{1p})^{-1}$, s^{-1}	$(fT_{2p})^{-1}$, s^{-1}
NCS^- (^{13}C)	1.0×10^4	5.0×10^5	2.7×10^2	2.2×10^4
(^{14}N)		1.6×10^7		
NCO^- (^{13}C)	5.3×10^3	5.6×10^5	2.8×10^2	6.9×10^4
(^{14}N)		5.0×10^6		

[a]From Ref. 18.

nism which has been suggested for the reaction is the following

$$Cu(II)(His^-)(HisH)_3 + O_2^- \xrightarrow{H^+} Cu(I)(HisH)_4 + O_2$$

$$Cu(I)(HisH)_4 + O_2^- \xrightarrow{H^+} Cu(II)(His^-)(HisH)_3 + H_2O_2$$

Recent ^{113}Cd NMR investigations on the Cu_2Cd_2SOD derivative suggest that the histidinate becames protonated on the copper side during the catalytic cycle [14].

The ESR spectrum of the enzyme is typical of a copper ion in a distorted five coordinate geometry [15] showing $g_\parallel = 2.27$, $A_\parallel = 143 \times 10^{-4}$ cm^{-1} and a large anisotropy in the normal region.

Anions are known to bind to BSOD, the site of attack being the copper atom. For example cyanide binds in a stoichiometric ratio, the 1:1 adduct showing $g_\parallel = 2.21$, $A_\parallel = 190 \times 10^{-4}$ cm^{-1} and an electronic absorption spectrum with a maximum at 19.3×10^3 cm^{-1} [16]. A further point is that addition of CN^- reduces the ^1H T_1^{-1} enhancement of water solutions containing the enzyme to the diamagnetic value [16]. A substantially similar behavior is observed in the case of N_3^-, the ^1H T_1^{-1} enhancement being again reduced upon the ion binding. The behavior of these two ions has been generalized, and it has been stated and commonly accepted that binding of anions in BSOD occurs at the copper atom with substitution of the bound water molecule. We recently demonstrated however that this opinion is wrong [17, 18] and that the problem of the interaction of anions with BSOD is much more complicated. The necessity of characterizing the site of attack of the anions is relevant not only to the comprehension of the mechanism of inhibition of the enzyme but probably also for explaining the interaction of the substrate, which is again an anion, with the enzyme.

In particular we studied the interaction of the enzyme with the anions thiocyanate and cyanate by means of ESR, electronic spectroscopy and ^1H, ^{13}C, ^{14}N, ^{15}N NMR. NMR measurements on different nuclei allowed us to ascertain that the formation of the copper–anion adducts is not accompanied by water removal [17, 18]. In Fig. 3 the ^1H T_{1p}^{-1} enhancement of water solutions containing BSOD in the presence of increasing amounts of NCS^- is reported. No decrease in the ^1H T_{1p}^{-1} value is observed up to a $[NCS^-] \cong 0.1$ *M* although at this point more than 80% of the BSOD–NCS adduct is formed, as estimated from ^{13}C NMR measurements [17]. Furthermore a comparison of the relaxation data between the BSOD-adducts and those of the model complex (1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane)copper(II), where anions coordinate in axial position, strongly suggests that in the enzyme adducts the anions coordinate in an equatorial rather than axial position. Indeed, the large difference in the fT_{2p} values for the same ion in the enzyme adduct with respect to the model adduct (Table II) should be ascribed to a larger contact contribution being

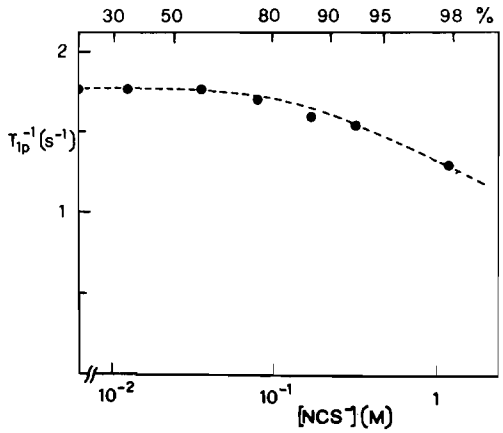


Fig. 3. T_{1p}^{-1} values of the ^1H NMR signal of water solutions containing 6.0×10^{-4} *M* BSOD as a function of thiocyanate concentration. The amount of enzyme–thiocyanate adduct formed in the same range of concentration is also reported. (From Ref. 17).

TABLE III. ESR Parameters of some BSOD Derivatives.[a]

	$g_\parallel$ ($cm^{-1} \times 10^{-4}$)	$g_\perp$	$A_\parallel$
Cu_2Zn_2SOD	2.262	2.080	135
Cu_2Zn_2SOD–NCS	2.259	2.065	155
Ag_2Cu_2SOD	2.311	2.120	105
Ag_2Cu_2SOD–NCS	2.260	2.065	154
Cu_2Cu_2SOD–NCS	2.262	2.065	155

[a]From Ref. 19.

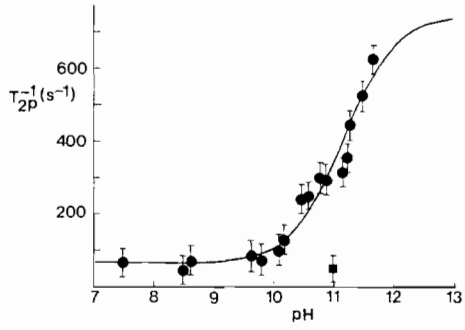


Fig. 4. T_{2p}^{-1} values of $H_2{}^{17}O$ in 1.33×10^{-3} *M* BSOD solutions as a function of pH (•) and in the presence of stoichiometric amounts of cyanide (▪). (From Ref. 20).

operative in the BSQD derivatives, consistent with equatorial coordination to the copper. As both the ESR parameters and the electronic spectra of the enzyme derivatives are very similar to those of the native enzyme, the coordination number of the copper ion is probably unchanged. The only possibility therefore is that the anion removes one of the bound histidines. This is a quite strange result as protein ligands are believed to be strongly bound to the metal and not easily removed by simple anions like NCS^- or NCO^-.

Recently, Lippard has been able to provide a more direct proof to our proposals. Working on the Cu_2-Cu_2SOD derivative he showed that upon addition of thiocyanate the triplet spectrum disappears, and the features typical of a copper(II) into a diamagnetic host are obtained [19]. This means that the anion removes the bridging histidine through whom the superexchange mechanism occurs. What is surprising in Lippard's experiment is that only a single ESR signal is observed whereas the two copper atoms occupy two different binding sites, one pseudotetrahedral and the other five-coordinated. As a matter of fact the spectral parameters of copper in the two derivatives Cu_2Zn_2BSOD and Ag_2Cu_2BSOD are very different (Table III). A possible suggestion is that thiocyanate binds also to the copper in the zinc site, replacing the aspartyl ligand and giving rise to a chromophore substantially similar to that of the copper–thiocyanate adduct of the native site.

We studied also the effect of increasing pH on BSOD [20]. Rotilio *et al.* found that at alkaline pH the ^1H NMR relaxation rate of water solutions containing BSOD increases, the values being fitted to a single equilibrium with a pKa of 11.5 that they attributed to the dissociation of the axial bound water molecule [21].

In our laboratory the ^{17}O T_{2p}^{-1} values of ^{17}O enriched water solutions containing the enzyme have been measured as a function of pH. The values also increase with increasing pH (Fig. 4), and again the points can be fitted to a single equilibrium, with a $pK_a = 11.3 \pm 0.3$. The major difference with the ^1H T_{1p}^{-1} values is that while the proton relaxation rates approximately double on going from neutral to alkaline pH [21], the ^{17}O T_{2p} values show a ten fold increase in the same pH range. The temperature dependence of the ^{17}O linewidth has been investigated in the range 5–35 °C at different pH values. In all cases the linewidth decreases by increasing the temperature, indicating fast exchange.

Whereas the T_2 values are strongly affected by the presence of the paramagnetic center (addition of cyanide results in the obtainment of the diamagnetic linewidth, see Fig. 4), the ^{17}O T_1 value is not appreciably affected, a T_1 of 2.9 ms being measured for both pure and reduced enzyme [20]. T_{2p}^{-1} values much larger than T_{1p}^{-1} values are indicative of dominant contact contributions operative in the former relaxation process. The problem remains of the small paramagnetic effect on the ^{17}O NMR linewidth at pH 9 where ^1H NMR relaxation data indicate the presence of a copper-coordinated water molecule. We again suggest that these results are in agreement with the coordination at high pH of an hydroxide ion in the equatorial plane with remotion of one histidine. A simple ionization of the coordinated water molecule to a hydroxide ion would hardly be consistent with so a large increase in the T_{2p}^{-1} values. On the other hand addition of OH^- in an equatorial position would leave the axial coordinated water molecule and would satisfactorily explain both the ^1H and the ^{17}O relaxation data.

In conclusion the histidinato bridge between the copper and zinc ions in BSOD appears to be removed by ions like NCS^-, NCO^- and OH^-, an effect which has also been observed in simple dinuclear inorganic complexes containing imidazolato bridges [19].

Blue Copper Oxidases

Blue copper oxidases constitute systems much more complex than the previously discussed BSOD.

TABLE IV. Molecular Weight and Copper Content of some Blue Oxidases.[a]

	MW	Type 1	Type 2	Type 3
Polyporus versicolor laccase	64500	1	1	2
Rhus vernicifera laccase	110000–140000	1	1	2
Podospora laccase II	390000	4	4	8
Podospora laccase I (tetrameric)	70000	1	1	2
Podospora laccase III	80000	1	1	2

[a]From Ref. 43 and references therein.

TABLE V. ESR Data of Some Bis(salicylaldiminato)copper(II) Complexes.[a]

Compound	$g_{\parallel}$	$g_{\perp}$	$A_{\parallel}$ ($cm^{-1} \times 10^{-4}$)
(Cu,Zn)-5-Me-Sal-*i*-Pr	2.27	2.02–2.11	113
(Cu,Pd)-5-Me-Sal-*i*-Pr	2.22	2.04	186
(Cu,Zn)-3-Me-Sal-*i*-Pr	2.27	2.03–2.11	120
(Cu,Pd)-3-Me-Sal-*i*-Pr	2.22	2.05	186
(Cu,Ni)Sal-Et	2.22	2.05	194
(Cu,Zn)Sal-*i*-Pr	2.28	2.095–2.014	117
(Cu,Zn)Sal-*t*-Bu	2.29	2.08–2.03	117

[a]From Ref. 25.

However I summarize here the most recent knowledge on these systems, because the 'blue proteins' have attracted for a long time the interest of chemists because of their unusual spectroscopic properties. Progress in theoretical chemistry now allows us to rationalize most of these unusual properties. Furthermore (also from the biological point of view) these systems have been better characterized and now a possible functional scheme for the catalyzed reaction is taking shape largely due to the contribution of the Swedish School of Malmström and Reinhammar.

The blue oxidases reduce the oxygen molecule to water by transferring four electrons (and four protons) from reducing substrates like phenols or polyphenols. These enzymes have a molecular weight ranging between 60,000 and 140,000 [22] and contain several copper ions per molecule, which according to their spectroscopic properties are usually referred as type 1, type 2 and type 3 copper (Table IV). It is interesting to observe that the type 3 coppers are always even.

Type 1 Copper

The type 1 copper is responsible for the intense blue color which gives the name to this class of enzymes. All the blue proteins show an intense absorption at *ca.* 610 nm with a molar absorption *ca.* 5000 M^{-1} cm^{-1}. This band originates from a sulfur to metal charge transfer [23]. The other very unusual spectroscopic property of type 1 copper is

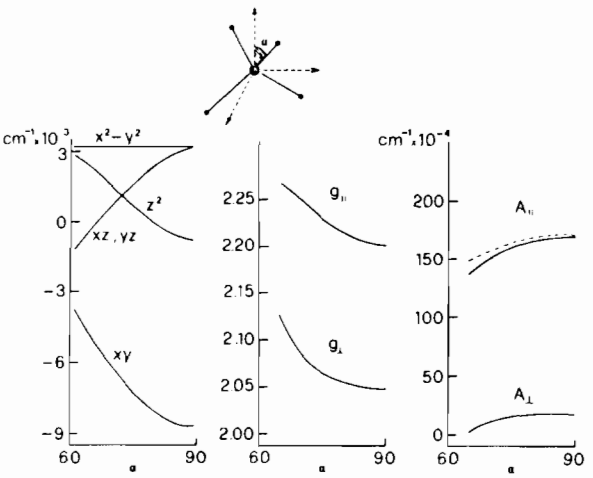


Fig. 5. Dependence of the energy levels, g and A parameters for a Cu(II) ion in D_{2d} symmetry as a function of the deviation from planarity. (From Ref. 25).

represented by the anomalously low values of the hyperfine splitting constant A_Z of the ESR spectra, which can be as low as 30×10^{-4} cm^{-1} [24]. This peculiarity is now well rationalized in terms of a pseudotetrahedral geometry of the copper chromophore, coupled with the presence of heavy atoms among the donors.

Figure 5 and Table V illustrate clearly the effect of a pseudotetrahedral distortion upon the ESR

TABLE VI. ESR Parameters of Two Simple Pseudotetrahedral Copper(II) Complexes.[a]

	$[CuCl_4]^{2-}$	$Cu[Ph_3PO]_2Cl_2$
$g_\parallel$	2.38	2.43
$g_\perp$	2.09	2.08
$A_\parallel$[b]	25	40
$A_\perp$[b]	48.5	25

[a]From Ref. 26. [b]$cm^{-1} \times 10^{-4}$.

parameters of a copper(II) chromophore. For example the same complex bis(N-i-propyl)5-methylsalicylaldiminato copper(II) shows $g_\parallel = 2.22$ and $A_\parallel = 190 \times 10^{-4}$ cm^{-1} when doped into the analogous Pd complex (which has a planar geometry), and $g_\parallel = 2.27$ and $A_\parallel = 113 \times 10^{-4}$ cm^{-1} when doped into the Zn complex where it assumes a pseudotetrahedral geometry [25]. Gatteschi *et al.* [26] pointed out that a further contribution to the reduction of the hyperfine splitting parameter A_z originates from the ligand spin orbit coupling interaction. This contribution is particularly sizeable in the case of heavy atoms, like sulfur, chlorine *etc.* owing to their larger spin orbit coupling constant. Simple inorganic complexes which fulfill the above requirements show A values similar to those of the blue proteins (Table VI).

The resolution of the X-ray structure of plastocyanin [27], an electron-carrier containing one type 1 copper, showing the copper atom coordinated to two nitrogens and two sulfur atoms in a pseudotetrahedral geometry, gave a definitive answer to the several hypotheses on the structure of a type 1 copper center.

Type 1 centers do not interact with anions and therefore are supposed to be located inside the enzyme molecule.

Type 2 Copper

This type of copper shows spectroscopic properties similar to those of simple tetragonal copper complexes, the A_z values being in the usual range $150–200 \times 10^{-4}$ cm^{-1} [28]. This type of copper has the property of interacting with anions like CN^-, N_3^- and especially F^-, the reaction with the fluoride ion being taken as distinctive property of this type of center [29].

Water has been demonstrated to be a ligand of this center through proton relaxation rate measurements [30]. For the other ligands there is more uncertainty; in several cases ESR spectra have shown superhyperfine splitting arising from three or four nitrogen atoms [31]. Type 2 copper has been shown to be essential for the catalytic activity and in some cases there is also evidence that it is alternatively reduced and oxidized during the catalytic cycle [31].

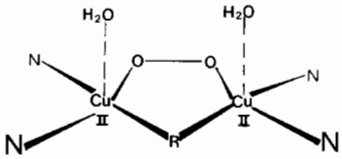


Fig. 6. A proposed structure of the ESR non-detectable copper center in oxyhemocyanin. (From Ref. 34).

Type 3 Copper

Structural information regarding this center is still scanty. This type of copper is ESR silent and diamagnetic, and shows an electronic absorption at 330 nm. After the discovery that this center can accept two electrons in a cooperative manner [32], there is general consensus that this center is represented by pairs of copper(II) ions antiferromagnetically coupled. This suggestion has found further support by recent studies on hemocyanin, a protein which contains ESR non-detectable copper. Solomon suggested for the oxyhemocyanin a structure in which two copper(II) ions are sharing a peroxide group and a ligand of the protein, probably a tyrosine (Fig. 6) [33, 34]. EXAFS studies on hemocyanin indicate a copper–copper distance of 367 pm [35].

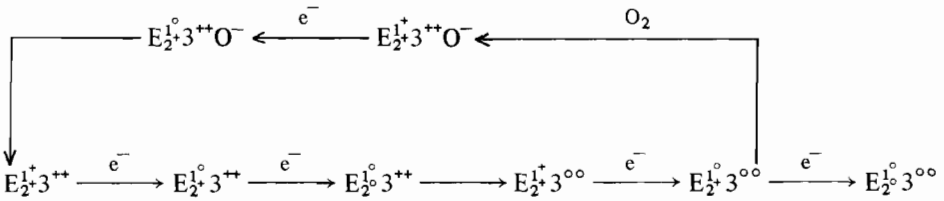
Recently new ESR signals which have been attributed to type 3 coppers have been observed in type 2 copper depleted laccases, or after reoxidation by peroxide of the fully reduced native laccases [36, 37]. It appears therefore that the cooperative two electron reduction of a type 3 center is somewhat altered when the type 2 copper is absent or reduced, in such a way that it is possible to obtain an intermediate state of the type 3 center in which the two copper ions have different oxidation numbers. These ESR spectra are similar to the spectrum of native BSOD, suggesting a five coordinate geometry for the type 3 center also.

The strategy for studying the blue oxidases from a catalytic point of view consists essentially in monitoring spectroscopically the different copper centers in the presence of only one substrate, following the reduction in anaerobic conditions and then studying the reoxidation pattern. Type 1 centers are monitored through the absorption at 610 nm, whereas type 3 copper is followed through the absorption at 330 nm. Type 2 copper can be easily followed through ESR spectroscopy.

The laccases are the best understood among the oxidases and a brief summary of the findings on this class will help to show which are the connections among the different copper centers from the functional point of view.

Anaerobic reduction experiments of Andreasson [38], and Malmström [39] in Polyporus Laccase showed that type 1 copper is reduced by the substrate with a second order reaction. EPR studies coupled with rapid freeze techniques have also shown that type 2 copper is reduced subsequent to the reduction of type 1 copper, and then re-oxidized simultaneously to the reduction of the type 3 center [31]. Type 2 copper therefore plays an important role in transferring electrons from reducible substrates to oxygen. As a matter of fact binding of F^- (or OH^-) to this center prevents reduction of type 3 copper but not that of type 1 [38]. Probably type 2 center is near to type 3 center whereas there are indications that the distance of type 1 copper from both type 2 and type 3 centers is larger than 1 nm [31, 40, 41].

The oxygen molecule is likely to interact as the type 3 center, but the structure of the adduct is still unknown. Reoxidation studies on Polyporus and Rhus laccases have shown the existence of a paramagnetic oxygen intermediate which forms after types 1 and 3 copper have been reoxidized [42]. Relaxation studies suggest that this intermediate is a O^- ion [43]. *In vitro* the reduction of this radical occurs through type 2 copper, but this is a slow process which probably does not have any catalytic causation. It has been suggested therefore that the electron necessary for the reduction of the O^- ion comes from type 1 copper after it is reduced again from the substrate. The following scheme [44], in which the different centers are labelled with + or ∘ depending if they are oxidized or reduced, summarizes the above reported observations:



This scheme shows clearly that all the centers participate in the exchange of the electrons in a chain which connects the substrate to the oxygen molecule. The lack of structural data unfortunately leaves without answer very important questions regarding for example the pathway of the electron transfer between the different copper centers and between the substrate and the type 1 copper, pathways which should also involve the organic part of the molecule.

Progress in structural knowledge will allow us to clarify these points, and will help the coordination chemists to synthetize polynuclear complexes which mimic the structure of the copper centers of the blue oxidases with the aim of obtaining inorganic compounds with catalytic efficiencies similar to the natural systems.

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