

5.3: Copper-zinc Superoxide Dismutase

Background

Two families of metalloproteins are excellent catalysts for the disproportionation of superoxide (Reaction 5.95).



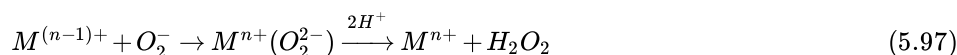
These are (1) the copper-zinc superoxide dismutases, CuZnSOD,¹⁰⁰⁻¹⁰² found in almost all eukaryotic cells and a very few prokaryotes, and (2) the manganese and iron superoxide dismutases, MnSOD and FeSOD, the former found in the mitochondria of eukaryotic cells, and both found in many prokaryotes.¹⁰³ Recent studies of bacterial¹⁰⁴ and yeast¹⁰⁵ mutants that were engineered to contain no superoxide dismutases demonstrated that the cells were unusually sensitive to dioxygen and that the sensitivity to dioxygen was relieved when an SOD gene was reintroduced into the cells. These results indicate that the superoxide dismutase enzymes play a critical role in dioxygen metabolism, but they do not define the chemical agent responsible for dioxygen toxicity (see Section III).

Enzymatic Activity

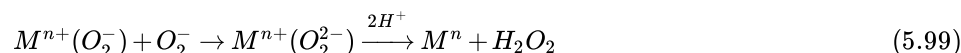
Several transition-metal complexes have been observed to catalyze superoxide disproportionation; in fact, aqueous copper ion, Cu²⁺, is an excellent SOD catalyst, comparable in activity to CuZnSOD itself!³⁷ Free aqueous Cu²⁺ would not itself be suitable for use as an SOD *in vivo*, however, because it is too toxic (see Section III) and because it binds too strongly to a large variety of cellular components and thus would not be present as the free ion. (Most forms of complexed cupric ion show much less superoxide dismutase activity than the free ion.) Aside from aqueous copper ion, few other complexes are as effective as the SOD enzymes.

Two mechanisms (Reactions 5.96 to 5.99) have been proposed for catalysis of superoxide disproportionation by metal complexes and metalloenzymes.³⁷

Mechanism I: $M^{n+} + O_2^- \rightarrow M^{(n-1)+} + O_2$ \tag{5.96}

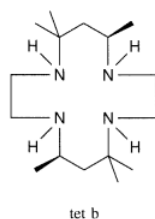


Mechanism II: $M^{n+} + O_2^- \rightarrow M^{n+}(O_2^-)$ \tag{5.98}



In Mechanism I, which is favored for the SOD enzymes and most redox-active metal complexes with SOD activity, superoxide reduces the metal ion in the first step, and then the reduced metal ion is reoxidized by another superoxide, presumably via a metal-peroxo complex intermediate. In Mechanism II, which is proposed for nonredox metal complexes but may be operating in other situations as well, the metal ion is never reduced, but instead forms a superoxo complex, which is reduced to a peroxo complex by a second superoxide ion. In both mechanisms, the peroxo ligands are protonated and dissociate to give hydrogen peroxide.

Analogues for each of the separate steps of Reactions (5.96) to (5.99) have been observed in reactions of superoxide with transition-metal complexes, thereby establishing the feasibility of both mechanisms. For example, superoxide was shown to reduce Cu^{II}(phen)₂²⁺ to give Cu^I(phen)₂⁺ (phen = 1,10-phenanthroline),¹⁰⁶ a reaction analogous to Reaction (5.96). On the other hand, superoxide reacts with Cu^{II}(tet b)₂²⁺ to form a superoxo complex¹⁰⁷ (a reaction analogous to Reaction 5.98), presumably because Cu^{II}(tet b)₂²⁺ is not easily reduced to the cuprous state, because the ligand cannot adjust to the tetrahedral geometry that Cu^I prefers.⁵³



(5.100)

Reaction of superoxide with a reduced metal-ion complex to give oxidation of the complex and release of hydrogen peroxide (analogous to Reaction 5.97) has been observed in the reaction of $\text{Fe}^{\text{II}}\text{EDTA}$ with superoxide.¹⁰⁸ Reduction of a Co^{III} superoxo complex by free superoxide to give a peroxo complex (analogous to Reaction 5.99) has also been observed.¹⁰⁹

If a metal complex can be reduced by superoxide and if its reduced form can be oxidized by superoxide, both at rates competitive with superoxide disproportionation, the complex can probably act as an SOD by Mechanism I. Mechanism II has been proposed to account for the apparent catalysis of superoxide disproportionation by Lewis acidic nonredox-active metal ions under certain conditions.³⁷ However, this mechanism should probably be considered possible for redox metal ions and the SOD enzymes as well. It is difficult to distinguish the two mechanisms for redox-active metal ions and the SOD enzymes unless the reduced form of the catalyst is observed directly as an intermediate in the reaction. So far it has not been possible to observe this intermediate in the SOD enzymes or the metal complexes.

Structure

The x-ray crystal structure of the oxidized form of CuZnSOD from bovine erythrocytes shows a protein consisting of two identical subunits held together almost entirely by hydrophobic interactions.¹⁰⁰⁻¹⁰² Each subunit consists of a flattened cylindrical barrel of β -pleated sheet from which three external loops of irregular structure extend (Figure 5.15). The metal-binding region of the protein binds Cu^{II} and Zn^{II} in close proximity to each other, bridged by the imidazolate ring of a histidyl side chain. Figure 5.16 represents the metal-binding region. The Cu^{II} ion is coordinated to four histidyl imidazoles and a water in a highly distorted square-pyramidal geometry with water at the apical position. The Zn^{II} ion is coordinated to three histidyl imidazoles (including the one shared with copper) and an aspartyl carboxylate group, forming a distorted tetrahedral geometry around the metal ion.

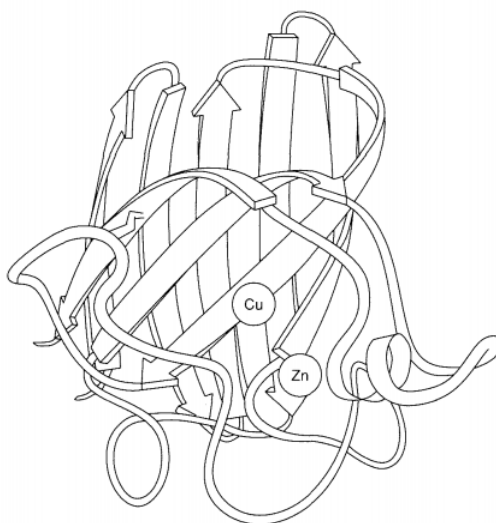


Figure 5.15 - Schematic drawing of the polypeptide backbone of one of the two subunits of bovine CuZnSOD. The strands of the β structure are shown as arrows. The active-site channel provides access to the copper site from the direction of the viewer. (From J. A. Tainer et al., *J. Mol. Biol.* **160** (1982), 181-217.)

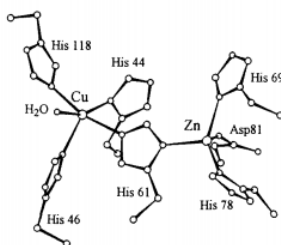


Figure 5.16 - Representation of the metal-binding region of bovine CuZnSOD.

One of the most unusual aspects of the structure of this enzyme is the occurrence of the bridging imidazolate ligand, which holds the copper and zinc ions 6 Å apart. Such a configuration is not unusual for imidazole complexes of metal ions, which sometimes form long polymeric imidazolate-bridged structures.



(5.101)

However, no other imidazolate-bridged bi- or polymetallic metalloprotein has yet been identified.

The role of the zinc ion in CuZnSOD appears to be primarily structural. There is no evidence that water, anions, or other potential ligands can bind to the zinc, so it is highly unlikely that superoxide could interact with that site. Moreover, removal of zinc under conditions where the copper ion remains bound to the copper site does not significantly diminish the SOD activity of the enzyme.¹¹⁰ However, such removal does result in a diminished thermal stability, i.e., the zinc-depleted protein denatures at a lower temperature than the native protein, supporting the hypothesis that the role of the zinc is primarily structural in nature.¹¹¹

The copper site is clearly the site of primary interaction of superoxide with the protein. The x-ray structure shows that the copper ion lies at the bottom of a narrow channel that is large enough to admit only water, small anions, and similarly small ligands (Figure 5.17). In the lining of the channel is the positively charged side chain of an arginine residue, 5 Å away from the copper ion and situated in such a position that it could interact with superoxide and other anions when they bind to copper. Near the mouth of the channel, at the surface of the protein, are two positively charged lysine residues, which are believed to play a role in attracting anions and guiding them into the channel.¹¹² Chemical modification of these lysine or arginine residues substantially diminishes the SOD activity, supporting their role in the mechanism of reaction with superoxide.¹⁰⁰⁻¹⁰²

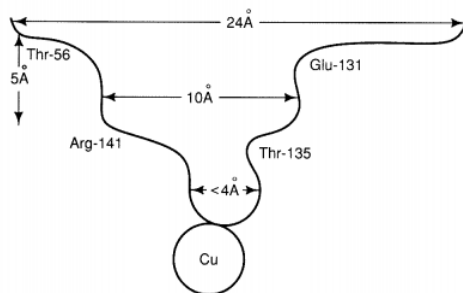


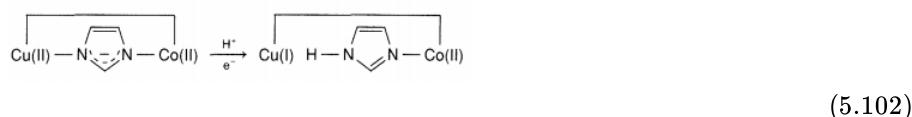
Figure 5.17 - Schematic diagram of a cross section of the active-site channel in CuZnSOD. The diameter of the channel narrows as the Cu^{II} center is approached, and only small ligands can actually reach that site. In addition to the positively charged Cu^{II} ion at the bottom of the channel, the positively charged side chain of Arg-141 is part of the walls of the channel. Two positively charged lysine side chains, not shown in this diagram, are close to the mouth of the channel. (From E. D. Getzoff, R. A. Hallewell, and J. A. Tainer, in M. Inouye, ed., *Protein Engineering: Applications in Science, Industry, and Medicine*, Academic Press, 1986, pp. 41-69.)

The x-ray structural results described above apply only to the oxidized form of the protein, i.e., the form containing Cu^{II} . The reduced form of the enzyme containing Cu^{I} is also stable and fully active as an SOD. If, as is likely, the mechanism of CuZnSOD-catalyzed superoxide disproportionation is Mechanism I (Reactions 5.96-5.97), the structure of the reduced form is of critical importance in understanding the enzymatic mechanism. Unfortunately, that structure is not yet available.

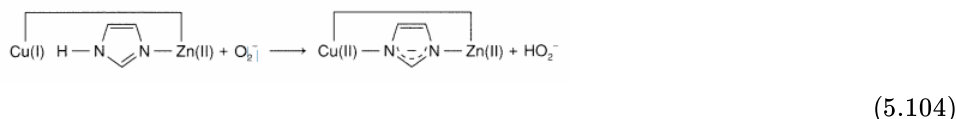
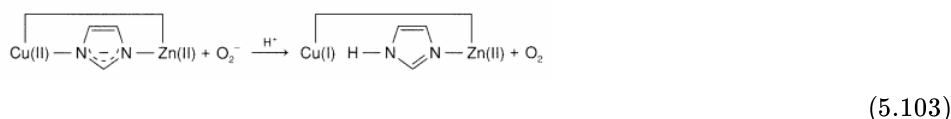
Enzymatic Activity and Mechanism

The mechanism of superoxide disproportionation catalyzed by CuZnSOD is generally believed to go by Mechanism I (Reactions 5.96-5.97), i.e., reduction of Cu^{II} to Cu^{I} by superoxide with the release of dioxygen, followed by reoxidation of Cu^{I} to Cu^{II} by a second superoxide with the release of HO_2^- or H_2O_2 . The protonation of peroxide dianion, O_2^{2-} , prior to its release from the enzyme is required, because peroxide dianion is highly basic and thus too unstable to be released in its unprotonated form. The source of the proton that protonates peroxide in the enzymatic mechanism is the subject of some interest.

Reduction of the oxidized protein has been shown to be accompanied by the uptake of one proton per subunit. That proton is believed to protonate the bridging imidazolate in association with the breaking of the bridge upon reduction of the copper. Derivatives with Co^{II} substituted for Zn^{II} at the native zinc site have been used to follow the process of reduction of the oxidized Cu^{II} form to the reduced Cu^{I} form. The Co^{II} in the zinc site does not change oxidation state, but acts instead as a spectroscopic probe of changes occurring at the native zinc-binding site. Upon reduction (Reaction 5.102), the visible absorption band due to Co^{II} shifts in a manner consistent with a change occurring in the ligand environment of Co^{II} . The resulting spectrum of the derivative containing Cu^{I} in the copper site and Co^{II} in the zinc site is very similar to the spectrum of the derivative in which the copper site is empty and the zinc site contains Co^{II} . This result suggests strongly that the imidazolate bridge is cleaved and protonated and that the resulting imidazole ligand is retained in the coordination sphere of Co^{II} (Reaction 5.102).¹⁰¹



The same proton is thus an attractive possibility for protonation of peroxide as it is formed in the enzymatic mechanism (Reactions 5.103 and 5.104).



Attractive as this picture appears, there are several uncertainties about it. For example, the turnover of the enzyme may be too fast for protonation and deprotonation of the bridging histidine to occur.¹¹³ Moreover, the mechanism proposed would require the presence of a metal ion at the zinc site to hold the imidazole in place and to regulate the pK_a of the proton being transferred. The observation that removal of zinc gives a derivative with almost full SOD activity is thus surprising and may also cast some doubt on this mechanism. Other criticisms of this mechanism have been recently summarized.¹⁰²

Studies of CuZnSOD derivatives prepared by site-directed mutagenesis are also providing interesting results concerning the SOD mechanism. For example, it has been shown that mutagenized derivatives of human CuZnSOD with major differences in copper-site geometry relative to the wild-type enzyme may nonetheless remain fully active.¹¹⁴ Studies of these and similar derivatives should provide considerable insight into the mechanism of reaction of CuZnSOD with superoxide.

Anions as Inhibitors

Studies of the interaction of CuZnSOD and its metal-substituted derivatives with anions have been useful in predicting the behavior of the protein in its reactions with its substrate, the superoxide anion, O_2^- .^{101,102} Cyanide, azide, cyanate, and thiocyanate bind to the copper ion, causing dissociation of a histidyl ligand and the water ligand from the copper.¹¹⁵ Phosphate also binds to the enzyme at a position close to the Cu^{II} center, but it apparently does not bind directly to it as a ligand. Chemical modification of Arg-141 with phenylglyoxal blocks the interaction of phosphate with the enzyme, suggesting that this positively charged residue is the site of interaction with phosphate.¹¹⁶

Electrostatic calculations of the charges on the CuZnSOD protein suggest that superoxide and other anions entering into the vicinity of the protein will be drawn toward and into the channel leading down to the copper site by the distribution of positive charges on the surface of the protein, the positively charged lysines at the mouth of the active-site cavity, and the positively charged

arginine and copper ion within the active-site region.¹¹² Some of the anions studied, e.g., CN^- , F^- , N_3^- , and phosphate, have been shown to inhibit the SOD activity of the enzyme. The source of the inhibition is generally assumed to be competition with superoxide for binding to the copper, but it may sometimes result from a shift in the redox potential of copper, which is known to occur sometimes when an anion binds to copper.^{100,101}

Metal-ion Substitutions

1. SOD Activity

In the example described above, studies of a metal-substituted derivative helped in the evaluation of mechanistic possibilities for the enzymatic reaction. In addition, studies of such derivatives have provided useful information about the environment of the metal-ion binding sites. For example, metal-ion-substituted derivatives of CuZnSOD have been prepared with Cu^{II} , Cu^{I} , Zn^{II} , Ag^{I} , Ni^{II} , or Co^{II} bound to the native copper site, and with Zn^{II} , Cu^{II} , Cu^{I} , Co^{II} , Hg^{II} , Cd^{II} , Ni^{II} , or Ag^{I} bound to the native zinc site.^{100,101,117} The SOD activities of these derivatives are interesting; only those derivatives with copper in the copper site have a high degree of SOD activity, whereas the nature of the metal ion in the zinc site or even its absence has little or no effect.^{100,101}

2. Spectroscopy

Derivatives of CuZnSOD are known with Cu^{II} ion bound either to the native copper site or to the native zinc site. The electronic absorption spectra of these derivatives indicate that the ligand environments of the two sites are very different. Copper(II) is a d^9 transition-metal ion, and its d-d transitions are usually found in the visible and near-IR regions of the spectrum.⁵³ Copper(II) complexes with coordinated nitrogen ligands are generally found to have an absorption band between 500 and 700 nm, with an extinction coefficient below $100 \text{ M}^{-1}\text{cm}^{-1}$. Bands in the absorption spectra of complexes with geometries that are distorted away from square planar tend to be red-shifted because of a smaller d-d splitting, and to have higher extinction coefficients because of the loss of centrosymmetry. Thus the optical spectrum of CuZnSOD with an absorption band with a maximum at 680 nm ($14,700 \text{ cm}^{-1}$; see Figure 5.18A) and an extinction coefficient of $155 \text{ M}^{-1}\text{cm}^{-1}$ per Cu is consistent with the crystal structural results that indicate that copper(II) is bound to four imidazole nitrogens and a water molecule in a distorted square-pyramidal geometry. Metal-substituted derivatives with Cu^{II} at the native copper site but with Co^{II} , Cd^{II} , Hg^{II} , or Ni^{II} substituted for Zn^{II} at the native zinc site all have a band at 680 nm, suggesting that the substitution of another metal ion for zinc perturbs the copper site very little, despite the proximity of the two metal sites. The absorption spectra of native CuZnSOD and these CuMSOD derivatives also have a shoulder at 417 nm ($24,000 \text{ cm}^{-1}$; see Figure 5.18A), which is at lower energy than normal imidazole-to- Cu^{II} charge-transfer transitions, and has been assigned to an imidazolate-to- Cu^{II} charge transfer, indicating that the imidazolate bridge between Cu^{II} and the metal ion in the native zinc site is present, as observed in the crystal structure of CuZnSOD. Derivatives with the zinc site empty, which therefore cannot have an imidazolate bridge, are lacking this 417 nm shoulder.

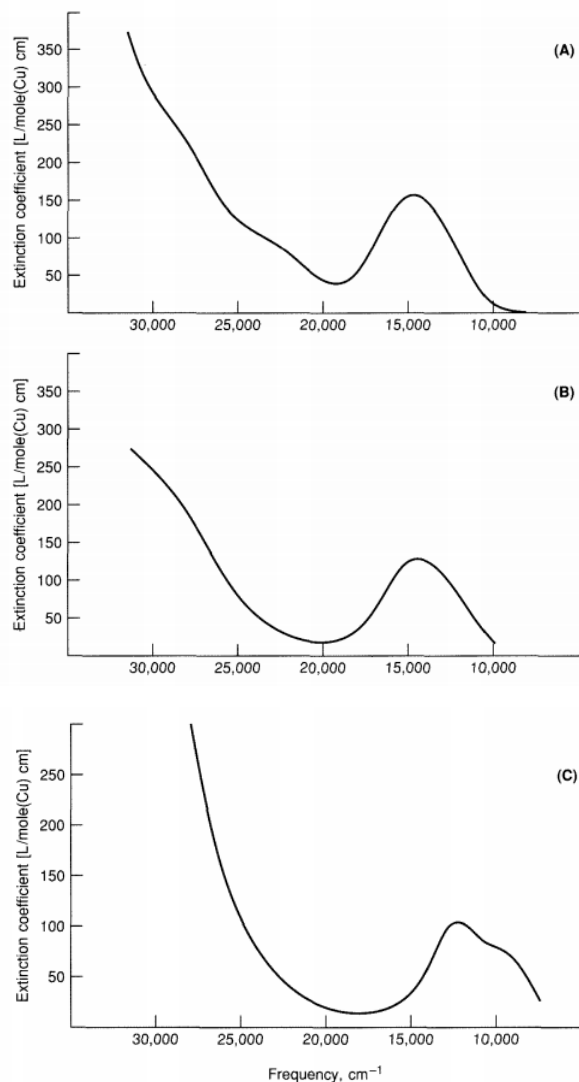


Figure 5.18 - Vis-UV spectra of (A) CuZnSOD, (B) copper-only SOD (zinc site empty), and (C) Ag^ICuSOD. In all three spectra, the low-energy band is the Cu^{II} d-d transition. Note that the spectrum of CuZnSOD contains a shoulder at 24,000 cm⁻¹ (417 nm) that is assigned to the imidazolate-to-copper(II) charge-transfer transition. This shoulder is not present in the copper-only derivative. For Ag^ICuSOD, the d-d transition is red-shifted because of the change in the ligand geometry when Cu^{II} is moved from the copper site to the zinc site (see text), (From M. W. Pantoliano, L. A. Nafie, and J. S. Valentine, *J. Am. Chem. Soc.* **104** (1982), 6310-6317.)

Small but significant changes in the absorption spectrum are seen when the metal ion is removed from the zinc site, e.g., in copper-only SOD (Figure 5.18B). The visible absorption band shifts to 700 nm (14,300 cm⁻¹), presumably due to a change in ligand field strength upon protonation of the bridging imidazolate. In addition, the shoulder at 417 nm has disappeared, again due to the absence of the imidazolate ligand.

The spectroscopic properties due to copper in the native zinc site are best observed in the derivative Ag^ICuSOD, which has Ag^I in the copper site and Cu^{II} in the zinc site (see Figure 5.18C), since the d¹⁰ Ag^I ion is spectroscopically silent. In this derivative, the d-d transition is markedly red-shifted from the visible region of the spectrum into the near-IR, indicating that the ligand environment of Cu^{II} in that site is either tetrahedral or five coordinate. The EPR properties of Cu^{II} in this derivative are particularly interesting (as discussed below).

The derivative with Cu^{II} bound at both sites, CuCuSOD, has a visible-near IR spectrum that is nearly a superposition of the spectra of CuZnSOD and Ag^ICuSOD (see Figure 5.19), indicating that the geometry of Cu^{II} in each of these sites is little affected by the nature of the metal ion in the other site.

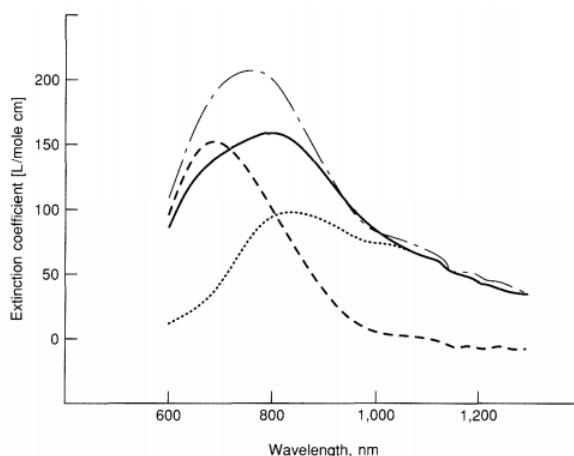


Figure 5.19 - Comparison of the visible absorption spectrum of CuCuSOD, -----, with that of CuZnSOD, ———, and of Ag^ICuSOD, A digital addition of the spectra of CuZnSOD and of Ag^ICuSOD generated the other spectrum, -.-.-.-. Note that the spectrum of CuCuSOD, which has Cu^{II} ions in both the copper and the zinc sites, closely resembles a superposition of the spectra of CuZnSOD, which has Cu^{II} in the copper site, and Ag^ICuSOD, which has Cu^{II} in the zinc site. (From M. W. Pantoliano, L. A. Nafie, and J. S. Valentine, *J. Am. Chem. Soc.* **104** (1982), 6310-6317.)

EPR spectroscopy has also proven to be particularly valuable in characterizing the metal environments in CuZnSOD and derivatives. The EPR spectrum of native CuZnSOD is shown in Figure 5.20A. The g_{\parallel} resonance is split by the hyperfine coupling between the unpaired electron on Cu^{II} and the $I = \frac{3}{2}$ nuclear spin of copper. The A_{\parallel} value, 130 G, is intermediate between the larger A_{\parallel} typical of square-planar Cu^{II} complexes with four nitrogen donor ligands and the lower A_{\parallel} observed in blue copper proteins (see Chapter 6). The large linewidth seen in the g_{\perp} region indicates that the copper ion is in a rhombic (i.e., distorted) environment. Thus, the EPR spectrum is entirely consistent with the distorted square-pyramidal geometry observed in the x-ray structure.

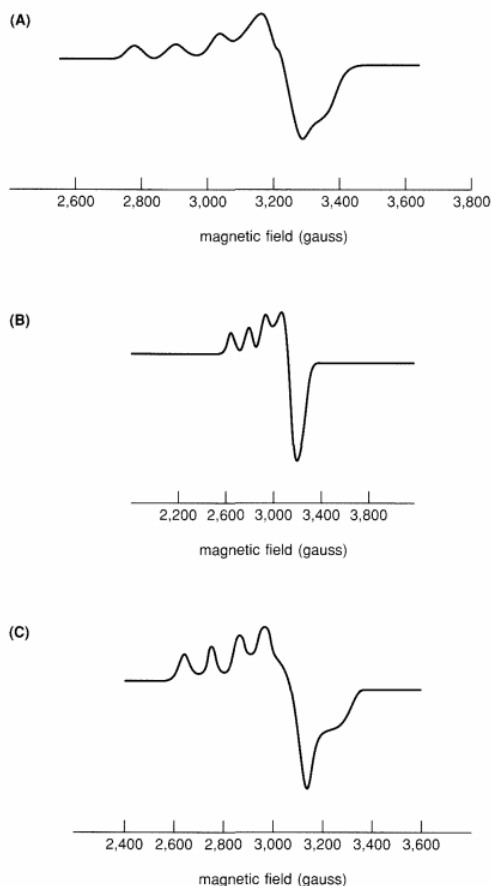


Figure 5.20 - Frozen solution EPR spectra of (A) CuZnSOD, (B) copper-only SOD (zinc site empty), and (C) Ag^ICuSOD. See text for discussion. (Adapted from References 100 and 101.)

Removal of zinc from the native protein to give copper-only SOD results in a perturbed EPR spectrum, with a narrower g_{\perp} resonance and a larger A_{\parallel} value (142 G) more nearly typical of Cu^{II} in an axial N_4 environment (Figure 5.20B). Apparently the removal of zinc relaxes some constraints imposed on the geometry of the active-site ligands, allowing the copper to adopt to a geometry closer to its preferred tetragonal arrangement.

The EPR spectrum due to Cu^{II} in the native Zn^{II} site in the $\text{Ag}^{\text{I}}\text{CuSOD}$ derivative indicates that Cu^{II} is in a very different environment than when it is in the native copper site (Figure 5.20C). The spectrum is strongly rhombic, with a low value of A_{\parallel} (97 G), supporting the conclusion based on the visible spectrum that copper is bound in a tetrahedral or five-coordinate environment. This type of site is unusual either for copper coordination complexes or for copper proteins in general, but does resemble the Cu^{II} EPR signal seen when either laccase or cytochrome c oxidase is partially reduced (see Figure 5.21).

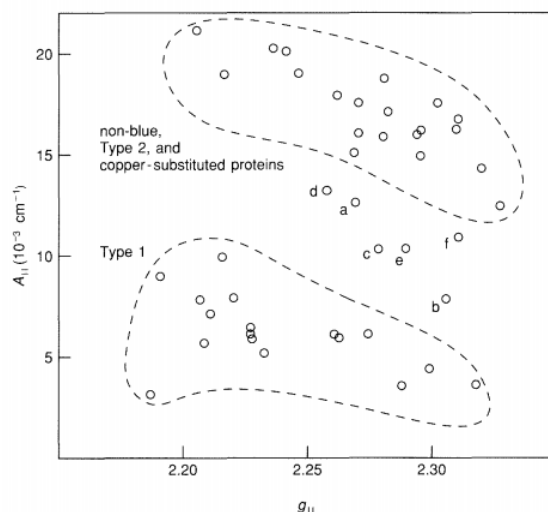


Figure 5.21 - Relationship between g_{\parallel} and A_{\parallel} for naturally occurring copper proteins and copper-substituted metalloproteins. Points labeled a-f are for laccases (a and b), cytochrome c oxidase (c), CuZnSOD (d), $\text{Ag}^{\text{I}}\text{CuSOD}$ (e), and $\text{Cu}^{\text{I}}\text{Cu}^{\text{II}}\text{SOD}$ (f). See text for discussion. (From M. W. Pantoliana, L. A. Nafie, and J. S. Valentine, *J. Am. Chem. Soc.* **104** (1982), 6310-6317.)

Partial reduction disrupts the magnetic coupling between these Cu^{II} centers that makes them EPR-silent in the fully oxidized protein.

The EPR spectrum of CuCuSOD is very different from that of any of the other copper-containing derivatives (Figure 5.22) because the unpaired spins on the two copper centers interact and magnetically couple across the imidazolate bridge, resulting in a triplet EPR spectrum. This spectrum is virtually identical with that of model imidazolate-bridged binuclear copper complexes.¹⁰¹

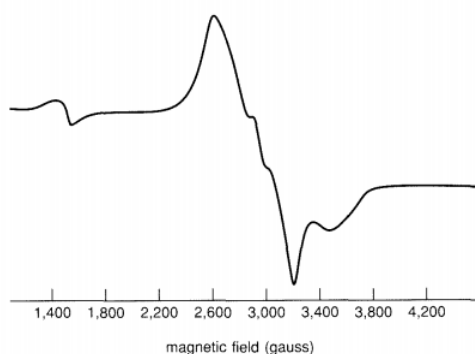


Figure 5.20. These differences are due to the fact that the two Cu^{II} centers are magnetically coupled across the imidazolate bridge.¹⁰⁰

Electronic absorption and EPR studies of derivatives of CuZnSOD containing Cu^{II} have provided useful information concerning the nature of the metal binding sites of those derivatives. ^1H NMR spectra of those derivatives are generally not useful, however, because the relatively slowly relaxing paramagnetic Cu^{II} center causes the nearby proton resonances to be extremely broad. This difficulty has been overcome in two derivatives, CuCoSOD and CuNiSOD , in which the fast-relaxing paramagnetic Co^{II} and Ni^{II} centers at the zinc site interact across the imidazolate bridge and increase the relaxation rate of the Cu^{II} center, such that well-

resolved paramagnetically shifted ^1H NMR spectra of the region of the proteins near the two paramagnetic metal centers in the protein can be obtained and the resonances assigned.^{118,119}

The use of ^1H NMR to study CuCoSOD derivatives of CuZnSOD in combination with electronic absorption and EPR spectroscopies has enabled investigators to compare active-site structures of a variety of wild-type and mutant CuZnSOD proteins in order to find out if large changes in active-site structure have resulted from replacement of nearby amino-acid residues.¹²⁰

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