Zn in Superoxide Dismutase: An Enigmatic Metal

While essential to aerobic life, oxygen poses a great risk to organisms in the form of superoxide, $O_2^-$. This radical attacks fatty acids to form more radicals and damage tissue in the body.\(^1\) Reactive oxygen species like superoxide in the cell result in oxidative stress. Fortunately, the protein superoxide dismutase catalyzes the reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

eliminating the danger of the oxygen radical. The rate of this reaction is diffusion limited, up to $10^{-9}$ M$^{-1}$ s$^{-1}$.\(^2\)

Several types of superoxide dismutase (SOD) exist.\(^3\) Fe and Mn SOD are both single metal active sites coordinated by an Asp, three histidines, and water. The Fe is found mainly in prokaryotes, and the Mn is found in both prokaryotes and eukaryotes. Ni SOD, found in cyanobacteria and some Streptomyces species, was only recently found to contain an active site with three cysteines and a histidine coordinated.\(^4\)

While all the SODs are interesting, for our purposes, the Cu/Zn SOD is particularly interesting for several reasons, including its dual metal center. In this active site, the Cu(II) and Zn(II) are coordinated by the same histidine. Additionally, the Cu is bound by a solvent molecule and three other histidines, and the Zn is coordinated to two other histidines and an Asp. Once the Cu is reduced, the shared His ligand is released and the Cu moves about 1 Å to react with superoxide.\(^5\)

The Zn does not participate in the mechanism for the disproportionation of superoxide, and it is believed it undergoes no redox reaction. The Zn has been shown to be important for the stabilization of the protein, but the delivery mechanism remains unknown. Zn(II) is a d$^{10}$ molecule, and as such, very little probing of the Zn site can be done. The Cu, however, undergoes an oxidation state change and can be examined more easily. Due to the difficulty of probing Zn and the convenience of a second metal site (Cu), studies to determine the role of Zn are often paired with Cu, or the Zn is replaced with the typically paramagnetic Co (d$^7$). Here, what little is known about the Zn is put forth, as well as a discussion of the better understood Cu.

Methods of Analysis and Results

Circular Dichroism

Light can be circularly polarized by a rotating electric field.\(^5\) The rotation of this light (either left or right) can be absorbed differently by a molecule, and the resulting difference provides spectra like that discussed in this paper. A Cary 61 spectropolarimeter at ambient temperature or a JobinYvon CD6 Dichrograph and a π*-180 spectrophotometer at 23°C were used for the following section.\(^6,7\)

While Zn may be difficult to probe, it is possible to examine spectra with and without that metal center present. Consider Figure 1a and 1b, which show CD spectra for Cu/Zn SOD and Cu/Zn-free SOD. Without the Zn the shoulder at 24000 cm$^{-1}$ disappears and the intensity of the 17,000 cm$^{-1}$ diminishes considerably.\(^7\)
Apo and holo SOD were compared under cysteine reducing conditions in increasing concentrations of guanidinium chloride (GdmCl), a chaotropic agent. This is seen in Figure 2(upper), where the holo form requires twice as much GdmCl to denature. Figure 2 also shows the difference between two preparation methods of holo SOD, these in an environment promoting cysteine disulfide bonds. The upper is shown at 216 nm, the lower at 230 nm, both in the range of secondary protein structure.6

**Electron Paramagnetic Resonance**

Here, EPR was used to observe Cu(I) reconstitution into the SOD active site. A Bruker ESP-300 X-band instrument at 100K was used, operating at 9.42 GHz.8 Figure 3a shows what Cu-free/Zn SOD looks like in EPR, but due to the zinc d₁₀ state, nothing useful can be seen.8 For this series of experiments, the EPR signal came about as Cu(I) complexed with glutathione (GSH) and was reconstituted. The Cu(I), in this case, doesn’t appear immediately in the EPR spectra either, because like the zinc, it is diamagnetic. Only after it is incorporated into the active site and oxidized to Cu(II) does it become paramagnetic and the signal appears.
Nuclear Magnetic Resonance

NMR aligns magnetic nuclei and perturbs them in order to probe structure. For the data presented here, $^1$H NMR was used at 400 MHz with a Bruker AM-400 spectrometer. For superoxide dismutase, NMR is another method of examining the SOD active site by replacing the zinc with a cobalt atom. The Co has a short relaxation time, preventing the protons of the bound histidine from broadening – instead they are enhanced by isotropic shifts.\(^8\) Figure 4 shows what the Cu-free/Co SOD looks like, and how that changes with the addition of the Cu(I) \cdot GSH complex. This was done aerobically, similar to the EPR mentioned above, so that the Cu could be oxidized, which helps in metal reconstitution.

Discussion and Analysis

Circular Dichroism

According to Pantoliano, the broad peak at 16,400 cm\(^{-1}\) (a positive Cotton effect) and the low point at 13,000 cm\(^{-1}\) have been assigned a $d \rightarrow d$ transition. Based on the data provided, the difference between the left and right circularly polarized light ($\Delta \varepsilon$ or $\varepsilon_l - \varepsilon_r$) at 16,400 cm\(^{-1}\) (or 610 nm) is about 0.75 (M Cu)\(^{-1}\) cm\(^{-1}\). This is with the Cu and the Zn coordinated normally.

When the Zn has been removed from superoxide dismutase, the spectra in Figure 1b results. The shoulder at 26,000 cm\(^{-1}\) is lost, and the $\Delta \varepsilon$ decreases dramatically. This is where the histidine coordinating the Cu and the Zn comes into play – the copper fluctuates between being attached to the histidine depending on where it is in the actual disproportionation of superoxide. The Zn, however, maintains its configuration the whole time. When it isn’t there to stabilize the histidine, the ability of the copper to bind normally would be affected, and its geometry could go from a typical Cu(II) distorted square pyramidal to a Cu(II) trigonal planar, similar to its reduced conformation. This explains the decrease in intensity.

CD was also used to analyze the stability of holo versus apo forms of superoxide dismutase. For this experiment the data collection was fairly simple. CD was used to determine the $\theta$ value at a fixed wavelength and known concentrations of GdmCl, where\(^6\)

$$\theta = \frac{\Delta \varepsilon \lambda - \sigma r}{2.303(4500 / \pi)}$$

Not surprisingly, the holo form was more stable than apo form. Both forms were denatured eventually, but they were in a cysteine reduced environment where disulfide bonds were prevented from forming.

EPR

The coordination environment of the zinc does not change, and so is constantly Zn(II), which is the spectroscopically silent $d^{10}$. This is the heart of the difficulty in studying Zn in superoxide dismutase. The Cu-free/Zn SOD is shown, but more to
confirm that there is nothing to look at. What the EPR does confirm is reduced Cu can be added to the copper deficient SOD with the help of GSH. This is confirmed by Figure 3d, which looks fairly typical for a Cu EPR spectra. The Cu is a Cu(II), d⁹, with s = 1/2. Additionally, the Cu has nuclear spin, which can couple with electron spin. It is distinguished by a g⊥ first derivative peak and four g‖ peaks. The nuclear spin (I) = 3/2. For I, there is a superhyperfine splitting of 2I+1, which in this case corresponds to the 4 g‖ peaks.

NMR

There was little change in the spectra in Figure 4, but enough to observe the loss of a peak, which Ciriolo speculates is due to rapid proton exchange between the histidine protons and the solvent. Three histidines coordinate at the Co site, but it is difficult to assign the peaks from ¹H NMR due to the protein environment. There are conceivably three hydrogens from each histidine that could be represented by the NMR. The two non-Cu coordinating His protons could have identical spectra, but it is difficult to say without looking in depth at the second coordination sphere. The substitution of Zn with Co is an interesting tool to use in clarifying the activity of the zinc site.

Conclusion

The Zn center of superoxide dismutase is difficult to characterize, but having the Cu center nearby allows for a large amount of research. Zn at d¹⁰ and Cu at d⁹ may only have one electron difference, but that lack of an electron opens doors to a variety of spectroscopy. Currently, Cu/Zn SOD generates interest due to its role in Amyotrophic lateral sclerosis, usually known as ALS or Lou Gehrig’s disease. The specific cause of this neurodegenerative disease is not entirely known, but mutations of SOD are known to be involved. It has been shown that zinc deficient SOD rapidly results in cell toxicity. While Zn is not the only metal that can be in that coordination site, it is the optimal metal for the protein.
References