Biochemistry **1981**, *20*, 7488–7493

Anion Binding to the Four-Copper Form of Bovine Erythrocyte Superoxide Dismutase: Mechanistic Implications[†]

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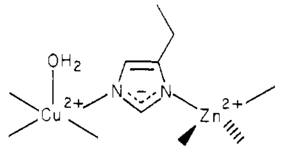
ABSTRACT: The binding of thiocyanate, azide, and other anions to superoxide dismutase (SOD) containing copper in both the copper and zinc sites of the native enzyme (Cu_2Cu_2SOD) has been studied. Electron spin resonance spectroscopy was used to show that binding of SCN^- to Cu_2Cu_2SOD breaks the imidazolate bridge between the two copper centers. Thiocyanate displaces the bridging histidine from the Cu^{2+} ion in the copper site and also replaces the aspartic acid ligand from the Cu^{2+} ion in the zinc site. This conclusion is supported by studies with Ag_2Cu_2SOD, where silver is in the native copper site and copper is in the native zinc site. At low concentrations, N_3^- ion displaces axially coordinated water from Cu_2Cu_2SOD. At higher concentrations, N_3^- also breaks the imidazolate bridge. Parallel behavior was observed for SCN^- and N_3^- binding to a model compound for Cu_2Cu_2SOD, showing that the difference between the two anions is a consequence of their copper binding properties and is not due to secondary interactions with the protein active site. The SCN^- complex of Cu_2Cu_2SOD is catalytically active, indicating that the intact imidazolate bridge is not essential to the mechanism of superoxide dismutase action. Phosphate ion breaks the histidine bridge in Cu_2Cu_2SOD, whereas cyanate, formate, and fluoride ions do not. At about pH 11, hydroxide ion promotes the irreversible formation of a copper complex of deprotonated peptide nitrogen atoms for Cu_2Cu_2SOD but not for Cu_2Zn_2SOD in the same pH range.

The copper–zinc superoxide dismutase (SOD)[1] found in the cytosol of eukaryotic cells (Cu_2Zn_2SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) has been studied extensively (Fridovich, 1975; Lippard et al., 1977; Valentine & Pantoliano, 1981). The enzyme from bovine erythrocytes is composed of two identical subunits, each of which contains one copper(II) and one zinc(II) ion. The X-ray structure shows the two metal ions to be separated by about 6 Å (Richardson et al., 1975). The copper is coordinated to four histidines, one of which it shares with the zinc ion. The zinc is also bonded to two additional histidines and an aspartic acid. The geometry around the copper is roughly square planar while the zinc is at the center of a distorted tetrahedron. The copper is also believed to have a coordinated water molecule (Gaber et al., 1972; Boden et al., 1979; Tainer et al., 1980).



Various derivatives of the protein have been prepared in which other metals are substituted for copper and/or zinc. One of these contains copper in both metal binding sites, Cu_2Cu_2SOD, and shows a moderately strong antiferromagnetic interaction with $-J \sim 26$ cm^{-1} (Fee & Briggs, 1975). The same dicopper(II) active site is formed reversibly when the pH of the zinc-free protein, Cu_2E_2SOD, is raised above ~ 8 (Valentine et al., 1979). Low-temperature ESR spectra of the imidazolate-bridged dicopper(II) form of SOD differ dramatically from spectra of forms of the enzyme having magnetically isolated copper ions, making it easy to determine whether both copper ions are bound to the bridging histidine ligand.

Spectroscopic and kinetic studies of anion complexes of a metalloenzyme can provide information about the environment of the metal ions and the catalytic mechanism. Because the potential sixth coordination site on copper in SOD is blocked by the protein folding, anion binding must involve displacement of H_2O or one of the histidine ligands. Much of the work on anion binding to SOD has focused on this point. Since the substrate for SOD is itself an anion, an understanding of how anions interact with the copper center may shed light on the nature of the initial complex of superoxide with the enzyme and, ultimately, the enzyme mechanism.

Several investigations have reported the effects of anion binding to Cu_2Zn_2SOD. Azide, cyanide, and halides bind to the copper site (Bertini et al., 1980; Rotilio et al., 1972; Fee & Gaber, 1972; Haffner & Coleman, 1973) and are competitive inhibitors (Rigo et al., 1977). NMR proton relaxation measurements indicate that these anions displace the coordinated water molecule. Electron spin-echo spectroscopic studies have shown that the bridging imidazolate group remains bonded to the copper of Cu_2Zn_2SOD when azide and cyanide are bound (Fee et al., 1981). In contrast, thiocyanate binds to the copper in SOD without affecting the water relaxation rate (Fee & Gaber, 1972; Bertini et al., 1980) and without inhibiting the enzyme (Rigo et al., 1977). Thiocyanate is therefore thought to displace a histidine ligand from the copper ions. If the histidine displaced by thiocyanate were the bridging imidazolate, it would be of considerable interest since breaking of the imidazolate bridge between Cu and Zn is a key feature of a proposed mechanism for SOD (Hodgson & Fridovich, 1975; Lippard et al., 1977).

ESR studies of the interaction of anions with Cu_2Cu_2SOD (Fee & Briggs, 1975) should indicate clearly whether the

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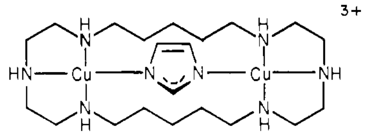
[1] Abbreviations: SOD, superoxide dismutase; Cu_2Zn_2SOD, native superoxide dismutase having copper and zinc in their normal binding sites (other derivatives of SOD are indicated as X_2Y_2SOD, meaning metal X is in the normal copper sites and metal Y is in the normal zinc sites; when Y = E, the zinc sites are empty); A′, 1,4,7,13,16,19-hexaazacyclotetracosan; im, imidazolate; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-7488$01.25/0

bridging ligand is displaced, since the magnetic interactions between the copper ions would be eliminated. We have carried out such experiments on Cu_2Cu_2SOD and a model compound, $[Cu_2(im)\subset A'](ClO_4)_3\cdot H_2O$ (Coughlin et al., 1980), having properties analogous to those of the active site of Cu_2Cu_2SOD.



$[Cu_2(im)CA']^{3+}$

Materials and Methods

SOD from bovine erythrocytes was isolated as previously described (McCord & Fridovich, 1969) and stored as a lyophilized powder at −20 °C after exhaustive dialysis against distilled, deionized water. The protein concentration of Cu_2Zn_2SOD was determined from its absorbance at 258 nm by using a molar extinction coefficient of 1.03×10^4 (McCord & Fridovich, 1969). Protein concentrations of other derivatives of SOD were measured either by dye binding (Bradford, 1976) or by microbiuret (Itzhaki & Gill, 1964) methods, using Cu_2Zn_2SOD as the standard.

All metal ion concentrations were determined by atomic absorption spectroscopy using a Varian AA-375 instrument. The average metal content of Cu_2Zn_2SOD for three preparations was 1.03 ± 0.02 Cu ions and 0.92 ± 0.03 Zn ions per subunit. Literature procedures were followed to prepare Cu_2E_2SOD, Cu_2Cu_2SOD (Fee & Briggs, 1975; Valentine et al., 1979), and Ag_2Cu_2SOD (Beem et al., 1977). The compound $[Cu_2(im)\subset A'](ClO_4)_3\cdot H_2O$ was synthesized as previously described (Coughlin et al., 1980). $KSC^{15}N$ was prepared from $KC^{15}N$ and sulfur (Chang & Ulbricht, 1958) and assayed for purity by Ag^+ and I_2 titrations (Rosin, 1961). It was determined to be free of cyanide and sulfides.

$KC^{15}N$ (99% ^{15}N) was obtained from Stohler Isotope Chemicals. Chelex 100 was a product of Bio-Rad Laboratories. All solutions were prepared with distilled, deionized water.

ESR spectra were obtained on a Varian E-line X-band spectrometer equipped with a temperature controller. All spectra were recorded at 140 K, and Mn(II), naturally present as an impurity in strontium oxide, was used to calibrate the magnetic field (Bolton et al., 1972). Anaerobic experiments were carried out by using ESR tubes previously described (O'Young & Lippard, 1980). Double integration of ESR spectra employed a Digital Equipment Corp. MINC system interfaced to the spectrometer (O'Young, 1980), using Cu_2Zn_2SOD as a standard. ESR parameters were calculated as previously described (Malmström & Vänngård, 1960). Ultraviolet and visible spectra were recorded on a Cary 118C spectrophotometer.

Enzymatic activity was measured at 24 °C with the 6-hydroxydopamine assay (Heikkila & Cabbat, 1976) in 0.1 M 2-picoline–$HClO_4$ and 0.5 mM EDTA, pH 6.0, containing 125 mM KSCN, NaN_3, or $NaClO_4$. The activity in the presence of $NaClO_4$ was used as a reference in comparing the activity of SOD in KSCN or NaN_3 solution.

Results

Thiocyanate Binding to Cu_2Cu_2SOD. Figure 1 shows the ESR spectra of Cu_2Cu_2SOD in H_2O at pH 5.7 before and after the addition of KSCN. The spectrum of Cu_2Cu_2SOD is characteristic of antiferromagnetically coupled binuclear copper complexes (Smith & Pilbrow, 1974). It consists of a broad absorption in the region 2000–4000 G and a weaker signal at 1200–1700 G, which arises from the $\Delta M_S = \pm 2$ transition. On addition of SCN^-, the ESR spectrum changes to that typical of a mononuclear copper complex having axial symmetry. This result clearly indicates the absence of a magnetic interaction between the copper ions in the SCN^- complex. ESR spectral parameters for this and all other protein samples studied in this investigation are given in Table I. The value of $A_{\parallel}$ for the SCN^- complex of Cu_2Cu_2SOD is considerably larger than that of the native enzyme, indicating a more axially symmetric geometry around the copper in the presence of SCN^-.

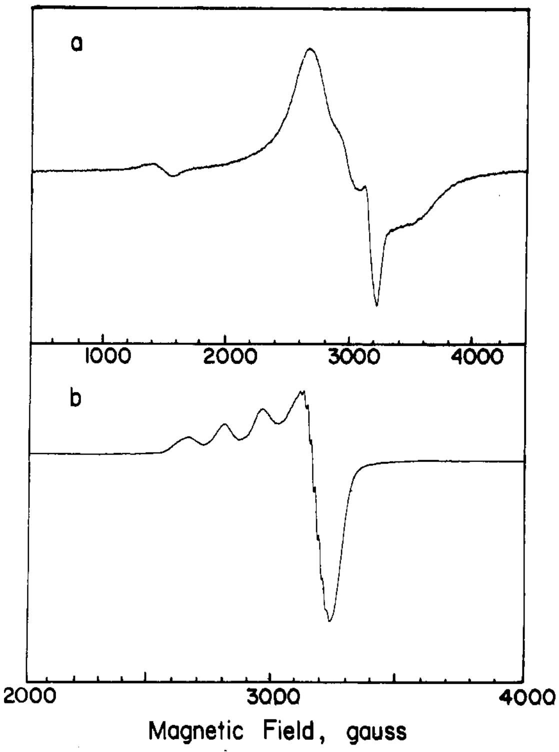


FIGURE 1: ESR spectrum of 1.3 mM Cu_2Cu_2SOD in H_2O, pH 5.7, at 140 K (a). The spectrum was recorded with 100-kHz field modulation at a modulation amplitude of 10 G. The microwave power was 50 mW at a frequency of 9.12 GHz, and the gain was 1250. Addition of 235 mM KSCN to Cu_2Cu_2SOD gave the spectrum shown in (b). Spectrometer conditions were the same as in (a) except that the modulation amplitude was 5 G and the gain was 250.

Table I: ESR Spectral Parameters of SOD Derivatives

protein sample	$g_{\perp}$ (or g_m)	$g_{\parallel}$	$A_{\parallel}$ (cm^{-1})
Cu_2Zn_2SOD	2.080	2.262	0.0135
$Cu_2Zn_2SOD\text{-}SC^{14}N^-$ complex	2.065	2.259	0.0155
$Cu_2Cu_2SOD\text{-}SC^{14}N^-$ complex	2.065	2.262	0.0155
$Cu_2Cu_2SOD\text{-}SC^{15}N^-$ complex	2.067	2.266	0.0156
Ag_2Cu_2SOD	2.120	2.311	0.0105
$Ag_2Cu_2SOD\text{-}SC^{14}N^-$ complex	2.065	2.260	0.0154
$Cu_2Cu_2SOD\text{-}N_3^-$ complex	2.063	2.254	0.0142
Cu_2Cu_2SOD, pH 12.0	2.046	2.176	0.0200
Cu_2Zn_2SOD, pH 12.3	2.053	2.244	0.0154

The spectrum of the SCN^- complex of Cu_2Cu_2SOD is unusual in that both copper ions appear to be in virtually identical environments. There is no evidence of two overlapping signals from chemically different copper ions as might have been expected, since the copper and zinc binding sites of SOD have different ligands and geometries. This result could be explained if only half of the copper were contributing to the ESR spectrum or if copper had been removed from the protein. The following experiments show that neither of these explanations is correct. Double integration of the ESR signal of two samples of the SCN^- complex of Cu_2Cu_2SOD and comparison with

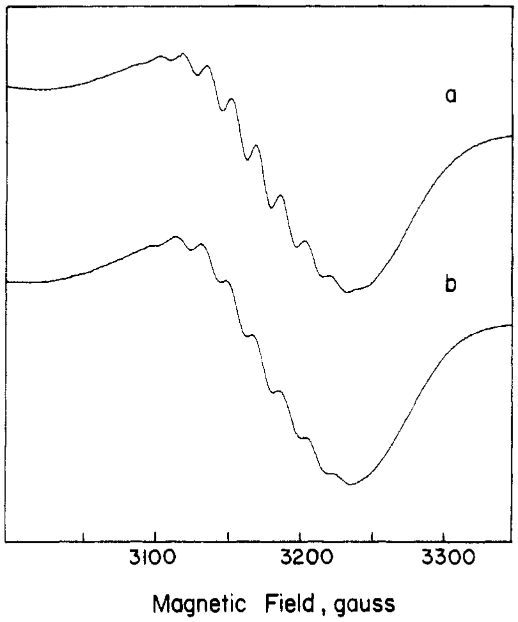


FIGURE 2: ESR spectra in the $g_\perp$ region of Cu_2Cu_2SOD plus $KSC^{14}N$ (a) and $KSC^{15}N$ (b). Spectra were recorded as described in Figure 1 except the modulation amplitude was 2.5 G. The gain was 500 for (a) and 630 for (b).

a series of standards consisting of different concentrations of Cu_2Zn_2SOD gave 98 ± 17% of the expected amount of copper based on the concentration of Cu_2Cu_2SOD used. All of the copper present in Cu_2Cu_2SOD therefore contributes to the ESR spectrum of the thiocyanate-treated samples. The ESR spectrum of Cu_2Cu_2SOD reacted with KSCN under anaerobic conditions is identical with that obtained from the aerobic reaction product. Dialysis of the SCN^- complex of Cu_2Cu_2SOD vs. either H_2O or 125 mM KSCN did not result in any loss of copper from the protein. Treatment of the SCN^- complex of Cu_2Cu_2SOD with Chelex 100 also failed to remove any copper from the protein, and the ESR spectrum was unchanged. In contrast, Chelex 100 completely removed all of the copper from a solution of $Cu(ClO_4)_2 \cdot 6H_2O$ and KSCN at the same concentration as used in the enzyme experiment. The color and ESR spectrum of copper(II) in the presence of excess SCN^- also differ from those observed with the Cu_2Cu_2SOD. These results indicate that the copper of Cu_2Cu_2SOD remains tightly bound to the protein in the presence of SCN^-. Removal of SCN^- from Cu_2Cu_2SOD by dialysis restores the characteristic ESR spectrum of the imidazolate-bridged dicopper unit.

The ESR spectrum of the SCN^- complex of Cu_2Cu_2SOD also shows considerable hyperfine structure in $g_\perp$, although no splitting of the lowest field component of $g_\parallel$ could be detected. Figure 2 displays the $g_\perp$ region of Cu_2Cu_2SOD in the presence of $SC^{14}N^-$ and $SC^{15}N^-$. The ESR parameters of these two complexes are the same (Table I). The spectrum obtained with $SC^{15}N^-$ shows one less peak than the $SC^{14}N^-$ spectrum. On this basis, we tentatively ascribe these splittings to nitrogen superhyperfine interactions, although we realize that it is not possible to assign definitively the splittings in $g_\perp$ to specific ligand superhyperfine interactions without observing similar features in $g_\parallel$. Further discussion of this point appears below.

Thiocyanate Binding to Cu_2Zn_2SOD and Ag_2Cu_2SOD. Figure 3 shows the ESR spectra of the SCN^- complexes of Cu_2Zn_2SOD and Ag_2Cu_2SOD. The rhombic distortion evident in the ESR spectrum of Cu_2Zn_2SOD (Rotilio et al., 1972) vanishes when SCN^- is added. From Table I, it can be seen that the ESR spectral parameters of this derivative are the same as those of Cu_2Cu_2SOD plus SCN^-. The fine structure in $g_\perp$ is similar to that seen with Cu_2Cu_2SOD, although it is not as well resolved. Addition of larger amounts of SCN^- results in loss of the fine structure in $g_\perp$, which is consistent with coordination of a second SCN^- ion at high concentrations (Bertini et al., 1980).

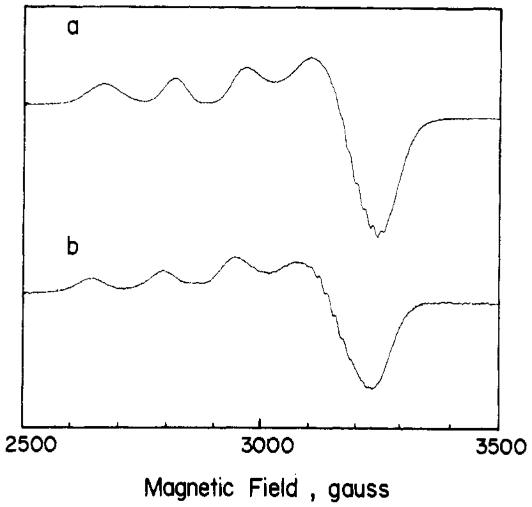


FIGURE 3: ESR spectra of 1.1 mM Cu_2Zn_2SOD in 300 mM KSCN (a) and 1.2 mM Ag_2Cu_2SOD in 500 mM KSCN (b). Spectral conditions are as described in Figure 1 except the gain was 1000 with a modulation amplitude of 2.5 G for (a) and 5 G for (b). The microwave frequency was 9.120 GHz in (a) and 9.099 GHz in (b).

The ESR spectrum of Ag_2Cu_2SOD is identical with that reported previously (Beem et al., 1977) and shows marked deviation from axial symmetry. Addition of SCN^- results in the appearance of an ESR spectrum very similar to that obtained with Cu_2Zn_2SOD, and having the same parameters (Table I). Chelex 100 does not remove the copper from solution, indicating that it is tightly bound to the protein. Silver ion is rapidly lost from Ag_2Cu_2SOD in the presence of SCN^-, however, as demonstrated by dialysis in conjunction with atomic absorption spectroscopic analyses. Loss of silver from the copper site might allow for migration of copper from the zinc site to the vacant site. That this migration does not occur to any appreciable extent under our experimental conditions, 50 mM phosphate, pH 7.8, is shown by the ESR spectrum of the protein after removal of the SCN^- by dialysis. The ESR parameters ($g_m = 2.096$, $g_\parallel = 2.308$, $A_\parallel = 0.0114$ cm^{-1}) are close to those of Ag_2Cu_2SOD and very different from those of Cu_2E_2SOD (Beem et al., 1977). A small amount of copper having different ESR parameters was also visible in the spectrum. The ESR spectrum of the complex of Ag_2Cu_2SOD, obtained within minutes of the addition of KSCN to the protein, therefore must arise from the SCN^- complex of copper in the zinc site. The fact that the ESR spectra of the SCN^- complexes of Cu_2Zn_2SOD and Ag_2Cu_2SOD are the same and identical with that of the SCN^- complex of Cu_2Cu_2SOD is nicely consistent with the finding that the latter has an ESR spectrum showing only one type of copper.

Azide Binding to Cu_2Cu_2SOD. Addition of N_3^- to Cu_2Cu_2SOD substantially changes the near-ultraviolet and visible absorption spectrum of the protein, as shown in Figure 4. A new absorption band appears at 375 nm. From the linear double-reciprocal plot of absorbance vs. azide concentration, the formation constant of the 1:1 complex is found to be 3.5×10^2 M^{-1}. The extinction coefficient of the complex is 2.8×10^3 M^{-1} cm^{-1}. Addition of N_3^- to Cu_2Zn_2SOD produces a complex having an absorption band at 373 nm with a molar

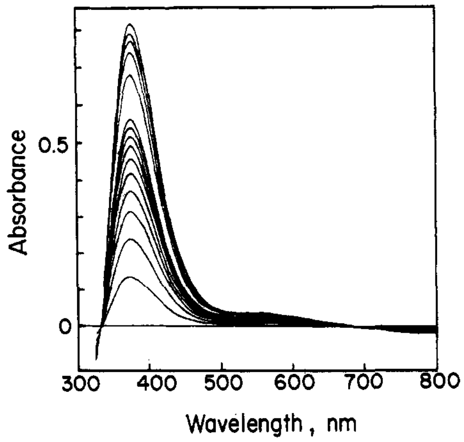


FIGURE 4: UV-visible difference spectra of Cu_2Cu_2SOD in H_2O, pH 5.7, titrated with NaN_3. The initial protein concentration in the reference and sample cuvettes was 0.313 mM, and water was added to the reference cell during the titration to keep the concentration of protein in both solutions equal. The concentration of azide was 0, 0.535, 1.07, 1.60, 2.13, 2.65, 3.18, 3.70, 4.22, 4.74, 5.25, 10.2, 15.1, 20.0, 24.9, and 34.6 mM. The temperature was 22 °C.

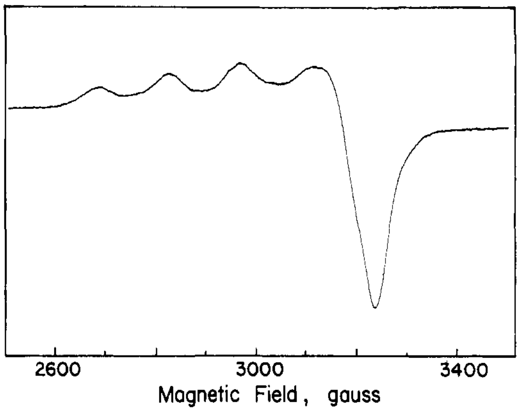


FIGURE 5: ESR spectrum of 0.43 mM Cu_2Cu_2SOD in 125 mM NaN_3. Spectral parameters are as described in Figure 1 except that the modulation amplitude was 10 G and the gain 630.

extinction coefficient of 2.7×10^3 M^{-1} cm^{-1} and a formation constant of 1.6×10^2 M^{-1} (Fee & Gaber, 1972). Formation of this N_3^- complex of Cu_2Cu_2SOD is not accompanied by any change in the ESR spectrum, indicating that the bridging imidazolate structure remains intact. At higher concentrations, the bridge is broken, as evident from the ESR spectrum (Figure 5). The resulting ESR spectrum is not the same as that obtained with SCN^- (Table I), although again both copper ions appear to be in very similar environments.

Other Anion Binding and Activity Studies. ESR spectra show that the imidazolate bridge in Cu_2Cu_2SOD is also broken by 0.3 M phosphate, pH 7.0, but that the bridge remains intact in the presence of 0.1 M cyanate, 0.1 M formate, pH 5.5, and 1.0 M fluoride. The activity of Cu_2Zn_2SOD, Cu_2Cu_2SOD, and Cu_2E_2SOD in the presence of 125 mM SCN^- or N_3^- in 0.1 M 2-picoline-$HClO_4$, pH 6.0, was measured relative to the activity of the same amount of enzyme in the same buffer containing 125 mM $NaClO_4$. The results are given in Table II. Cu_2E_2SOD is strongly inhibited by SCN^- and N_3^-. Thiocyanate is a relatively weak inhibitor of Cu_2Zn_2SOD and Cu_2Cu_2SOD. The concentration of SCN^- used is sufficient to break the imidazolate bridge in Cu_2Cu_2SOD, indicating that the intact bridge is not essential for activity. Azide is a stronger inhibitor of both Cu_2Zn_2SOD and Cu_2Cu_2SOD than thiocyanate. Ag_2Cu_2SOD has only 7.5% of the specific activity of Cu_2Zn_2SOD in 0.10 M 2-picoline-$HClO_4$, 0.5 mM EDTA, and 125 mM $NaClO_4$, pH 6.0. This small amount of activity probably results from a fraction of protein having Cu^{2+} in the native copper sites (Beem et al., 1977). The activity of Ag_2Cu_2SOD is unchanged when 125 mM KSCN is substituted for $NaClO_4$ in the buffer. The SCN^- complex of Ag_2Cu_2SOD is therefore inactive.

Table II: Inhibition of SOD Activity by Thiocyanate and Azide Anions

	% inhibition[a] by	
protein (nM)	125 mM KSCN	125 mM NaN_3
Cu_2Zn_2SOD (0.40)	31	83
Cu_2Cu_2SOD (0.40)	48	83
Cu_2E_2SOD (0.80)	98	93

[a] Percent inhibition calculated from the difference between the activity of the enzyme in 125 mM $NaClO_4$ and the activity in the presence of an equal concentration of KSCN or NaN_3. The buffer was 0.1 M 2-picoline-$HClO_4$ and 0.5 mM EDTA, pH 6.0.

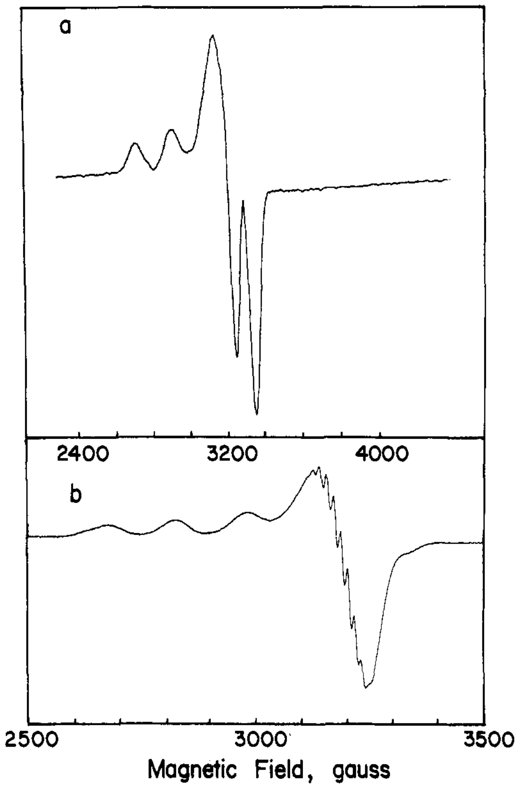


FIGURE 6: ESR spectra of Cu_2Cu_2SOD at pH 12.0 (a) and Cu_2Zn_2SOD at pH 12.3 (b). In (a), the modulation amplitude was 20 G and the gain 500. In (b), the modulation amplitude was 5 G and the gain 320. Other spectrometer conditions were as described in Figure 1.

No change occurs in the ESR spectrum of Cu_2Cu_2SOD when an aqueous solution is titrated with NaOH until the pH rises above ~10.5-11.0. At this point, the spectrum alters irreversibly to that shown in Figure 6a. The color of the protein also changes from green to pink in the same pH range. The ESR spectrum has parameters (Table I) in good agreement with those reported for an alkaline solution of a copper-triglycylglycine complex [$g_\perp = 2.041$, $g_\parallel = 2.172$, $A_\parallel = 0.0209$ cm^{-1} (Falk et al., 1967)] and copper-acetylglycylglycylhistidine [$g_\perp = 2.045$, $g_\parallel = 2.179$, $A_\parallel = 0.0203$ cm^{-1} (Bryce, 1966)], where the copper is coordinated to three to four deprotonated peptide nitrogen atoms. The pink color of the solution is also consistent with formation of a copper-peptide complex (Koltun et al., 1963). In contrast, Cu_2Zn_2SOD is stable over the same pH range (Figure 6b), although the spectrum becomes more axial and fine structure in $g_\perp$,

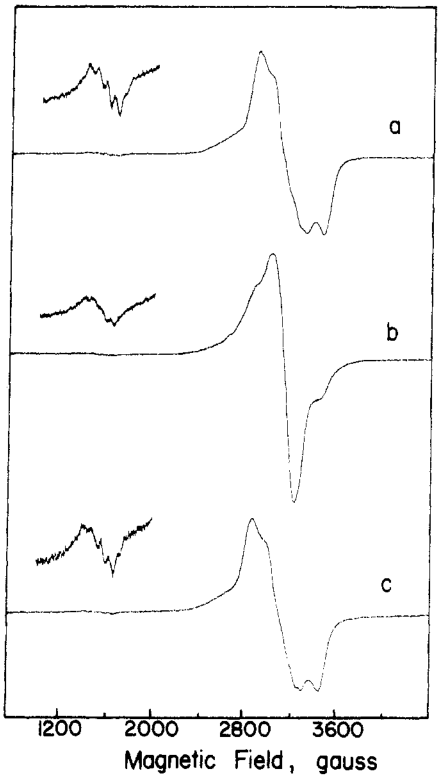


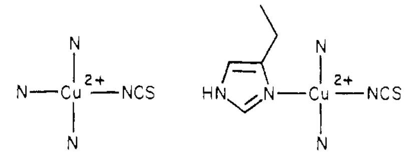
FIGURE 7: ESR spectra of 4.9 mM $[Cu_2(im)\subset A']^{3+}$ in 50% aqueous dimethyl sulfoxide (a) and with the addition of 1.5 M KSCN (b) and 1.5 M NaN_3 (c). The microwave power was 20 mW, and the modulation amplitude was 5 G. The gain was 500 in (a) and (b) and 630 in (c). The region 1100–2000 G is shown at 10-G modulation amplitude and a gain 10 times that of the complete spectrum. Other spectral conditions were the same as those described in Figure 1.

similar to that observed for the SCN^- complex, is apparent. The active site of Cu_2Cu_2SOD is not as stable to high pH as that of Cu_2Zn_2SOD.

Studies with a Model Complex. Figure 7a shows the ESR spectrum of the binuclear imidazolate-bridged copper complex $[Cu_2(im)\subset A']^{3+}$, the basic features of which are identical with those of Cu_2Cu_2SOD. Addition of SCN^- alters the spectrum (Figure 7b) as revealed by the increase in the signal at 3000–3200 G and the decrease at 3400 G. These changes result from conversion of the imidazolate-bridged structure, where the copper(II) ions are antiferromagnetically coupled, to one in which the copper ions are not interacting magnetically. Further addition of SCN^- results in continued decrease of the features characteristic of the imidazolate-bridged complex. Thiocyanate breaks the imidazolate bridge in $[Cu_2(im)\subset A']^{3+}$, as it does in Cu_2Cu_2SOD. Figure 7c shows the spectrum of $[Cu_2(im)\subset A']^{3+}$ plus N_3^-. It is evident that N_3^-, at the same concentration as SCN^- in Figure 7b, does not break the imidazolate bridge. Thiocyanate and azide show the same behavior toward $[Cu_2(im)\subset A']^{3+}$ as they do toward Cu_2Cu_2SOD. The propensity for SCN^- to displace the bridging imidazolate group in Cu_2Cu_2SOD, relative to N_3^-, is therefore attributed to a difference in the interaction of the two anions with copper and not to some other feature of the active site of Cu_2Cu_2SOD.

Discussion

It is clear that when SCN^- binds to Cu_2Cu_2SOD, the imidazolate bridge between the two copper ions is broken. The ESR spectrum of the SCN^- complex indicates that both copper ions are in nearly identical environments having axial symmetry. A model for the SCN^- complex of Cu_2Cu_2SOD is shown below, where N stands for a histidine imidazole nitrogen. In this model, thiocyanate displaces the imidazolate ligand from Cu^{2+} in the copper site and also replaces the aspartic acid ligand on the Cu^{2+} in the zinc site. Breaking the imidazolate bridge and replacing the aspartic acid allow the copper in the zinc site to relax from a tetrahedral (Beem et al., 1977) to a more favorable square planar geometry. The Cu^{2+} in the copper site is also in a more axial geometry than in Cu_2Zn_2SOD, which shows considerable rhombic distortion in its ESR spectrum (Rotilio et al., 1972). The SCN^- complex of Cu_2Zn_2SOD has an ESR spectrum with parameters identical with those of the SCN^- complex of Cu_2Cu_2SOD, indicating that the rhombic distortion of the copper site is eliminated when SCN^- displaces the bridging imidazolate. The SCN^- complex of Ag_2Cu_2SOD gives an ESR spectrum virtually identical with those of Cu_2Cu_2SOD and Cu_2Zn_2SOD, providing independent evidence that SCN^- converts the geometry of the copper in the zinc site from tetrahedral to square planar. These results account for the single ESR signal from Cu_2Cu_2SOD in the presence of SCN^-. The SCN^- complex of Cu_2Zn_2SOD very likely has a water molecule in the axial position (Bertini et al., 1980), and the same may be true for Cu_2Cu_2SOD.



The fine structure in the $g_\perp$ region of the SCN^- complex of Cu_2Cu_2SOD and Cu_2Zn_2SOD probably results from nitrogen superhyperfine splitting since the number of lines is reduced when $SC^{15}N^-$ is used in place of $SC^{14}N^-$. The actual number of lines expected will depend on whether the thiocyanate nitrogen is magnetically equivalent to the imidazole nitrogens. Given the uncertainty in assigning splittings in $g_\perp$ to ligand superhyperfine interactions, we have not attempted a detailed analysis of these features. It should be noted, however, that the proposed nitrogen-bonded structure of the SCN^- complex of Cu_2Cu_2SOD is consistent with the $g_\perp$ splittings arising from nitrogen superhyperfine interactions.

At high concentrations of SCN^-, the coordinated H_2O of Cu_2Zn_2SOD is displaced by SCN^- (Bertini et al., 1980). The relatively weak inhibition of activity of Cu_2Zn_2SOD and Cu_2Cu_2SOD by SCN^- observed in the present work may result from this secondary binding. Azide is a stronger inhibitor of both Cu_2Zn_2SOD and Cu_2Cu_2SOD and at low concentrations is thought to bind by displacing the water molecule. This interpretation is consistent with the idea that superoxide ion coordinates to copper by displacing the water. Azide is a competitive inhibitor of SOD (Rigo et al., 1977), indicating that azide and superoxide bind at the same site on the enzyme.

Our results also clearly indicate that an azide complex of Cu_2Cu_2SOD is formed at low concentrations of N_3^- without breaking the imidazolate bridge. The N_3^- must displace either the H_2O or one of the imidazole ligands, with the H_2O being the most likely possibility. At higher concentrations, N_3^- will also displace the bridging imidazolate ligand.

The initial coordination of SCN^- to Cu_2Cu_2SOD involves binding in the equatorial plane by displacement of the bridging ligand while N_3^- enters the axial site by replacing the bound water. Similar modes of coordination of these two anions to Cu_2Zn_2SOD have been proposed (Bertini et al., 1980). The difference between SCN^- and N_3^- may be either the result of their different abilities to displace the imidazolate group or the result of secondary interactions between the anion and amino acid side chains in the active site. An insight into this question is provided by the results of anion binding to $[\mathrm{Cu_2(im)}\subset \mathrm{A'}]^{3+}$, which is a model for the active site of Cu$_2$Cu$_2$SOD. Thiocyanate breaks the imidazolate bridge in this complex while azide does not, a result that parallels the behavior of the two anions toward Cu$_2$Cu$_2$SOD. This finding suggests that the different behavior of the two anions is a direct result of their interaction with the primary coordination sphere of the copper and not with more distant parts of the protein's active site.

Since Cu$_2$Cu$_2$SOD is catalytically active in the presence of SCN$^-$ under conditions where the imidazolate bridge is broken, reversible displacement and protonation of the bridging imidazolate group during turnover cannot be an essential feature of the enzyme mechanism, as previously suggested. Control experiments with Ag$_2$Cu$_2$SOD in the presence of thiocyanate show that the activity is not due to copper in the zinc site. Copper may still become 3-coordinate in the reduction step by losing the imidazolate or some other ligand to provide a vacant coordination site for inner sphere reoxidation by superoxide anion (Lippard et al., 1977). The activity of Cu$_2$E$_2$SOD, observed previously and in this work, indicates that attenuation by zinc of the pK_a of the pyrrole nitrogen of the bridging imidazolate group is not essential to the catalytic mechanism, however.

The instability of Cu$_2$Cu$_2$SOD relative to Cu$_2$Zn$_2$SOD at high pH may result from the less favorable coordination geometry of the zinc site for copper, making it easier to remove copper than zinc from the zinc site. Since the ESR spectrum of Cu$_2$Cu$_2$SOD at high pH shows that copper has been lost from the copper site, the stability of the copper site appears to be dependent on the presence of zinc, or presumably any other metal favoring tetrahedral coordination, in the zinc site. The increased inhibition of Cu$_2$E$_2$SOD relative to Cu$_2$Zn$_2$SOD and Cu$_2$Cu$_2$SOD by N$_3^-$ and SCN$^-$ may also result from a change in the active site of the enzyme when the zinc site is vacant. Thus, in addition to whatever other functions it may have, zinc clearly stabilizes the active-site structure of SOD.

Acknowledgments

We thank Peter K. Coughlin for providing a sample of $[\mathrm{Cu_2(im)}\subset \mathrm{A'}](\mathrm{ClO_4})_3\cdot\mathrm{H_2O}$, and also for modifying the computer program for integrating ESR spectra, and Judith Plust and David Prager for excellent technical assistance.

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