Metal Sites of Copper–Zinc Superoxide Dismutase†

Karl M. Beem, David C. Richardson, and K. V. Rajagopalan*

ABSTRACT: Silver–copper and silver–cobalt proteins have been prepared in which Ag^+ resides in the native copper site of superoxide dismutase and either Cu^{2+} or Co^{2+} reside in the zinc site. The electron paramagnetic resonance (EPR) spectrum of the copper and the visible absorption spectrum of the cobalt greatly resemble those of either Cu_4 or Cu_2,Co_2 proteins, respectively, in which the copper of the native copper sites has been reduced. It was found that, unlike cyanide, azide anion would not perturb the EPR spectrum of Ag_2,Cu_2 protein. Since azide produces the same perturbation upon the EPR spectrum of native and Cu_2 proteins, it must bind to the copper and not the zinc of superoxide dismutase. A model of the metal sites of the enzyme has been fitted to a 3-Å electron-density map using an interactive molecular graphics display. The model shows that histidine-61, which appears to bind both copper and zinc, does not lie in the plane of the copper and its three other histidine ligands, but occupies a position intermediate between planar and axial. This feature probably accounts for the rhombicity of the EPR spectrum and the activity of the enzyme.

The green copper–zinc protein from the cytosol of eukaryotic cells, superoxide dismutase, has been extensively studied in order to determine the roles of the metals in its enzymatic activity. Copper has been shown to be absolutely essential for activity (McCord and Fridovich, 1969), while the role of zinc is less understood. The enzyme containing copper alone has been found to have variable activity (Beem et al., 1974). Divalent zinc, cobalt, and mercury have been shown to enhance the thermal stability of Cu_2 protein, with zinc allowing the recovery of full activity (Forman and Fridovich, 1973). A method has been found which allows complete reconstitution of the enzyme and also allows the preparation of highly active enzyme containing divalent mercury, cobalt, or cadmium in place of zinc (Beem et al., 1974). Using a similar procedure, Fee and Briggs (1975) have been able to replace zinc with cupric ion. No successful attempts at substituting for the native copper have been reported.

The crystal structure of the protein, as determined by x-ray diffraction, has been solved at 3-Å resolution (Richardson et al., 1975a,b). The copper and zinc of each subunit are found to be about 6 Å apart, sharing a common histidine ligand. Copper has four histidine ligands, while zinc has three, along with an aspartate.

The EPR[1] properties of the enzymatic copper have been extensively investigated. It is apparent that copper experiences a ligand field of less than axial symmetry, and superhyperfine splittings from at least some of the histidyl nitrogens are observed (Rotilio et al., 1972; Beem et al., 1974). Several anions, notably cyanide and azide, modify the spectrum of native enzyme by removing the rhombic distortion (Rotilio et al., 1971, 1972; Fee and Gaber, 1972). In addition, cyanide by binding to copper via the carbon atom (Haffner and Coleman, 1973) inactivates the enzyme, causes a color change from green to pink, and enhances the intensity of the superhyperfine pattern. The EPR changes resulting from azide treatment are less dramatic. This adduct is yellowish green and active (Hodgson and Fridovich, 1975). Fee and Gaber (1972) have suggested that azide binds to the zinc, but this has yet to be definitely proven. The structure of the cyanide adduct has also not been completely resolved.

We report here on the preparation of inactive Ag_2,Cu_2 and Ag_2,Co_2 proteins, in which monovalent silver occupies the copper sites and either Cu^{2+} or Co^{2+} occupy the zinc sites. Azide does not attack this form of protein-bound copper, rather it binds to the copper of native and Cu_2 proteins. A model of the metal sites of the protein has been fitted to the 3-Å electron-density map. This model has been correlated with the spectral data and implications have been drawn as to the structures of the cyanide and azide adducts.

Materials and Methods

Bovine erythrocyte superoxide dismutase was purchased from Truett Laboratories and stored frozen until use. The pure isotope of ^{63}Cu was purchased from Oak Ridge National Laboratories as the oxide. The compound was dissolved in aqueous HCl and then diluted to 200 ppm of ^{63}Cu.

Activity assays were performed as described by McCord and Fridovich (1969), using the xanthine–xanthine oxidase system. Reaction rates were measured on a Gilford Model 2000 single-beam spectrophotometer with the cuvette compartment maintained at 25 °C. Acrylamide gel electrophoresis was conducted at room temperature and pH 8.9 using the method of Davis (1964). Protein concentrations were determined by the method of Lowry et al. (1951) and by the difference in absorbance at 215 and 225 nm, where a difference of 0.35 results from a 50-μg protein solution/mL (Murphy and Kies, 1960). Absorption spectra and rates of absorbance changes were monitored with a Cary 14 spectrophotometer.

EPR measurements were made on a Varian E-9 spectrophotometer with a variable temperature accessory and capability for modulation at 100 kHz and, alternatively or simultaneously, at lower frequencies. Mathematical manipulations of the data were performed on a Nicolet Model 1072 computer interfaced with the E-9. Magnetic-field positions were determined using diphenylpicrylhydrazide as the internal standard. EPR parameters are defined as shown by Malmström and Vänngård (1960). The uncertainty in the g values is estimated to be ± 0.004. The value of the hyperfine parameter $A_\parallel$ in $cm^{-1} \times 10^{-3}$ is given by the relation $0.467 \times g_\parallel \times A_\parallel$ (gauss).

Atomic absorption measurements were performed on a

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received July 1, 1976.* This work was supported in full by Research Grants GM-15000 and GM-00091 from the National Institutes of Health.

[1] Abbreviations used are: EPR, electron paramagnetic resonance; EDTA, (ethylenedinitrilo)tetraacetic acid.

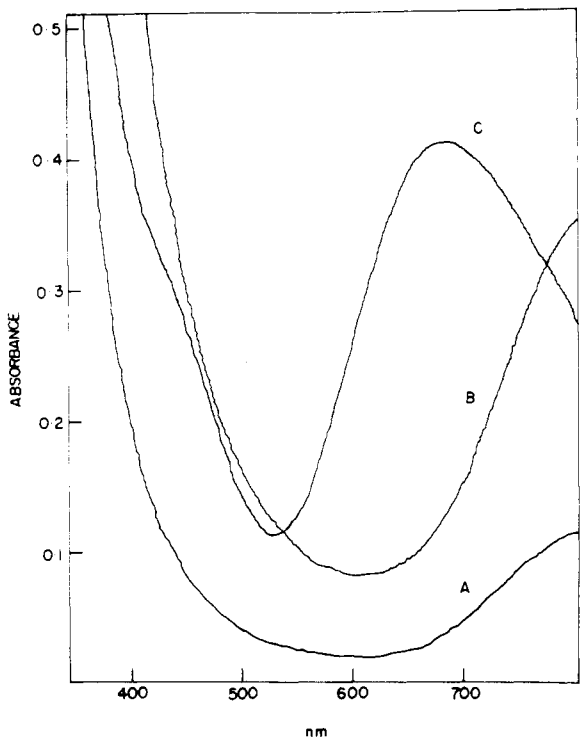

FIGURE 1: Absorption spectra of (A) Ag_2Cu_2 protein, preparation I, 0.5 mM; (B) $Ag_2{}^{63}Cu_2$ protein, preparation II, 1.1 mM; and (C) native enzyme, 1.5 mM. The samples were in 50 mM potassium phosphate, pH 7.8.

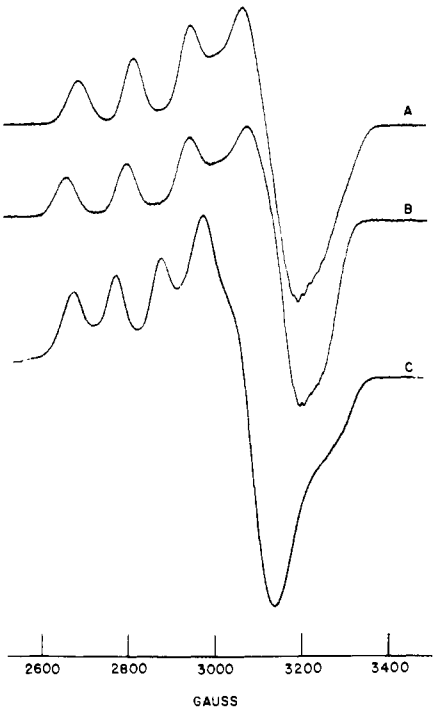

FIGURE 2: First derivative EPR spectra of (A) native enzyme, 1.5 mM; (B) Cu_2 protein, 1.3 mM; and (C) Ag_2Cu_2 protein, 0.5 mM. The instrument settings were microwave frequency 9.13 GHz, power 20 mW, modulation amplitude 4 G, scan rate 125 G/min, time constant 0.3 s, and temperature −100 °C with the exceptions that in C the modulation amplitude was 10 G and the time constant was 1 s. The samples were in 50 mM potassium phosphate, pH 7.8.

Perkin-Elmer Model 107 spectrophotometer equipped with a heated graphite atomizer (HGA-2000). A Sage Model 355 variable-speed syringe pump was used to infuse copper and silver into solutions of apoprotein. The anions cyanide, azide, and fluoride were added to protein solutions in the form of freshly prepared 40 mM solutions of the sodium salts in 100 mM potassium phosphate, pH 7.8. The actual pHs of the solutions were, respectively, 9.5, 7.8, and 7.8.

An electron-density map of native bovine erythrocyte superoxide dismutase at 3-Å resolution was obtained by standard x-ray crystallographic heavy-atom replacement methods (Thomas et al., 1973; Richardson et al., 1975b). Model-to-map fitting was done using the interactive molecular graphics system developed in the Computer Science Department of the University of North Carolina at Chapel Hill.

Results

Except where otherwise noted, apoprotein was prepared as described previously (Beem et al., 1974). In one experiment, apoprotein in 10 mM sodium acetate at pH 3.8 was diluted to approximately 0.03 mM with deionized water. Two equivalents of Ag^+ as aqueous 9 mM silver nitrate were infused into the sample over 30 min. The resulting solution was concentrated by ultrafiltration to 0.2 mM protein and then dialyzed overnight against 25 mM potassium phosphate at pH 7.8 and 4 °C. At this point, the sample was colorless. The sample was then diluted to 0.03 mM with the phosphate buffer and infused with 2 equiv of Cu^{2+} over 30 min. The sample was then concentrated by ultrafiltration to 0.3 mM protein, the solution becoming noticeably yellow. Two equivalents of Zn^{2+} were then directly added and, after overnight storage at 4 °C, the sample was dialyzed for a day at 4 °C against 50 mM potassium phosphate, 0.1 mM EDTA at pH 7.8. The resulting sample was then concentrated by ultrafiltration to 0.5 mM. The visible absorption and EPR spectra of this sample are displayed in Figures 1 and 2. The specific activity was determined to be 180 units/mg, which is approximately 5% of the native activity. The sample was found to be 0.80 mM Ag, 0.85 mM Cu, and no more than 0.05 mM Zn, representing about 80% occupancy of the metal sites. It could be argued that, after the addition of Cu^{2+}, the sample contained about 50% Ag_2,Cu_2 protein, 25% Cu_2 protein, and 25% Ag_2 protein. Were this so, the subsequent addition of Zn^{2+} should have resulted in the incorporation of Zn^{2+} into about 50% of the sites, yielding 25% Cu_2,Zn_2 protein and 25% Ag_2,Zn_2 protein. The fact that the final preparation contained less than 5% Zn^{2+} showed that this was clearly not the case. It was, therefore, apparent that the above procedure gave Ag_2,Cu_2 protein in about 80% yield.

In another preparation, 2 equiv of Co^{2+} were directly added to a sample of the above 0.2 mM silver–protein complex at pH 7.8. A pink color appeared within a few minutes. The visible absorption spectrum is shown in Figure 3 along with that of reduced Cu_2Co_2 protein, the preparation of which is described elsewhere (Beem et al., 1974). The molar absorbance at 583 nm of the protein containing Ag^+ was found to be 440 $(MCo)^{-1}$ cm^{-1}, while that of the Cu^+ protein was 420 $(MCo)^{-1}$ cm^{-1}.

In the following experiments, apoprotein was prepared with dialysis at room temperature replacing the column steps. Native protein was first dialyzed against 10 mM EDTA, pH 3.8, for 2 days with several changes of buffer, then against 10 mM $MgCl_2$ at pH 3.8 for 1 day. The resulting preparation was found to contain less than 1% of the original copper.

Earlier it was found that the yields of copper dismutases could be increased by adding copper to dilute solutions of apoprotein at pH 3.8. Accordingly, 2 equiv of Ag^+ were infused over a 40-min period to a 0.03 mM apoprotein sample in pH

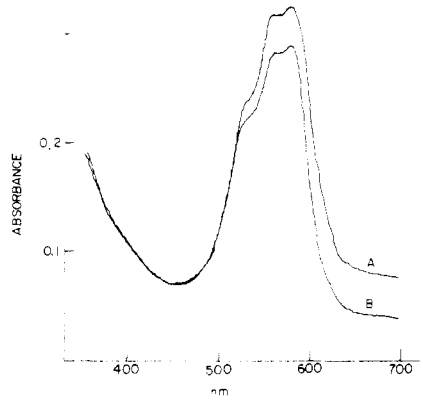

FIGURE 3: Absorption spectra of (A) reduced Cu_2Co_2 protein, 0.67 mM Co and (B) Ag_2Co_2 protein, 0.77 mM Co. The copper of the Cu_2Co_2 protein was reduced by adding an eightfold excess of ascorbate. The samples were in 50 mM potassium phosphate, pH 7.8.

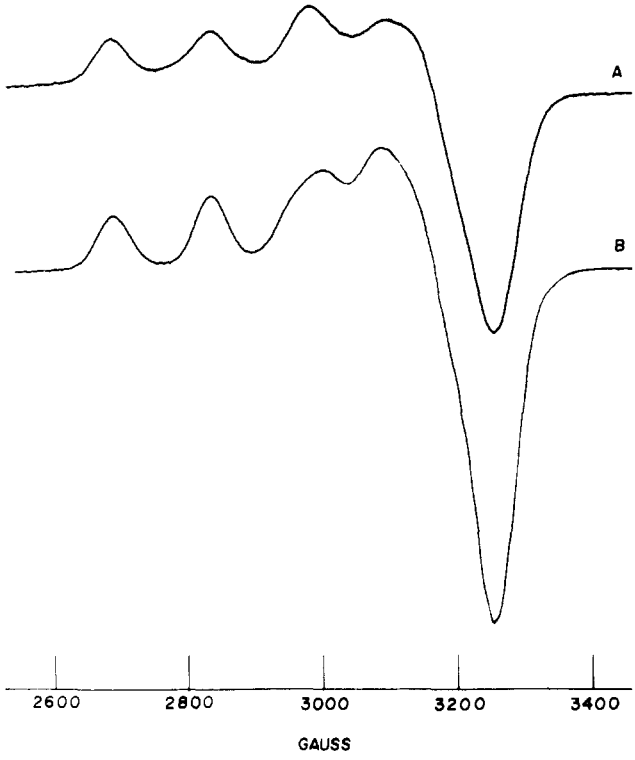

FIGURE 4: First derivative EPR spectra of azide adducts of superoxide dismutases (A) $^{63}Cu_2$ protein, 1 mM, treated with 4 equiv of azide, and (B) native enzyme, 3 mM, treated with 2 equiv of azide. The instrument settings were microwave frequency 9.13 GHz, power 20 mW, modulation amplitude 4 G, scan rate 125 G/min, time constant 1 s, and temperature −100 °C. The samples were in 50 mM potassium phosphate, pH 7.8.

3.8 acetate buffer at room temperature. Following overnight storage at 4 °C, separate samples of this silver–protein complex were treated with direct addition of 2 equiv of either Zn^{2+} or Co^{2+}. Following dialysis against 50 mM potassium phosphate at pH 7.8, the proteins were found to have approximately 1% of native activity. Upon concentration by ultrafiltration, the cobalt sample was found to have an absorption spectrum identical to that of the Ag_2Co_2 protein shown in Figure 3. When copper was directly added to a concentrated sample of the silver protein in the pH 3.8 acetate buffer, a blue color immediately developed. Dialysis against 50 mM potassium phosphate at pH 7.8 gradually elicited a green and then greenish-yellow color. The absorption spectrum of this sample appeared to be a composite of the spectra (Figure 1) of the

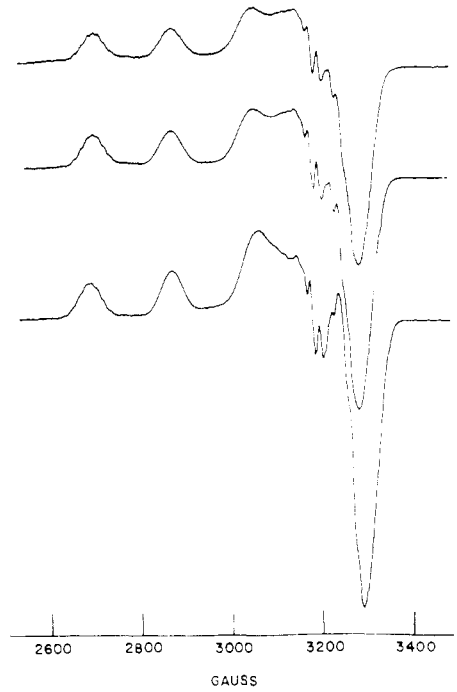

FIGURE 5: First derivative EPR spectra of the cyanide adducts of, from the top, $Ag_2,^{63}Cu_2$ protein, 1.1 mM; $^{63}Cu_2$ protein, 1 mM; and native enzyme, 3 mM, first treated with 2 equiv of azide. All samples were treated with 2 equiv of cyanide and were in 50 mM potassium phosphate, pH 7.8. The instrument settings were as described in Figure 4.

earlier Ag_2,Cu_2 protein and protein-containing copper in its native site. The ratio of the absorbance at 680 nm to that at 800 nm was 0.50, as compared to related values of 1.52 for native protein and 0.30 for the Ag_2,Cu_2 protein of preparation I. Another sample of Ag_2 protein was prepared as above but then dialyzed against 50 mM potassium phosphate at pH 7.8 and 4 °C for 1 day. Copper was infused into the 0.03 mM solution with no noticeable color change. After 4 h of storage at room temperature, the sample was concentrated by ultrafiltration. The absorption spectrum of this yellow sample, preparation II, is shown in Figure 1. This preparation is the Ag_2Cu_2 protein of subsequent experiments.

Fee and Briggs (1975) prepared a Cu_4 protein possessing 100% native activity by a method similar to that described here. They found that ferrocyanide or H_2O_2 reduced approximately half of the copper giving an EPR spectrum quite different from that of native enzyme. A sample of $^{63}Cu_2$ protein was prepared as above with infusion of $^{63}Cu^{2+}$ replacing that of Ag^+. After dialysis against 50 mM potassium phosphate at pH 7.8 and concentration by ultrafiltration to about 1 mM, 2 additional equiv of $^{63}Cu^{2+}$ were directly added to the sample. The EPR spectra obtained before and after addition of 4 equiv of ferrocyanide reproduced the results of Fee and Briggs. The EPR spectrum of the reduced Cu_4 protein was essentially identical to that of the Ag_2,Cu_2 protein shown in Figure 2.

The Effects of Anions. Two equivalents of sodium azide were added to an EPR tube containing a 3 mM sample of native enzyme. The solution became more yellow immediately upon mixing and was quickly frozen. A similar change in color resulted when a 1 mM sample of $^{63}Cu_2$ protein was treated with 4 equiv of azide. The EPR spectra of these adducts are shown in Figure 4. Treatment with azide produced no effect on the EPR spectrum of a 1.1 mM sample of Ag_2,Cu_2 protein. The EPR spectrum remained unchanged after a week's storage at 4 °C.

TABLE I: EPR Parameters of Superoxide Dismutases and Adducts.[a]

Adduct	g_m	$g_\parallel$	$A_\parallel$ ($10^{-3}cm^{-1}$)	Protein Concn (mM)
Native	2.083	2.259	13.5	3.0
Cu_2	2.076	2.269	15.0	1.0
$^{63}Cu_2$	2.080	2.266	14.3	1.0
Ag_2Cu_2	2.125	2.312	10.5	0.5
$Ag_2{}^{63}Cu_2$	2.120	2.306	10.6	1.1
2 N_3^-: Native	2.060	2.238	15.4	3.0
4 N_3^-: Native	2.052	2.235	16.4	2.0
4 N_3^-: $^{63}Cu_2$	2.061	2.244	16.1	1.0
2 CN^-: Native		2.211	18.6	2.8
2 CN^-: 2 N_3^-: Native		2.202	18.4	3.0
2 CN^-: $^{63}Cu_2$		2.207	18.1	1.0
2 CN^-: $Ag_2{}^{63}Cu_2$		2.208	18.0	1.1

[a] All samples were in 50 mM potassium phosphate, pH 7.8.

In similar experiments, 2 equiv of cyanide were added to samples of $^{63}Cu_2$ and $Ag_2,{}^{63}Cu_2$ protein, as well as to the above azide adduct of native protein. In all cases, the samples became pink immediately upon mixing. The EPR spectra of the samples are shown in Figure 5. As can be seen, the cyanide adduct of the Ag_2,Cu_2 protein is indistinguishable from that of the Cu_2 protein and is quite similar to the derivative of the native enzyme. The EPR parameters of the proteins and adducts are collected in Table I. The direct addition of 2 equiv of fluoride had no effect on the spectrum of native enzyme.

When observed with a K-band (35 GHz) spectrometer, the EPR spectrum of native enzyme clearly shows the lack of full axial symmetry, while the spectra of the cyanide and azide adducts are axial (Rotilio et al., 1972). The rhombic distortion in the spectrum of native enzyme is not resolved with x-band radiation. However, if second derivative spectra are obtained, resolution is improved somewhat. In Figure 6 are shown the second derivative spectra of native enzyme, $^{63}Cu_2$ protein and its azide adduct, and $Ag_2{}^{63}Cu_2$ protein and its cyanide adduct. Rhombic effects, if any, may be found beyond about 3200 G.

Ligand Superhyperfine Splittings. The barely resolved structure near 3200 G in the first derivative spectra of copper superoxide dismutases has been shown to result at least in part from magnetic interactions of the unpaired copper electron with the nuclear moments of nitrogenous ligands (Rotilio et al., 1971). The number of ligands contributing to these patterns cannot be determined with certainty, since copper hyperfine splittings in this region may be of similar magnitude. The binding of cyanide to native enzyme has been found to enhance the intensities of the superhyperfine lines so that patterns may be observed at lower field where copper hyperfine is well resolved. Azide does not bring about such an enhancement. Rotilio et al. (1972) have suggested that the splittings in the spectrum of the cyanide adduct of protein reconstituted with ^{63}Cu arise from three magnetically similar nitrogenous ligands. In earlier work, a method was described which allowed the extraction of a superhyperfine pattern from the gross part of a second derivative EPR spectrum (Beem et al., 1974). Patterns resulting from three or four magnetically similar nitrogens would contain up to seven or nine lines, respectively. Natural copper consists of approximately 75% ^{63}Cu and 25% ^{65}Cu. The magnetic moments of the two isotopes differ by about 10% (Griffith, 1964). Considering that the copper hyperfine splitting in this region is about 170 G and the superhyperfine splittings are about 15 G, the two isotopes should give patterns which are staggered by one line. Indeed, the cyanide adduct of native enzyme possesses an eight-line pattern, while only seven lines are observed when proteins reconstituted with ^{63}Cu are examined. Thus, the pattern results from only three nitrogens. In Figure 7 the results of computer integration of the patterns of spectra resulting from adding stoichiometric amounts of cyanide to native enzyme and its azide adduct are presented. It is apparent that azide does not alter the pattern of hyperfine interaction. While the spectrum of Ag_2,Cu_2 protein does not possess superhyperfine structure in the $g_\perp$ region, its cyanide adduct displays the hyperfine structure similar to those of cyanide adducts of native enzyme and Cu_2 protein in both $g_\perp$ and $g_\parallel$ regions.

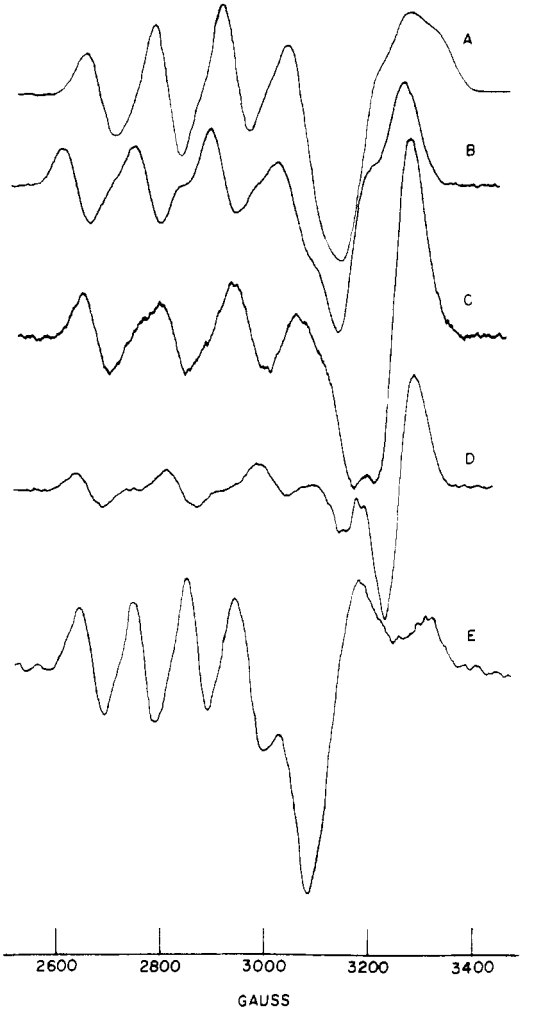

FIGURE 6: Second derivative EPR spectra of (A) native enzyme, 1.5 mM; (B) $^{63}Cu_2$ protein, 1 mM; (C) the azide adduct of $^{63}Cu_2$ protein, 0.6 mM; (D) the cyanide adduct of $^{63}Cu_2$ protein, 1 mM; and (E) Ag_2,Cu_2 protein, 0.5 mM. The instrument settings were microwave frequency 9.3 GHz, power 20 mW, scan rate 250 G/min, and temperature −110 °C. The settings for the 100 kHz mode were modulation amplitude 4 G and time constant 1 s, while the settings for the 1 kHz mode were 10 G and 1 s, respectively. All samples were in 50 mM potassium phosphate, pH 7.8.

The previously reported x-ray crystal structure of bovine copper–zinc superoxide dismutase (Richardson et al., 1975a,b) showed that the copper and zinc in a single subunit are approximately 6 Å apart, with the imidazole ring of His-61 bridging between them. The zinc also has His-69, His-78, and Asp-81 as ligands, in approximately tetrahedral arrangement,

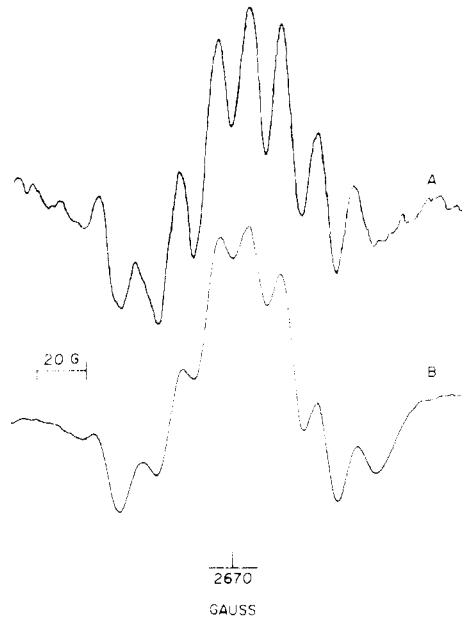

FIGURE 7: Superhyperfine patterns extracted from the feature at lowest magnetic field of the second derivative EPR spectra of the cyanide adducts of (A) native enzyme and (B) the azide adduct of native enzyme. The instrument settings were microwave frequency 9.13 GHz, power 20 mW, scan rate 200 G/min, and temperature −100 °C. The 100 kHz mode was set for modulation amplitude 4 G and time constant 0.3 s. The 1 kHz mode was initially set for 0.3-s time constant and 10 G modulation amplitude and finally for 40 G modulation amplitude with the spectrometer gain being reduced fourfold. The spectra obtained in the two cases were computer subtracted, then integrated, giving the displayed patterns.

and the other copper ligands are His-44, His-46, and His-118.

Detailed model to map fitting of the 3-Å resolution electron-density map on an interactive computer graphics system has now made it clear that the ring of His-61 is not directly on a line between the copper and zinc, although it is close enough to interact with both of them. The crystals of this enzyme have four subunits (two molecules) in the asymmetric unit, so that the electron-density map contains four independent pictures of the structure. The four subunits appear to have identical conformations within the expected resolution of the map, except where side-chain positions are perturbed by nonequivalent crystal packing contacts. The model to map fitting was done separately for each of the four subunits.

In general, the conformation of the fitted models agrees extremely well among the four subunits, although there is inaccuracy in such features as the orientation of the histidine rings, since this map is only at 3-Å resolution. There is some variation in the appearance of the electron density for the bridging histidine, although these differences are more likely to be due to phasing inaccuracies in the map than to a genuine conformational difference. In order to fit into the density in each of the subunits, the position of the bridging imidazole ring must be somewhat above the line between the two metals and considerably above the plane of the other three copper ligands. However, since the map does not show the orientation of the ring except indirectly through constraints imposed by the backbone position, the His-61 ring can either be fit with a symmetrical bending of the bonds to both metals, or it can be fit in an asymmetrical position where the proximal nitrogen sits above the copper. The latter possibility is illustrated in Figures 8 and 9. The map and superimposed model for one of the four independent subunits are shown in Figure 8 and a stick model for the copper and zinc and their seven ligand residues is shown in Figure 9. Both figures show the His-61 imidazole with extra "bonds" extending from the ring nitrogens. The one from the proximal nitrogen ends at the zinc position (metal positions can be located by the high local electron-density maxima, as well as by inference from the geometry of their surrounding ligands), so that His-61 is fit as a normal histidine ligand to a tetrahedrally coordinated zinc. The nitrogens of His-44, His-46, and His-118 lie approximately at three corners of a square plane centered around the copper, with the fourth corner unoccupied, and this fitting places the distal nitrogen of His-61 somewhat above that square plane (on the side accessible to solvent) in a position intermediate between planar and axial.

Discussion

Addition of Ag^+ to metal-free superoxide dismutase at pH 3.8 leads to binding of the metal at the native copper site. Subsequent addition of Cu^{2+} to Ag_2 protein at pH 3.8 apparently leads to the partial displacement of silver. However, if the pH of the Ag_2 protein is raised to 7.8 before the addition of copper, the latter metal preferentially occupies the native zinc site. Hybrid species were obtained by Moss and Fee (1975) when Cu_2,Co_2 protein was prepared at pH 5.5. The authors reported that the samples contained the species Cu_2,Co_2, Cu_4, and Co_2 proteins in equal amounts and displayed the EPR spectrum of Cu_4 protein. In contrast, the Cu_2,Co_2 protein prepared by Beem et al. (1974) by addition of Co^{2+} to Cu_2 protein had, at most, 10% Cu_4 protein based on EPR intensity and activity.

Until recently, divalent cobalt was the one paramagnetic cation known to occupy the zinc site of superoxide dismutase. The visible absorption spectrum characteristic of Co^{2+} is greatly enhanced on addition of Co^{2+} to Cu_2 protein. We have seen that the absorption spectra of Ag_2,Co_2 protein and reduced (by ascorbate) Cu_2,Co_2 protein are virtually identical. These results indicate that, although Cu^{2+} may prefer its native site at pH 3.8, it will occupy the zinc site at pH 7.8. Silver apparently has little affinity for the latter. All cations thus far known to replace Zn^{2+} are divalent.

The EPR spectrum of Ag_2,Cu_2 protein indicates that copper resides in a site of less than axial symmetry. The hyperfine splitting parameter, about 100 G, is considerably smaller than what is normally observed in square planar or distorted octahedral complexes of Cu^{2+}, and, in fact, agrees favorably with that of known tetrahedral complexes (Gould and Ehrenberg, 1968; Forster and Weiss, 1968). The g values of this protein, shown in Table I, also resemble those of distorted tetrahedral, as opposed to those of distorted octahedral, complexes. The Cu_4 protein prepared by Fee and Briggs (1975) contains copper in both metal sites of superoxide dismutase. When this protein is exposed to conditions which bring about the reduction of the copper of native enzyme, only about half of its copper is reduced. Such a treatment elicits an EPR spectrum identical to that of Ag_2,Cu_2 protein. This spectrum is thus characteristic of Cu^{2+} in the zinc site of superoxide dismutase with either Cu^+ or Ag^+ residing in the copper site.

With this in mind, the Ag_2,Cu_2 and Ag_2,Co_2 proteins would allow an examination of the zinc site in superoxide dismutase without interference from reducing agents or nearby paramagnetic ions. The 3-Å electron-density map shows that zinc is bound by one aspartate and three histidyl residues in an approximately tetrahedral arrangement. The EPR parameters of Ag_2,Cu_2 and half-reduced Cu_4 protein could result from copper in such a site. In the visible absorption spectrum of

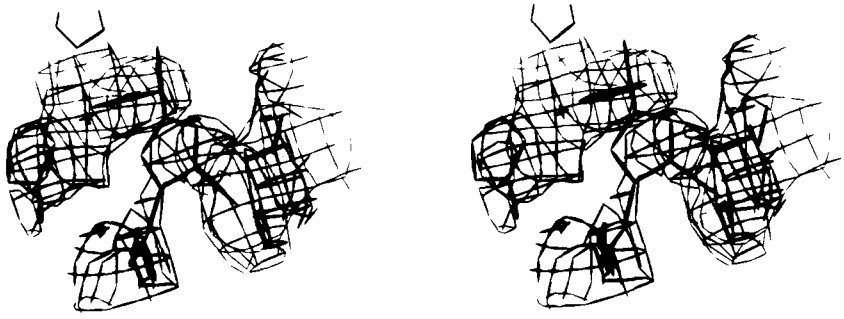

FIGURE 8: Stereo view of stick model superimposed on the electron-density map at 3-Å resolution for a small region around the copper and the bridging imidazole (His-61). Only one of the four crystallographically independent subunits is shown. Contours are "basket-weave" style: a single electron-density level is simultaneously displayed on the *xy*, *xz*, and *yz* planes. His-61 has extra "bonds" extending from its ring nitrogens; the Zn position is at the end of the one which points toward the lower right. The Cu position (chosen as the local density maximum seen at higher contour levels) is marked with a +. The photographs were taken directly from the screen of the computer graphics display.

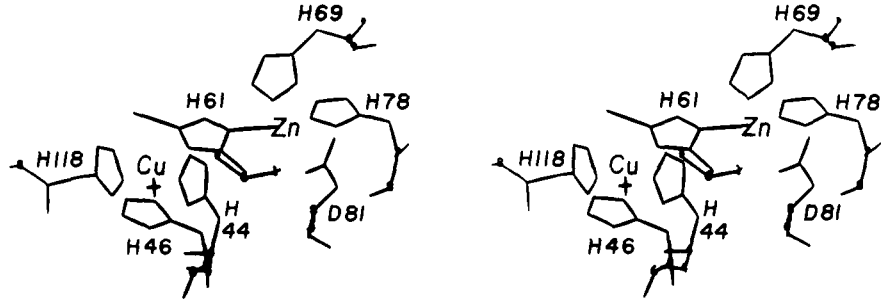

FIGURE 9: Stereophotograph of stick model showing the metal-ligand geometry in copper-zinc superoxide dismutase as determined by x-ray crystallography. His-61 (the bridging ligand) has extra "bonds" extending from its imidazole nitrogens. This picture shows the model as fit to the same subunit shown in Figure 8. This is an unrefined model fitting to an electron-density map at only 3-Å resolution; therefore, such features as ring orientations should be considered inexact.

Ag_2,Cu_2 protein, the band at 680 nm responsible for the blue color of native enzyme is not present. There appears to be a band centered at greater than 800 nm, beyond the visible range. This protein also significantly absorbs in the region of 450 nm, which accounts for its yellow color, just as do native enzyme and the reconstituted proteins which possess an occupied zinc site. Since Cu_4 protein is only 100% active, zinc-site copper is probably inactive and is fulfilling the structural role of native zinc, just as cadmium, mercury, and cobalt do in other reconstituted proteins (Beem et al., 1974). The mere trace activity of the Ag_2,Zn_2 and Ag_2,Co_2 proteins indicates that silver is most likely inactive.

The anions cyanide, azide, cyanate, thiocyanate, and fluoride under certain conditions modify the properties of superoxide dismutase (Fee and Gaber, 1972; Rotilio et al., 1972). Of this group, cyanide appears to be unique and certainly binds at the copper site (Rotilio et al., 1972) while inactivating the enzyme. Both cyanide and azide hasten the reoxidation of ferrocyanide-reduced enzyme (Rotilio et al., 1973). The azide adduct is active and reducible by H_2O_2, while the cyanide adduct is reduced much more slowly by H_2O_2 (Hodgson and Fridovich, 1975). The major modification of the visible absorption spectrum elicited by azide binding is the generation of an intense band at 373 nm, similar to the result of azide binding to the copper of ceruloplasmin (Kasper, 1968). Fee and Gaber have concluded, however, that the site of azide attack is the zinc. The following results of this study contradict that conclusion. Two equivalents of azide directly added to a concentrated sample of Ag_2,Cu_2 protein produced no effect, in comparison to the immediate changes in color and the EPR spectrum produced when the experiment was repeated with either native enzyme or Cu_2 protein.

The azide adducts unfortunately do not possess observable superhyperfine patterns in the $g_\parallel$ region and the evidence from the $g_\perp$ region is inconclusive. The EPR parameters are consistent with a planar complex in the adducts. Azide does not bind as tightly and is displaced by cyanide. At millimolar or greater protein concentrations, it was found that 2 equiv of azide did not produce a complete conversion of either native or Cu_2 protein as monitored by EPR, while conversion was essentially total with 4 equiv. No evidence was found for copper binding more than one molecule of either azide or cyanide.

Although Ag_2,Cu_2 protein contains copper bound almost totally to the zinc site, the EPR spectrum of its cyanide adduct is identical to the spectra of the adducts of native and Cu_2 protein. This result might be explained by a migration of Cu^{2+} from the zinc site to its native site on cyanide attack. However, the reaction is immediate and total at a pH where Cu^{2+} does not by itself displace Ag^+. Cyanide could displace the aspartate of the zinc site, producing initially a tetrahedral complex. Following a rearrangement, the end result of cyanide binding to zinc site copper could be a square planar complex similar to that of the adduct of native enzyme.

Histidine-61 would thus be involved in the copper complex of the cyanide adduct of Ag_2,Cu_2 protein. Hodgson and Fridovich (1975) have proposed that His-61 becomes protonated and no longer binds to copper when native protein is reduced during enzymatic catalysis. If so, then this ligand may not bind to Ag^+ in the Ag_2,Co_2 and Ag_2,Cu_2 proteins. This could explain the difference in the absorption spectrum of Cu_2,Co_2 protein obtained by reduction of copper (Moss and Fee, 1975).

The superhyperfine patterns extracted from the $g_\perp$ region of the spectra of native and Cu_2 protein are very similar (Beem et al., 1974). Though the number of nitrogenous ligands contributing to these patterns cannot be conclusively determined, it would appear that this number is the same in these two cases. The apparent rhombic distortion in the spectrum of native enzyme can be explained by the model based on the 3-Å electron-density map. Being out of the plane determined by copper and the other histidine ligands, His-61 is less accessible to the unpaired electron of copper and thus cannot give an equivalent superhyperfine interaction. In addition, an interaction between copper and His-61 must necessarily differ from the other copper–histidyl interactions as long as the bond between zinc and His-61 is maintained. Thus, His-61 would not participate in the superhyperfine pattern of native enzyme, yet its chemical modification due to binding by zinc can produce observable changes in the properties of the copper complex. The square planar complex $Cu^{2+}(imidazole)_4$ has been investigated in frozen solution and found to have the EPR parameters g_m = 2.06, $g_\parallel$ = 2.27, and $A_\parallel$ = 18 $10^{-3}cm^{-1}$ and an absorption maximum at 600 nm (Mälmstrom and Vänngård, 1960). While these g values compare favorably with those of Cu_2 protein, the hyperfine parameter, though of a typical value for square planar complexes, is much larger. Considering copper's propensity for forming such complexes, it appears that even in the absence of zinc the protein is preventing His-61 from forming a symmetrical planar complex along with His-44, -46, and -118. Since the copper complexes of the cyanide adducts of Ag_2,Cu_2 protein and native copper dismutases are identical in terms of EPR properties, cyanide probably displaces His-61 in forming the adduct of native enzyme. The initial attack may be axial to the plane of the three histidines. The known ability of cyanide to form π bonds with planar Cu^{2+} complexes may stabilize the initial formation of an axial adduct. A following rearrangement leaves a planar complex. It cannot be predicted with certainty whether this plane is parallel or perpendicular to the original plane, though the constraints on the three histidines as seen in the electron density map would suggest that the former is more likely. In this case, the inactivity of the adduct could result from the altered redox potential of copper and the weak attraction for axial ligands of planar copper complexes of strong-field ligands.

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