THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 249, No. 10, Issue of May 25, pp. 3157-3160, 1974
Printed in U.S.A.

Electron Paramagnetic Resonance Studies of Cobalt-Copper Bovine Superoxide Dismutase

(Received for publication, July 30, 1973)

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SUMMARY

1. EPR spectra of cobalt-copper bovine superoxide dismutase at liquid nitrogen and liquid helium temperature show that the two metal centers are magnetically coupled. The temperature dependence of the spectra indicates that this coupling arises from an exchange interaction.

2. The EPR spectrum of the Co(II) of the enzyme can only be seen after reduction of the Cu(II), at very low temperature. It is typical of tetrahedral coordination which is distorted in a particular way. The EPR parameters are $g_\perp \simeq 4$, $g_\parallel \simeq 2$, $D = 11.5\ cm^{-1}$. No feature indicating interaction between the two Co(II) centers is observed.

3. Anions such as CN^- and N_3^- do not affect the EPR spectrum of Co(II) significantly, but only modify the spectrum of Cu(II).

It is concluded that the Co(II) site (and presumably the native Zn(II) site) can be described as distorted tetrahedral, strongly spin-coupled to the Cu(II) and therefore very near to it, and noninteracting with the other Co(II) site and with solvent molecules.

Bovine superoxide dismutase (1, 2) contain 2 Cu(II) and 2 Zn(II) per molecule. The copper site, which is directly involved in the catalytic activity (1, 3, 4, 4a), has been extensively studied on the basis of the probe properties of Cu(II). Optical and CD spectra (5, 6), EPR spectra (5, 7), and proton relaxation rate (8, 9) measurements have contributed to the description of the Cu(II) site as composed of 3 nitrogens and a water molecule bound to copper in a field with less than axial symmetry. On the other hand, very little information is available about the zinc site, which is assumed to have a role in maintaining the protein structure (3). Recently, Co(II) has been introduced in the place of zinc in bovine superoxide dismutase (10) and this makes spectroscopic observations possible, due to the well known optical (11) and EPR (12, 13) properties of Co(II). A recent magnetic circular dichroism study of the substituted enzyme (14) has pointed out striking similarities of the Co(II) chromophore with the anion complexes of Co(II) carbonic anhydrase implying similar coordination geometries in the two cases. This paper reports EPR studies on the Co(II) site of the protein, which provide information on the zinc site of the native enzyme in terms of symmetry, relationship to the copper site, and exposure to the solvent.

EXPERIMENTAL PROCEDURE

All chemicals were reagent grade and were used without further purification. Co(II) superoxide dismutase was prepared as previously described (10) by an exchange method which results, in the best preparations, in 50 to 60% exchange of Co(II) for Zn(II). Different preparations are labeled by the per cent of Co(II) substitution with respect to the Zn content of the natural enzyme.

X band EPR spectra at liquid nitrogen temperature were performed with a V 4502-14 Varian spectrometer equipped with 100-KHz modulation within a Varian multipurpose cavity; microwave power was 20 milliwatts and modulation amplitude 10 G. X band EPR near liquid helium temperature was taken with a superheterodyne spectrometer described by Feher (15). Incident power levels of no more than 1 nanowatt were required to prevent partial saturation of the EPR spectra at the lowest temperature. Temperatures between 1.4 and 4.2 K were obtained by adjusting the vapor pressure over liquid helium; above 4.2 K the EPR cavity was placed in a controlled stream of gas obtained by boiling liquid helium at various rates.

Optical spectra were carried out in a Beckman DK2A ratio recording spectrophotometer. Metal analyses were performed by atomic absorption spectroscopy using a Hilger and Watts Atomr spek, model H 1170.

RESULTS

Fig. 1*a* shows EPR spectra at liquid nitrogen temperature of the enzyme copper in the native and Co(II)-containing protein. The samples have the same copper content as measured by atomic absorption spectroscopy. They also have the same visible and ultraviolet optical spectra as far as both absorbance and line shape are concerned (Fig. 1, *b* and *c*). The band centered at 680 nm and the absorbance at 260 nm reflect the Cu(II) content of the protein (3). Thus, it is evident that, in spite of having the copper present in the same amount and in the same valence state, Co(II) superoxide dismutase contains less EPR-detectable copper. By double integration of the signal it was apparent that the amount of EPR nondetectable copper corresponds to the amount of Co(II) introduced into the enzyme. EPR spectra at 1.5 K are reported in Fig. 2; the Co(II) protein shows a signal attributable to Co(II) only after reduction of copper with ferrocyanide (Fig. 2*b*); addition of azide (Fig. 2*c*) and cyanide (Fig. 2*d*) only affects the copper signal. Azide and cyanide also bring about an increase of the intensity of the copper EPR signal with

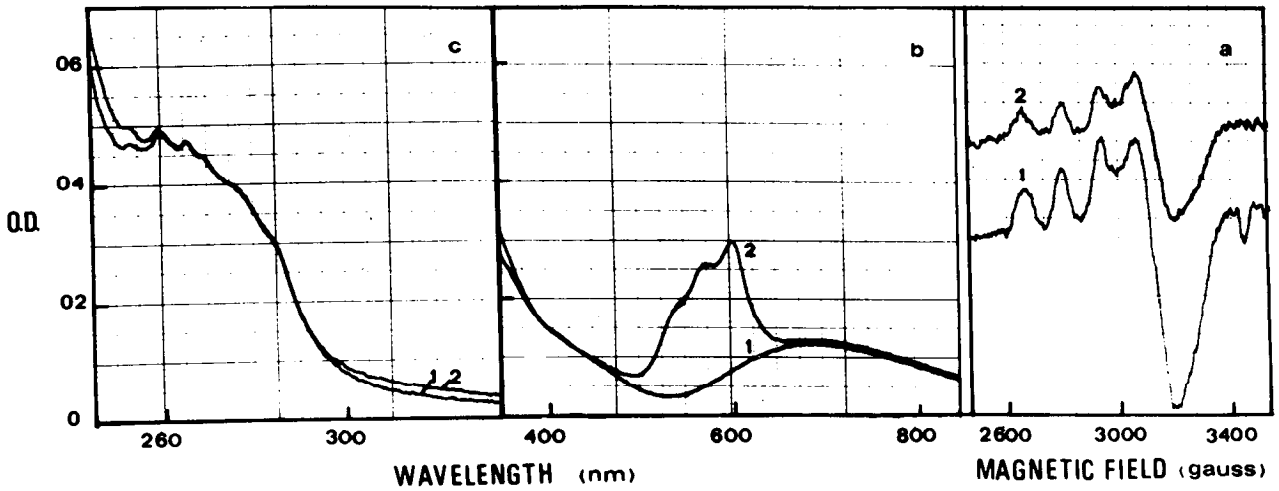

FIG. 1. Liquid nitrogen temperature EPR (*a*), visible (*b*), and ultraviolet (*c*) spectra of normal (*1*) and Co(II) (*2*) superoxide dismutase. Conditions: 5×10^{-4} M enzyme, 60% Co(II), water solutions.

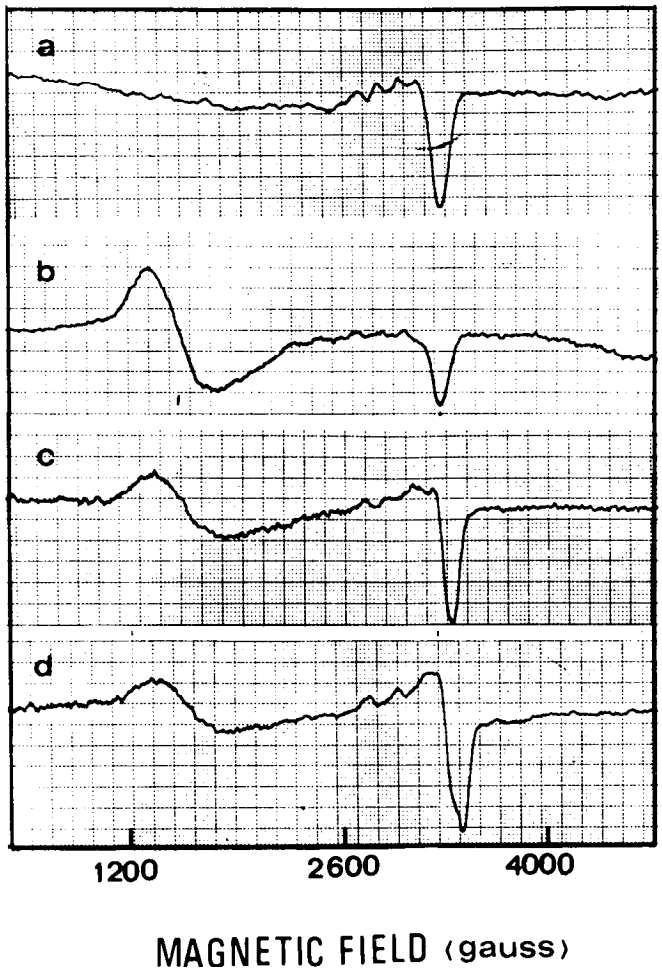

FIG. 2. Liquid helium temperature EPR spectra of Co(II) superoxide dismutase (*a*), plus ferrocyanide (*b*), plus azide (*c*), plus cyanide (*d*). Conditions: 5×10^{-4} M enzyme, 60% Co(II), 1 mM ferrocyanide, 20 mM azide, 10 mM cyanide, water solutions.

a parallel decrease of the intensity of the cobalt EPR signal. This is related to partial reoxidation of ferrocyanide-reduced enzyme in the presence of anions (16).

In the customary absorption derivative presentation of EPR spectra, the Co(II) appears to exhibit only a single, symmetric feature at approximately g = 4.3. However, when the EPR

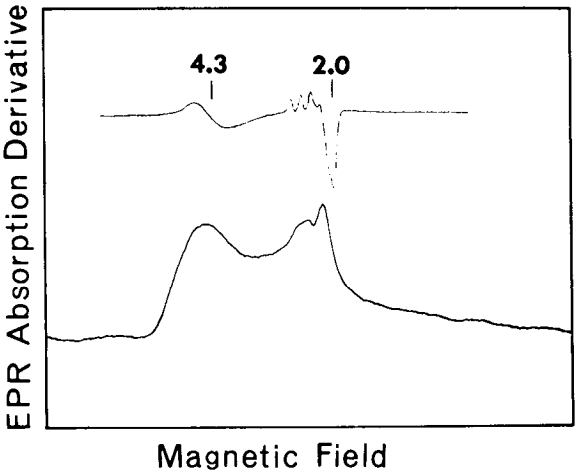

FIG. 3. EPR spectrum of partially reduced cobalt-substituted superoxide dismutase at 1.5 K taken under (*upper spectrum*) the usual absorption derivative conditions (incident power 10^{-8} watts, modulated 10 G) and (*lower spectrum*) nonadiabatic rapid passage conditions (incident power 10^{-4} watts, modulation 20 G, mixture of absorption and dispersion modes).

absorption is examined under certain nonadiabatic rapid passage conditions as discussed by Weger (17), it becomes apparent (Fig. 3) that the absorption extends from g = 4.3 to approximately g = 2. It proved impossible to adjust conditions such that the cobalt and copper absorption envelopes were simultaneously displayed. Therefore, the copper envelope is slightly distorted in the lower spectrum of Fig. 3.

Since the cobalt EPR absorption arises from the lower Kramers doublet of a $S = 3/2$ spin system, it is possible to determine the zero field splitting Z between the two Kramers doublets by observing the temperature dependence of the EPR absorption. In the absence of any interfering effects, such as spin coupling to neighboring paramagnetic atoms, the product of the EPR absorption intensity, A, times the absolute temperature, T, which is proportional to the population of the lower Kramers doublet, will be given by

$$A \times T = 1/(1 + e^{-Z/kT})$$

where k is the Boltzmann constant. Such a plot for reduced cobalt-substituted superoxide dismutase is given in the *top* of Fig. 4. From the best fit of the theoretical equation to the data, one obtains $Z = 23$ cm^{-1}.

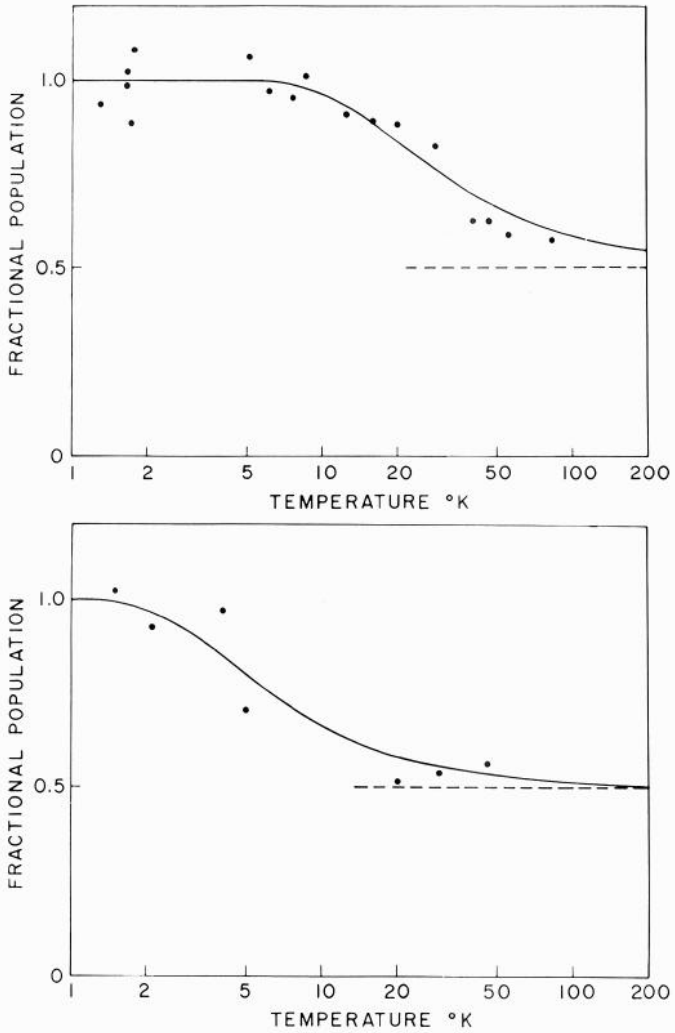

FIG. 4. The temperature dependence of the population of the lower Kramers doublet for *top*, the Co(II) in partially reduced cobalt-substituted superoxide dismutase, and *bottom*, the minority Co(II) species sometimes found in the oxidized enzyme. ——, best fits to the equation given in the text.

In some samples of the oxidized cobalt-substituted protein, especially noticeable in samples which had been reduced and reoxidized in air, a small cobalt EPR absorption could be observed. This never amounted to more than 10% of the amount of cobalt present but was indistinguishable from the cobalt which appeared upon reduction of the enzyme.

A study of the temperature dependence of the EPR absorption of this minority species, however, reveals that it has a smaller zero field splitting, $Z = 4.8$ cm^{-1} (*bottom*, Fig. 4).

DISCUSSION

We believe that the results reported above can be interpreted in terms of the native zinc and copper sites of superoxide dismutase. In fact, Co(II) substitution appears to have occurred at the zinc site without modifying the protein conformation and the occupancy of the copper sites by Cu(II). This is supported by the metal analyses of Co(II) protein (10) which show complementarity of the zinc and cobalt contents, by the substantial identity of ultraviolet spectra of native and Co(II) protein (Fig. 1*a*), as the aromatic spectrum of superoxide dismutase is highly sensitive to the amount of Cu(II) in the native site (3), and by the fact that the visible band of the copper is not modified in the Co(II) protein (Fig. 1*b*). Thus, we can reasonably assume that we probed the zinc site by Co(II) in a protein which maintains the native physicochemical properties, as well as its full catalytic activity (10).

The first consideration is that the copper and zinc sites must be close to each other, since both the Cu(II) and Co(II) EPR spectra are not observable even at very low temperatures (EPR-nondetectable copper in different samples is just equal to the amount of cobalt), and the Co(II) spectrum only appears on reduction of copper.

Clearly the copper and cobalt spin systems in the oxidized protein are coupled antiferromagnetically at the temperature used to observe the EPR. Since the spectra were unchanged, except for amplitude, in the range 1.4 to 80 K and no new spectral features appeared as a function of temperature, the magnitude of the coupling must be larger than about 100 cm^{-1}. Such a large coupling can only arise from an exchange interaction; it is too large for dipolar coupling alone. Since the copper site is surrounded by three nitrogenous groups and one water molecule as ligands to the metal, it is likely that this exchange coupling is brought about by binding of cobalt and copper to a common ligand. Recent photooxidation data (18) would in fact support the idea that zinc and copper share a histidine imidazole nucleus as a bridging group.

The second point is that EPR spectra of Co(II) in superoxide dismutase are not affected by treatments which usually modify spectra of Co(II) in other proteins, such as carbonic anhydrase (11) where the metal site is known to interact with solvent molecules. Neither azide nor cyanide affect the Co(II) spectrum of superoxide dismutase significantly. It is interesting to point out that azide does not interact with cobalt, thus making improbable that this anion binds to zinc with higher affinity than to copper, as suggested on the basis of proton relaxation rate titration data (8). These results indicate that the zinc site is not exposed to solvent molecules, as could be anticipated from its role in maintaining the protein structure (3).

Finally, EPR spectra of Co(II) in superoxide dismutase can be used to speculate about the symmetry of the zinc site. The molar absorbancies and ellipticities, the band pattern and the band energies of the optical absorption and magnetic circular dichroism spectra of bovine superoxide dismutase are very similar to those of the 1:1 CN^- complex of Co(II) human carbonic anhydrase B (14). On the basis of comparison with model complexes and theoretical consideration this kind of spectrum has been assigned to tetrahedral coordination of the cobalt (19). The EPR spectra observed at 1.5 K are also comparable to those reported for the CN^- complex of Co(II) carbonic anhydrase (20). The fact that the EPR spectrum of the Co(II) in the reduced, cobalt-substituted protein can be described by approximate g values $g_\perp = 4$, $g_\parallel = 2$ and has no resolved hyperfine structure from the ^{59}Co nuclear moment unambiguously points to a distorted tetrahedral arrangement of ligands to the Co(II) atom (12, 13). The axial second rank component of the ligand field, which in this case is given by $D = Z/2$ or 11.5 cm^{-1}, is positive, which indicated that the tetrahedral array of ligand atoms is distorted in a particular way. For example, such a field would arise from a tetrahedron in which the atom at one corner was less electron-donating than the other three. It is not possible to resolve any feature of the EPR spectrum which could be attributed to interaction between 2 Co(II) atoms.

The analysis of the small Co(II) EPR absorption which may be observed in the oxidized protein shows that it arises from a tetrahedral site similar to that discussed above but with less

distortion. In this case $D = 2.4\ cm^{-1}$. This site could be the remnants of the active site in partially denatured protein or it could be an entirely different site which is only populated by Co(II) to a small degree under the conditions employed.

In summary it may be stated that these EPR studies allow one to describe the Co(II) site (and presumably the native Zn(II)) as distorted tetrahedral, strongly spin-coupled to the Cu(II), and magnetically noninteracting with the other Co(II) site in the same molecule.

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