ON THE MAGNETIC PROPERTIES OF COBALT SUBSTITUTED BOVINE SUPEROXIDE DISMUTASE DERIVATIVES*†

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SUMMARY

We have measured the magnetic susceptibility in the temperature range 1.4-77°K of three derivatives of bovine superoxide dismutase in which Co^{2+} was substituted for Zn^{2+}: (1) $2Co^{2+}$ - in which Co^{2+} binds to the normal Zn^{2+} site and the Cu^{2+} site is unoccupied, (2) $2Co^{2+}2Cu^{2+}$ - in which the Zn^{2+} site is occupied by Co^{2+} and the copper sites contains Cu^{2+} and (3) $2Co^{2+}2Cu^{+}$ - which is the reduced form of the second derivative. The $2Co^{2+}$ protein exhibits Curie paramagnetism indicating $S' = 1/2$ and the zero-field splitting must be greater than $\gtrsim 20$ cm^{-1}. The same properties have been observed with the $2Co^{2+}2Cu^{+}$-protein. By contrast, the $2Co^{2+}2Cu^{2+}$-derivative exhibits relatively little paramagnetism, some of which arises from non-specifically associated metal ions. The lower susceptibility is due to antiferromagnetic coupling between Co^{2+} and Cu^{2+}, and the magnitude of the coupling constant is probably $>>5$ cm^{-1}.

The structure of bovine superoxide dismutase has recently been reported at a resolution of 3 Å (2,3). The zinc and copper atoms associated with each subunit were found to be approximately 6 Å apart and to share an imidazolium anion as a common, bridging ligand. Most of the previous physical and chemical observations (4-8) can be rationalized in terms of this structure as has been discussed elsewhere (9,10). Indeed, one prediction based on previous work was that the Zn^{2+} and

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Cu^{2+} ions were bound to the protein in close proximity to one another (11), and this has been borne out by the results of the x-ray crystallographic studies (2).

In this communication we report the presence of antiferromagnetic coupling between Co^{2+} (S = 3/2) and Cu^{2+} (S = 1/2) in cobalt substituted superoxide dismutase. This finding is consistent with the idea that the Co^{2+} in this derivative, and by analogy Zn^{2+} in the native protein, shares a common ligand with Cu^{2+}.

Materials and Methods

These were generally as detailed in previous communications (5,9,11). Apoprotein and $2Co^{2+}$-forms[1] were prepared as described in Reference 5. The $2Co^{2+}2Cu^{2+}$-derivative was formed either by slowly adding Cu^{2+} to $2Co^{2+}$-protein or by simultaneously infusing Co^{2+} and Cu^{2+} into an apoprotein solution maintained at room temperature and pH 5.5.

Magnetic susceptibility measurements were made as described previously except that de-oxygenation with the glucose glucose-oxidase system was omitted (12). The dissolved oxygen contribution for these samples was very small compared to the measured paramagnetism and was estimated from data for both buffer blanks. The magnetic moment was measured as a function of magnetic field from 0 to 3000 gauss at all temperatures, to check for ferromagnetic impurities or other anomalies. The susceptbility was taken from the linear, low-field part of these curves. Theoretically anticipated values of the magnetic susceptibility for various assumed metal ion configurations can be compared directly with the experimentally determined values without the need for corrections or assumptions concerning the diamagnetism of the protein or solvent.

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[1] The nomenclature used to identify the various derivatives referred to is based on the fact that the native protein contains two Zn-Cu pairs one on each subunit (2). Native protein is referred to as $2Zn^{2+}2Cu^{2+}$. Since only two Co^{2+} bind to the apoprotein (5) this is referred to as $2Co^{2+}$ protein, and it is assumed that Co^{2+} ions occupy the native Zn binding sites in this and other Co derivatives. The designation $2Co^{2+}2Cu^{2+}$ refers to an apoprotein solution containing two Meq each of Co^{2+} and Cu^{2+}. Similarly, $2Cu^{2+}2Cu^{2+}$- refers to a solution containing 4 Meq of Cu^{2+}.

Results

Chemical Properties

Optical absorption spectra typical of $2Co^{2+}2Cu^{2+}$ - and $2Co^{2+}2Cu^{+}$- are shown in Fig. 1. The spectrum of the former is identical with that published previously (5) and the spectrum of $2Co^{2+}2Cu^{+}$ -protein is qualitatively identical to that of the $2Co^{2+}$ derivative. The latter observation may suggest that Cu^{+} is no longer associated with the protein, and this was tested by measuring the removal of Cu^{+} from the dithionite reduced protein with bathocupreine sulfonate (BCS). Previous work (9) has shown that BCS does not react with dithionite reduced native protein at pH 7.8 and temperatures below 70°C, while all Cu^{+} can be removed by this chelating agent near 90°C.

The reactivity of BCS toward a representative sample of reduced 2Co2Cu deriva-

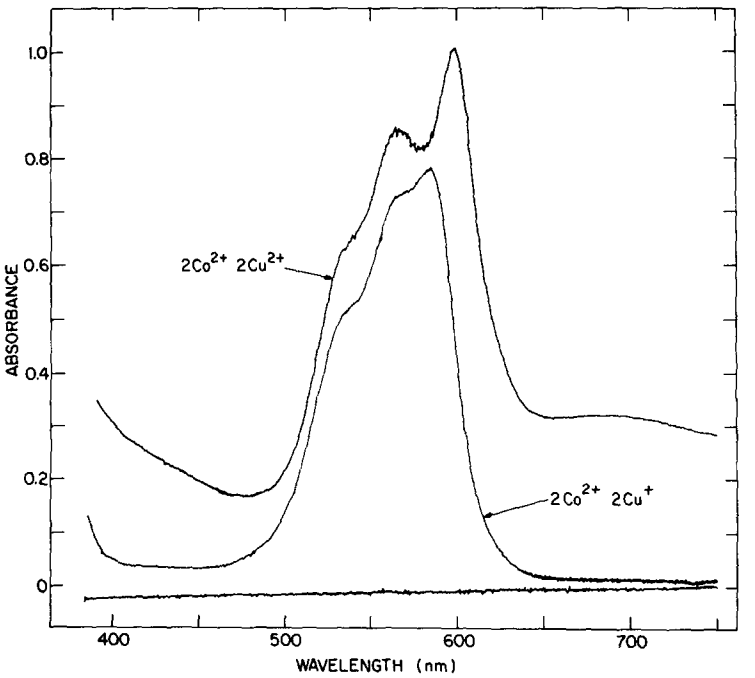

Figure 1 Optical absorption spectra of $2Co^{2+}2Cu^{2+}$- and $2Co^{2+}2Cu^{2+}$-superoxide dismutase derivatives. The concentration of Co and Cu were both 2.03 mM.

tive is shown in Fig. 2. About 25% of the total Cu^{+} reacted very rapidly with BCS, an additional 22.5% was chelated over a ten minute interval, and subsequent heating for 10 min. at 49°C caused only a small further release of Cu^{+} to BCS. These results are readily rationalized if part of the protein molecules of the original preparation before reduction exist as the $2Cu^{2+}2Cu^{2+}$ (or $4Cu^{2+}$) form, i.e., Cu^{2+} binding to both Zn and Cu sites (9,11). The properties of this derivative have been examined in some detail (9) and those relevant to this communication are listed:

(a) The four Cu^{2+} ions bind as two pairs exhibiting a broad featureless electron paramagnetic resonance (EPR) spectrum which arises from the excited triplet state.

(b) The magnetic coupling between the Cu^{2+} ions is on the order of $-50\ cm^{-1}$.

(c) Half the Cu^{+} of the fully reduced form reacts very rapidly with BCS while the

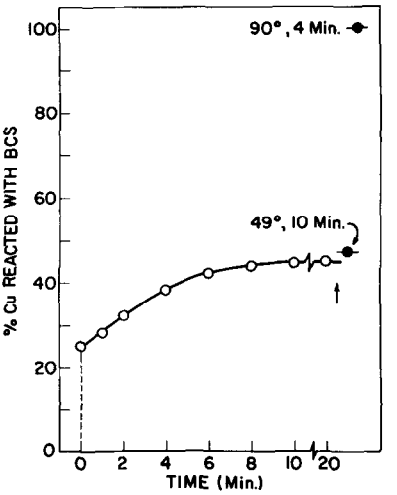

Figure 2 The removal of Cu^{+} from reduced 2Co2Cu superoxide dismutase with bathocupreine sulfonate. The reaction mixture consisted of 1.6 ml 0.7 M Tris-HCl pH 7.4, 0.4 ml, 1.53 mM BCS in H_2O, and 20µl $2Co^{2+}2Cu^{2+}$ dismutase being 2.9 mM in Cu. The reaction was initiated by adding a small amount of solid sodium dithionite on a Nichrome wire. The maximum absorption attained at 480 nm was 0.38 OD units. The absorption measurements were made at 22°C (○), and the sample was heated as indicated (●) in an external operation. In a separate experiment the BCS concentration was increased 33% at the time indicated by the arrow with no change in 480 nm absorbance. The total concentration of Cu determined using $\varepsilon_{480} = 1.25 \times 10^{4}\ M^{-1}\ cm^{-1}$ was within 10% of the anticipated value in all such experiments.

other half reacts over a period of about ten minutes. Since the reactivity of BCS toward $2Co^{2+}2Cu^{+}$ protein is qualitatively similar the $4Cu^{2+}$ derivative forms would appear to constitute a significant fraction of the original preparation. Examination of the EPR spectra of several $2Co^{2+}2Cu^{2+}$ samples at 100°K also showed the presence of the characteristic signal of the $4Cu^{2+}$-protein (9). The results shown in Fig. 2 suggest that this sample contained approximately 2.5% non-specifically associated Cu^{2+}, 45% $Cu^{2+}Cu^{2+}$ pairs (both evident in the EPR spectrum), and by difference 47.5% as $Co^{2+}Cu^{2+}$ pairs. Several samples of $2Co^{2+}2Cu^{2+}$ protein have been prepared by the two different procedures described in the Materials and Methods section and each shows a quantitatively similar reactivity toward bathocupreine sulfonate.

Single crystals of $2Co^{2+}2Cu^{2+}$ protein were obtained and x-ray crystallographic measurements carried out in collaboration with Dr. Martha Ludwig at the Biophysics Research Division, University of Michigan showed the crystallographic symmetry, space group (C2), and unit cell dimensions to be identical within error to those of the native protein (13). Further, EPR spectra of micro-crystalline suspensions and associated material in the mother liquor were identical.

The simplest conclusions which can be made from these observations regarding the heterogeneity of the $2Co^{2+}2Cu^{2+}$ preparations is that Cu^{2+} competes effectively with Co^{2+} for the Zn^{2+} binding site, but, as shown previously Co^{2+} does not appear to bind to the Cu^{2+} site (5). Thus our preparations of $2Co^{2+}2Cu^{2+}$ appear to contain a mixture of Co^{2+}-Cu^{2+} pairs in which Co^{2+} is occupying the Zn site and Cu^{2+} is in the Cu site, some Cu^{2+}-Cu^{2+} pairs in which Cu^{2+} occupies both sites, an amount of nonspecific Co^{2+} corresponding to the Cu-Cu pairs, and a very small amount of nonspecifically bound Cu^{2+}.

Magnetic Properties

The magnetic susceptibility of superoxide dismutase containing only cobalt and of the protein with both cobalt and copper are shown in Figure 3. It can be seen that the $2Co^{2+}$ protein yields data compatible with all the cobalt as fully paramagnetic high spin Co^{2+} with a large zero-field splitting (ZFS) between the S_z =

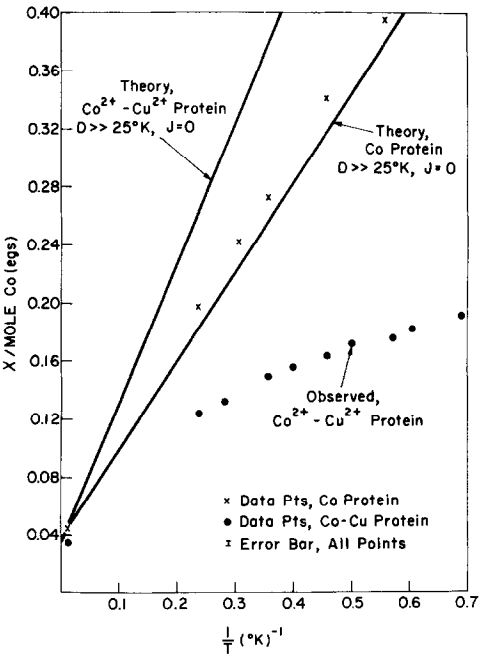

Figure 3 Magnetic susceptibility plotted against inverse temperature for samples of cobalt substitued superoxide dismutase: $2Co^{2+}$, 0.523 ml, 4.62 mM Co; $2Co^{2+}2Cu^{2+}$, 0.590 ml, 5.58 mM Co^{2+} and Cu^{2+}. The data are given per mole of Co. The expected contribution from added Cu^{2+} with no exchange coupling is expressed in the theoretical curve given for the $2Co^{2+}2Cu^{2+}$-protein.

$\pm 3/2$ and $S_z = \pm 1/2$ doublets. The data with Cu^{2+} present are in sharp contrast. Instead of observing an increased susceptibility due to the additional presence of paramagnetic Cu^{2+}, the total susceptibility is <u>decreased</u>, much below that expected even with Co alone having an arbitrarily large ZFS. Moreover, the susceptibility is no longer linear in inverse temperature in the 77° to 1.5°K temperature range. Results for two independently prepared $2Co^{2+}$-$2Cu^{2+}$ -samples were repeatable within 15% for the measured paramagnetism as a fraction of the paramagnetism expected from metal content alone. In a separate experiment, titration of $2Co^{2+}$ protein with Cu^{2+} showed an incrementally decreasing susceptibility approaching the limit of the $2Co^{2+}2Cu^{2+}$ samples. Additionally, Figure 4 shows that a sample of $2Co^{2+}$-$2Cu^{2+}$ protein exhibiting low, non-linear susceptiblity, and then treated to reduce

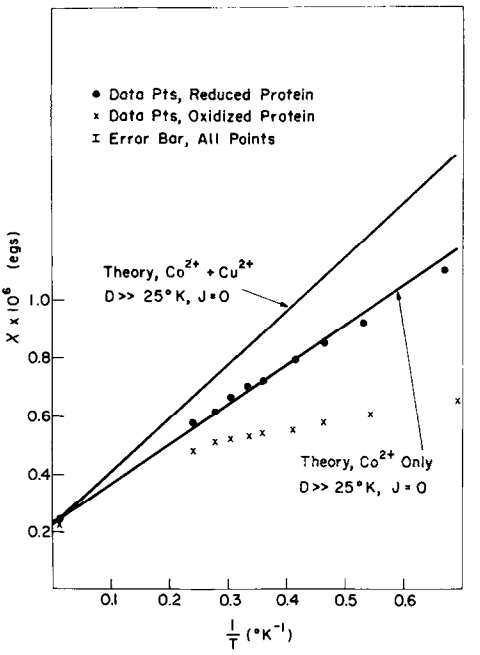

Figure 4 Magnetic susceptibility plotted against inverse temperature for a sample of 2Co2Cu superoxide dismutase in the oxidized and reduced forms. The concentration of Co and Cu were both 2.03 mM, and these were the same samples used in the experiment described in Fig. 1. The theoretical curve for Co^{2+} alone represents the assumption that the Cu ion is Cu^{+} in the reduced for of the protein.

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the paramagnetic Cu^{2+} to diamagnetic Cu^{1+}, subsequently shows a linear, increased paramagnetism, to a level expected for the Co content as independent, fully spin-free ions.

DISCUSSION

The normal high spin (S = 3/2) Co^{2+} ion has EPR g-values far from the free spin value of 2.0, and due to rapid spin relaxation and consequent broadening at higher temperatures, EPR signals are generally observed for this species only at temperatures lower than 77°K. This is the case for the $2Co^{2+}$ dismutase discussed here which has g-values near 4 and 2 (5) and thus labels those ions as S = 3/2 Co^{2+}. Such an ion will have a zero-field splitting (ZFS) between the $S_z = \pm 3/2$ and $S_z = \pm 1/2$ levels giving a ground state with effective spin $S_z = \pm 1/2$ which will, when the sign of the ZFS parameter is positive, be exclusively populated at temperatures

much lower than the magnitude of ZFS. Values of ZFS have been reported for Co^{2+} in a variety of environments; these range from ~-40 to +80 cm^{-1} (14-17). The observed susceptibility corresponds to that expected for an effective spin $S_z = \pm 1/2$, with the measured g-values, within the entire temperature region where we have good sensitivity ($1.5 < T \lesssim 20°K$). It appears, then, that the magnitude of ZFS must be quite large, probably greater than 20 cm^{-1}. Blumberg and co-workers (18) on the basis of EPR measurement have estimated that the ZFS of Co^{2+} in the $2Co^{2+}2Cu^{+}$ protein is 23 cm^{-1}. Further, the linear dependence of the susceptibility indicates the absence of significant magnetic exchange between the two Co^{2+} ions. This would be expected if the Zn^{2+} sites are occupied as these are known to be ~34Å apart in the dimer form of the molecule (2).

In contrast to the independence of the cobalt (zinc) sites from one another, each copper site seems closely coupled to a cobalt in the $2Co^{2+}2Cu^{2+}$ protein. The loss of Co^{2+} EPR signal on addition of Cu^{2+} to the $2Co^{2+}$ protein observed previously (5,18) had indicated at least magnetic dipole coupling between the Cu^{2+} and Co^{2+}, but the lowered susceptibility of the $2Co^{2+}2Cu^{2+}$ form shows actual exchange interactions, leading to antiferromagnetic coupling.[2] The molar susceptibility of the $2Co^{2+}2Cu^{2+}$ form at 1.5°K is less than 25% that of the $2Co^{2+}$ protein, even with the Co^{2+} all in the ground $S_z = \pm 1/2$ doublet. This indicates that exchange coupling must be invoked, and that, for example, a Cu-induced spin state change of the Co^{2+} to a configuration with isotropic g-values near 2.0 would not suffice to account for the data. Further confirmation of the Co^{2+}-Cu^{2+} coupling is obtained from the experiments (Fig. 4) in which the full expected Co susceptibility is restored by reducing the Cu^{2+} to diamagnetic Cu^{1+}. Similar (unpublished)

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[2] Blumberg (18) has stated that the Co^{2+} and Cu^{2+} must be antiferromagnetically coupled with a constant (-2J) larger than about 100 cm^{-1}. This estimate was based on the observation that no change in appearance or non-Curie dependence of the EPR spectrum of a mixture of $2Co^{2+}2Cu^{2+}$ and native protein was observed over the temperature range 1.4 to 80°K. While this might be consistent with the observations (5,18) the EPR behavior of a coupled 3/2-1/2 spin system having both J and the zero-field splitting parameters undefined is not easily predicted and a direct observation of antiferromagnetic coupling is therefore desireable.

results were obtained for a $2Co^{2+}2Hg^{2+}$ protein complex, in which diamagnetic Hg ions appear to occupy the Cu sites. Again, the full Co paramagnetism was measured, consistent with the lack of another paramagnetic ion in the cluster for the Co to couple with. In general, this means that the copper must be very closely linked with a Co site, separated at most by a bridging group.

The exact strength of the exchange coupling between the Co and Cu is difficult to ascertain from curve-fitting to the susceptibility data because of the presence of some non-specifically bound copper and cobalt in the samples. Since the $4Cu^{2+}$ protein is already known to be diamagnetic below 10°K (9), the residual paramagnetism evident in the 1/T 0.25 to 0.69 range must be presumed to arise from Co^{2+}-Cu^{2+} pairs and isolated non-specifically bound Co^{2+} and Cu^{2+} ions. While it is possible to make reasonable estimates of the concentrations of the various species present from EPR and the type of experiment described in Fig. 2 there are still several parameters which are unrestricted: ZFS of specific and non-specific Co^{2+}, and the Co-Cu exchange coupling constant. While these make curve fitting of dubious value, our tentative analyses along this line suggest that the coupling constant (-2J) between Co^{2+} and Cu^{2+} is at least 5 cm^{-1} and is probably much greater (19). It should be emphasized that these difficulties do not detract from the basic qualitative conclusion that a large fraction of the Cu^{2+} and Co^{2+} ions in these samples must participate in some form of magnetic exchange interaction.

In conclusion, the magnetic susceptibility data confirm predictions from previous EPR studies which suggested that there might be two pairs of metal sites in the dimer and they are consistent with the recent structural studies (2) which would predict that some degree of exchange interaction would occur between paramagnetic ions occupying both Cu and Zn sites. Zn/Cu superoxide dismutase can be confidently included among a growing list of metalloproteins which utilize "clusters" of metal ions in the performance of biological function: ferredoxins, hemerythrin, hemocyanins, tyrosinase, ribonucleotide reductase, and multicopper oxidases (20-25 and references therein).

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