A Study of the Electron Paramagnetic Resonance Properties of Single Monoclinic Crystals of Bovine Superoxide Dismutase

(Received for publication, June 16, 1981)

Robert A. Liebermant‡, Richard H. Sands§, and James A. Fee†
From the Biophysics Research Division and Departments of Physics and Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109

Monoclinic crystals of native bovine superoxide dismutase and its monocyano derivative were studied by means of electron paramagnetic resonance spectroscopy. Through computer simulation of the spectra, the directions of the principal axes of the magnetic tensors (g and A) have been found with respect to the crystal principal axes and with respect to the positions of atoms near the Cu(II) as previously determined by X-ray crystallography (Richardson, J. S., Thomas, K. A., and Richardson, D. C. (1975) Biochem. Biophys. Res. Commun. 63, 986-992; Tainer, J. A., Getzoff, E. D., Richardson, J. S., and Richardson, D. C. (1980) in 2SOD: Cu, Zn-Superoxide Dismutase Complete Atomic Coordinates (Richardson, D. C., and Richardson, J. S., eds) Brookhaven Protein Structure Data Bank). In the native protein, the direction of the g, axis of Cu(II) was found to lie perpendicular to the rough plane formed by the four imidazole nitrogen atoms coordinated to the Cu(II). The direction of g, is approximately along the His 44N-Cu-His 48N direction, and g, is in the direction of the Cu-His 61-Cu-N bond. The A is coaxial with g, within 15° C. A substantial shift occurs in the direction of g, when CN− binds to the Cu(II), suggesting a change in the coordination configuration of the metal.

Superoxide dismutases are metalloproteins which catalyze the reaction, 

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$

They are readily classified according to the metal ion cofactor necessary for this activity, Fe, Mn, or Cu (1). While the distribution of these catalysts in different life forms is quite variable, most eucaryotic cells express a dimeric protein binding one Zn(II) and one Cu(II) per identical 16,000-dalton subunit. This protein, which was first described in 1939 by Mann and Keilen (2) and termed hemocuprein, has had a somewhat turbulent history as indicated by the different appellations it has accrued over the years. Presently, it is called superoxide dismutase (3), but this has not been proven to be the physiological function (4), and the biological role of the protein remains controversial.

X-ray crystallographic analyses to a resolution of 3 Å (5-8)

* Summarizes the main results of work performed by R.A.L. in partial fulfillment of the requirements for the Ph.D. in Physics from the University of Michigan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Bell Laboratories, 600 Mountain Avenue, Murray Hill, NJ 07974.
§ Supported by United States Public Health Service Grants GM12176.
¶ Supported by United States Public Health Service Grant GM15319. To whom inquiries should be directed at the Biophysics Research Division, 2200 Bonita Boulevard, Ann Arbor, MI 48109.

† Supported by United States Public Health Service Grant GM15319. To whom inquiries should be directed at the Biophysics Research Division, 2200 Bonita Boulevard, Ann Arbor, MI 48109.

1 This drawing and the drawings in Figs. 6 and 7 were generated by a computer program which makes use of the NAMOD molecular display package which is available from the National Protein Data Bank (9, 10). Atomic coordinates used were those listed in the May, 1980 "DAPRT" tape.

2 D. C. Richardson and J. S. Richardson, personal communication.

tube using a narrow strip of filter paper, and the surrounding fluid was wicked out. The tube was then sealed with wax and cemented to a sample holder having the same dimensions as that used on a standard Charles Supper goniometer head. The monocrysal derivative was prepared by adding a small amount of mother liquor made 2 mM in KCN prior to sealing the tube. After 1 h at room temperature the crystal had turned a deep purple color, and it was subsequently handled in the same fashion as crystals of holoprotein.

The orientations of the crystal unit cell axes with respect to the sample mount (Fig. 1) were determined by means of x-ray diffraction using a Buerger precession camera. Fifteen-degree precession photos were then recorded with the x-ray beam along the [100] and along the [001] axis and compared with previously published photos (5) to ensure proper alignment. Subsequently the mounted crystal was transferred to the EPR goniometer system. This consisted of a 72-tooth "antibacklash" worm wheel mated to a four-turn worm drive as indicated diagrammatically in Fig. 1. The axis of the worm wheel is perpendicular to the axis of the rotating base of the magnet, thus permitting complete freedom in orienting the crystal relative to the DC field ($H_0$) of the EPR experiment. The spindle and two arc settings of the x-ray goniometer system which resulted in placing the x-ray beam along the crystal axes were used as input to a computer program (24, 25) which calculated the wormwheel and magnet base settings for any desired orientation of the crystal with respect to $H_0$. By using ruby crystals as standards it was determined that this system results in less than 0.5° overall alignment error.

Crystal EPR spectra were recorded using a home-built instrument (cf. Ref. 25). Briefly, the spectrometer utilizes a "hot carrier" diode as the detector in a reflection bridge in conjunction with a phase-sensitive detection system. The sample arm, which is mounted on the EPR goniometer system, terminates with a rectangular quartz-filled cavity operating in the TE00 resonance mode. This cavity is constructed from a quartz block (0.67 × 1.04 × 1.33 cm) which is plated with gold and coupled to the waveguide through a small hole in the plating. The block also has a 2-mm diameter hole drilled from one of the opposite faces perpendicular to the coupling hole to within 1 mm of the opposite face. The quartz tube containing the crystal is inserted into this hole such that the crystal lies in a region of high microwave magnetic field density. This cavity design was intended (27) to give a high signal to noise ratio for small samples and was found to be adequate for the crystals available to us.

The cavity and orientation gear system was maintained at 77 K by enclosure in a glass finger immersed in liquid nitrogen, the inside of which was flushed with dry nitrogen gas to avoid condensation. EPR spectra were recorded at 30 milliwatts microwave power ($H_0$ ~ 0.3 G, peak-to-peak (30), 6 kHz modulation frequency, 8 to 15 G modulation amplitude, and approximately 9.5 GHz microwave frequency. The signal was recorded on a Varian D-370 signal averager and subsequently transferred to magnetic tape for storage on files in the Michigan Terminal System for comparison with computed spectra.

Frozen solution EPR spectra were recorded on a Varian E-112 spectrometer at temperatures near 90 K.

Data

EPR spectra of $^{65}$Cu-substituted bovine superoxide dismutase in frozen buffer solution, a slurry of microcrystals in frozen mother liquor, and its monocrysal derivative in frozen buffer are shown in Fig. 2, a-c. It is evident that the spectrum of the native protein does not depend on the solvents used in these experiments. Thus the frozen solution spectra are identical in mother liquor and in phosphate buffer, and these spectra are indistinguishable from previously published spectra of the protein (13-15). Representative experimental spectra obtained from single crystals of native protein and the monocrysal derivative are shown in Figs. 3 and 4, respectively, and additional spectra are presented in Ref. 25. These data were analyzed as described in the following section, and the smooth lines shown in Figs. 2-4 are the computed spectra resulting from this analysis.

Computations

Calculation of EPR Spectra (General)—Both frozen solution and single crystal spectra were analyzed by means of computer simulation. The programs written for this purpose utilized a computational al-

---

* R. A. Lieberman, R. H. Sands, and J. A. Fee, manuscript in preparation.

---

**MATERIALS AND METHODS**

Bovine superoxide dismutase was prepared by the method of McCord and Fridovich (3) or purchased from Diagnostic Data, Inc. (Mountain View, CA). The protein was reconstituted with Zn(II) and $^{65}$Cu(II) as described previously (23).

Crystals of native and reconstituted protein were grown in small sealed bottles containing 0.56 ml of vacuum-distilled 2-methylpentanediol, 0.28 ml of 0.2 M KH$_2$PO$_4$, 0.02 ml of 0.2 M K$_2$HPO$_4$, and 0.14 ml of a protein solution (18 mg/ml) in glass-distilled water. The final apparent pH as measured with a glass electrode was 7.2. Several such preparations were prepared and was somewhat lower than that reported, and (b) the 3-methylpentanediol concentration was only 52% by weight compared to the previously used 58%.
Algorithm based on second order perturbation theory (27) treatment of the spin Hamiltonian.

$$H = \beta S \cdot \mathbf{g} \cdot \mathbf{H} + S \cdot \mathbf{A} \cdot \mathbf{i}$$  (1)

In the notation of Toriyama and Iwasaki (28), the four allowed \( \Delta m = 0 \) transitions occur at magnetic fields given by the expressions

$$H(\pm \frac{1}{2}) = g_{\text{free}} / g[H_0 \pm \frac{1}{2}K - A_{3/2}/8(H_0 \pm 3K/2)]$$  (2)

$$H(\pm \frac{1}{2}) = g_{\text{free}} / g[H_0 \pm \frac{1}{2}K - A_{1/2}/8(H_0 \pm K/2)]$$  (3)

where

$$g_{\text{free}} = \text{free electron } g \text{ value}$$  (4)

and

$$A_{3/2} = 3[\text{Tr } \mathbf{A}^2 + 2 \mathbf{A} \cdot \mathbf{K} - 3K^2]$$  (5)

$$A_{1/2} = 7[\text{Tr } \mathbf{A}^2 - 6 \mathbf{A} \cdot \mathbf{K} - K^2]$$  (6)

$$K = 1/g \sqrt{\mathbf{g} \cdot \mathbf{g}^2 - \mathbf{h} \cdot \mathbf{h}}$$  (7)

$$g = \sqrt{\mathbf{g} \cdot \mathbf{g}^2 - \mathbf{h} \cdot \mathbf{h}}$$  (8)

$$\mathbf{h} = H_0/|H_0|$$  (10)

From Equations 5 and 6 it can be seen that the values of \( A_{3/2} \) and \( A_{1/2} \) are always positive. Thus, the effect of the second order terms (the third terms in Equations 2 and 3) is to shift all resonances to slightly lower magnetic field values. This shift is on the order of 0–10 G, depending upon the orientation of \( \mathbf{h} \) with respect to the principal axes of \( \mathbf{g} \) and \( \mathbf{A} \).

Neither the nuclear quadrupole interaction \( (I \cdot \mathbf{p} \cdot \mathbf{I}) \) nor the nuclear Zeeman interaction \( (g_e \beta_e \mathbf{I} \cdot \mathbf{H}_0) \) were included in Equation 1. The maximum strength of the interaction \( |P_{11}| < 15 \text{ MHz} \) was estimated by electron-electron double resonance (ELDOR) measurements. Thus the shift in the resonance line position produced by this interaction produces \( |P_{11}|/K \) is an order of magnitude less than the shift due to the second order terms in Equations 2 and 3. The nuclear Zeeman interaction is not included in Equation 1 because it has no effect on the field values at which the \( \Delta m = 0 \) lines occur. Further, the simulation programs do not include the forbidden transitions \( (\Delta m \neq 0) \) since these have intensities which are estimated to be less than \( 10^{-7} \) of the intensity of the allowed transitions (25).

Line positions calculated via Equations 2 and 3 are convoluted with Gaussian lineshape functions. This approximates the broadening due to superhyperfine interactions between the electronic spin of the Cu(II) and the neighboring nitrogen atoms. The width of the lines is calculated by using a linewidth tensor, assumed to be coaxial with the

resonance properties of bovine superoxide dismutase crystals

**Cu(I1) in the sample** are randomly oriented with respect to the magnetic tensor axes of a particular Cu(I1) asymmetric unit contains two molecules each consisting of identical subunits related by a local (monocrystalllographic) 2-fold rotation axis, and (d) that the orientation of these rotation axes with respect to the crystal axes are as given in Ref. 6 and information available from the National Protein Data Bank. Assumption b is reasonable since spectra from randomly oriented microcrystals suspended in mother liquor are identical with frozen solution spectra in both mother liquor and buffer (Fig. 2b). Thus, the tensor principal values which were found from the analysis of the frozen solution spectra were used as input values to the computer program.

The objective of these calculations was to determine the orientation of the magnetic tensor axes with respect to a coordinate system fixed to the protein molecule. The coordinate system used was centered on the Cu(I1) (Fig. 6); the axes are called \( \hat{u} \) (parallel to the Cu-Cu vector within the molecule), \( \hat{v} \) (parallel to the noncrystallographic C2 axis), and \( \hat{w} \) (parallel to the noncrystallographic C2 axis). The angles \( \epsilon_s \), \( \omega \), and \( \theta \) which relate the tensor principal values to this coordinate system are the only free parameters used in the calculation.

It must be noted that, due to the presence of the local 2-fold axes, there are defined relationships between the axis sets used to describe g tensor directions within a particular dimer. For example, if the dimers are numbered as described in Footnote 8, then the local coordinate axes (Fig. 6) within an asymmetric unit have the following relationships: \( \hat{u}_1 = -\hat{u}_3 \), \( \hat{u}_2 = -\hat{u}_4 \), \( \hat{v}_1 = -\hat{v}_3 \), \( \hat{v}_2 = -\hat{v}_4 \), \( \hat{w}_1 = \hat{w}_3 \), and \( \hat{w}_2 = \hat{w}_4 \). In the simulation program, the direction cosines of the \( \hat{u} \) and \( \hat{w} \) axes for sites 1 and 3 within an asymmetric unit were used as input data along with \( \epsilon_s \), \( \omega \), and \( \theta \) chosen by the operator. The input angles are used to compute the direction cosines of the \( \hat{u} \) axes along \( \hat{u} \), \( \hat{v} \), and \( \hat{w} \) for Sites 1 and 3 (assumed to be the same). Next, another set of direction cosines, with \( \hat{u}_1 = -\hat{u}_3 \), \( \hat{v}_1 = -\hat{v}_3 \), \( \hat{w}_1 = \hat{w}_3 \), and \( \hat{w}_2 = \hat{w}_4 \), is formed, corresponding to the tensor directions of the second center in each dimer (2 or 4), as expressed in the axis system of the first dimer (1 or 3). This corresponds to performing the local symmetry operation. The two sets of direction cosines found in this way are then multiplied by the direction cosines relating \( \hat{u} \), \( \hat{v} \), and \( \hat{w} \) to the \( \alpha \), \( \beta \), and \( \gamma \) crystal axes, thus producing direction cosines of the \( \hat{g} \) axes with respect to the crystal axes for all four centers within an asymmetric unit. Finally, another four sets of direction cosines, representing the \( \gamma \) axis directions for the four sites in the other, orientationally inequivalent crystal asymmetric unit are found by changing the signs of the \( \alpha \) and \( \beta \) direction cosines. This represents to the crystallographic C2 operation. The simulation program uses the eight sets of direction cosines found by the above procedure, in conjunction with the known orientation of \( \hat{H} \) and \( \hat{H} \) with respect to the crystal axes, to compute EPR spectra. For each of the directions (Equations 1, 3, 6, 7) in both the orientationally inequivalent ions within the crystal unit cell. The program then convolves each position with Gaussian lineshapes of the proper intensity, creating eight single ion spectra which are summed to yield the simulated spectrum.

The presence of the local 2-fold axis introduces an unavoidable ambiguity in relating the magnetic tensor principal axis directions to the molecular coordinates. This is because the EPR experiment cannot distinguish between the tensor axes sets of the two different Cu(I1) ions within one protein molecule. Specifically, the tensor axes orientation with respect to the local C2 axis, \( \hat{u} \), \( \omega \), and \( \theta \), throughout the entire parameter space in increments of \( 10^5 \). Totally unacceptable fits (line positions off by more than 100 gauss) resulted.

The simulation program made use of the obtained X-ray structural data to determine the directions of the local reference axes. If there is an exact C2 relating the two halves of the dimer, then \( \hat{u} \) and \( \hat{w} \) should be rigorously perpendicular. In analyzing the data obtained from the National Protein Data Bank, it was found that the \( \hat{u} \) axes had to be shifted up to 3° from the indicated Cu-Cu vectors in order to make \( \hat{u} \) perpendicular to the indicated directions of the local C2. This indication of a slight error in the crystallographic data means that the tensor axis directions reported in this work are accurate to no more than ±3°.

---

**Note:** The colors are used merely to distinguish between identical protein subunits within the asymmetric unit of the crystal, and they do not imply any structural or spectral differences. In other work by Richardson and co-workers (6-8), numbers are used to distinguish between subunits. Thus 1 = "blue," 2 = "green," 3 = "orange," and 4 = "yellow."
when the \( g \) axis was outside the region \( 50^\circ < \epsilon_1 < 90^\circ, -50^\circ < \omega_2 < 20^\circ, \) and \( 60^\circ < \epsilon_2 < 100^\circ \). Within this range, angles were varied by amounts of \( 5^\circ \) or less in order to find the orientations which gave the best visual correspondence between calculated and experimental spectra.

RESULTS

The parameters used to obtain the simulated frozen solution spectra shown in Fig. 2 are listed in Table I. Differences between these and previously published values (also listed in the table) are primarily due to the fact that our program introduces the second order term of Equations 2 and 3. A significant result is that unless the Euler angles describing \( \bar{A} \) with respect to \( \bar{g} \) are kept small \((<15^\circ)\), satisfactory fits cannot be obtained to any of the spectra. Thus, it can be concluded that \( \bar{g} \) and \( \bar{A} \) are essentially coaxial for both the native and

**Fig. 5.** Representation of bovine superoxide dismutase molecules in the C2 unit cell. The arrows labeled 1-2 are the local (noncrystallographic) 2-fold axes relating the two subunits of a single molecule. The "blue," "green," and the "orange," "yellow" subunits correspond to dimer 1-2 and dimer 3-4, respectively, in the notation of Thomas et al. (7). The four asymmetric units are shown with the Cu(I) ions in the like shaded units being related by a simple translation, thus only 8 of the 16 Cu(I) ions in the unit cell are orientationally inequivalent. The double headed arrows along \( b \) represent crystallographic 2-fold axes, and the single headed arrows signify 2-fold screw axes.

**Fig. 6.** Local coordinate system used in simulating the single crystal EPR spectra. \( \hat{u} \) is defined as the Cu-Cu vector, \( \hat{w} \) is parallel to the local symmetry axis, and \( \hat{w} = \hat{u} \times \hat{w} \). The orientation of each 2-fold with respect to the crystal axes was taken from the results of Thomas et al. (7) and information deposited with the National Protein Data Bank (cf. Footnote 9). \( \alpha \), the \( \hat{u}, \hat{v}, \hat{w} \) coordinate system for a dimer. \( \beta \), indication of the two tensor orientations, \( \gamma \), which give identical EPR spectral patterns (see text).

**Table I**

| Magnetic tensor principal values of bovine superoxide dismutase |
|---------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Isotope | 13 Cu | 14 Cu | 15 Cu | 16 Cu | 17 Cu | Present work |
| \( g_0 \) | \( 2.03 \pm 0.01 \) | \( 2.03 \pm 0.01 \) | \( 2.03 \pm 0.01 \) | \( 2.03 \pm 0.01 \) | \( 2.03 \pm 0.01 \) | \( 2.03 \pm 0.01 \) |
| \( g_\epsilon \) | \( 2.11 \) | \( 2.108 \) | \( 2.051 \) | \( 2.11 \) | \( 2.103 \) | \( 2.09 \pm 0.01 \) |
| \( g_\omega \) | \( 2.265 \) | \( 2.265 \) | \( 2.259 \) | \( 2.259 \) | \( 2.257 \) | \( 2.26 \pm 0.01 \) |
| \( A_\| \) (MHz) | 112 | 112 | 112 | 116.9 \( \pm 10 \) | 168.1 \( \pm 10 \) | \( 103.7 \pm 10 \) | 112.1 \( \pm 10 \) | 112.1 \( \pm 10 \) |
| \( A_{\perp} \) (MHz) | 209 | 131 | 131 | 103.7 \( \pm 10 \) | 112.1 \( \pm 10 \) | 112.1 \( \pm 10 \) |
| \( A_{\perp} \) (MHz) | 434 | 412 | 420 | 417 | 417 | 426 \( \pm 3 \) | 451 \( \pm 3 \) |
| \( 1w_0 \) (g) | 25 | 25 | 25 | 50 \( \pm 8 \) | 50 \( \pm 8 \) | 25 \( \pm 8 \) |
| \( 1w_0 \) (g) | 25 | 25 | 25 | 24 \( \pm 3 \) | 24 \( \pm 3 \) | 24 \( \pm 3 \) |

<table>
<thead>
<tr>
<th>Cyano Protein</th>
<th>Native work</th>
</tr>
</thead>
<tbody>
<tr>
<td>( g_0 )</td>
<td>( 2.05 \pm 0.01 )</td>
</tr>
<tr>
<td>( g_\epsilon )</td>
<td>( 2.21 )</td>
</tr>
<tr>
<td>( A_| ) (MHz)</td>
<td>70.1 ( \pm 10 )</td>
</tr>
<tr>
<td>( A_{\perp} ) (MHz)</td>
<td>619 ( \pm 3 )</td>
</tr>
<tr>
<td>( 1w_0 ) (g)</td>
<td>30 ( \pm 8 )</td>
</tr>
<tr>
<td>( 1w_0 ) (g)</td>
<td>25 ( \pm 5 )</td>
</tr>
</tbody>
</table>
The site designation refers to single ion spectra created by the simulation program. As explained in the text, Site 1 may be the Cu(II) ion of either the blue or green subunit. If it is blue, then so is Site 5, and Sites 2 and 6 are green. Similar relationships exist between sites 2, 3, 7, 8, and the yellow and orange subunits.

### Table II

<table>
<thead>
<tr>
<th>Site</th>
<th>$\vec{g}$ axis</th>
<th>Crystal axis</th>
<th>Symmetry related pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$a^*$</td>
<td>$b$</td>
<td>$c$</td>
</tr>
<tr>
<td>Native protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$g_x$</td>
<td>0.0259</td>
<td>-0.438</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>-0.961</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>-0.058</td>
<td>-0.895</td>
</tr>
<tr>
<td>2</td>
<td>$g_x$</td>
<td>0.0512</td>
<td>0.0274</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>0.0827</td>
<td>0.0094</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.277</td>
<td>0.0955</td>
</tr>
<tr>
<td>3</td>
<td>$g_x$</td>
<td>0.0317</td>
<td>0.0063</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>-0.551</td>
<td>-0.830</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.0023</td>
<td>0.0764</td>
</tr>
<tr>
<td>4</td>
<td>$g_x$</td>
<td>0.0410</td>
<td>-0.073</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>0.0879</td>
<td>-0.231</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.241</td>
<td>-0.970</td>
</tr>
<tr>
<td>5</td>
<td>$g_x$</td>
<td>-0.259</td>
<td>-0.438</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>0.981</td>
<td>-0.065</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.058</td>
<td>-0.895</td>
</tr>
<tr>
<td>6</td>
<td>$g_x$</td>
<td>-0.512</td>
<td>0.0274</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>-0.827</td>
<td>0.0094</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.0227</td>
<td>0.0955</td>
</tr>
<tr>
<td>7</td>
<td>$g_x$</td>
<td>-0.179</td>
<td>0.0362</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>0.551</td>
<td>0.830</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.023</td>
<td>0.0764</td>
</tr>
<tr>
<td>8</td>
<td>$g_x$</td>
<td>-0.410</td>
<td>-0.073</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>0.879</td>
<td>-0.231</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.0241</td>
<td>-0.970</td>
</tr>
<tr>
<td>Cyanide-treated protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>$g_x$</td>
<td>0.138</td>
<td>-0.715</td>
</tr>
<tr>
<td></td>
<td>$g_y$</td>
<td>0.549</td>
<td>0.0803</td>
</tr>
<tr>
<td></td>
<td>$g_z$</td>
<td>0.0232</td>
<td>0.0529</td>
</tr>
<tr>
<td>2</td>
<td>$g_x$</td>
<td>0.551</td>
<td>-0.830</td>
</tr>
</tbody>
</table>

*Table II Direction cosines of the g tensors with respect to the crystal axes of the eight orientationally inequivalent Cu(II) in the C2 unit cell of bovine superoxide dismutase*
up to 32 resonance lines (four hyperfine lines per ion). The fact that only eight lines appear implies that the ions are so arranged that pairs of ions have magnetic tensors with $g_x$ axes nearly parallel to one another. Before the crystal EPR fitting results became available, it was thought that this apparent pairing of centers might be due to the dimeric nature of the protein. This would imply that the $g_x$ axes of the two Cu(II) within a dimer might be perpendicular to the local 2-fold axis. The data of Table II, however, show that Sites 1 and 3 have $g_x$ axis directions differing by 14°, Sites 2 and 8 differ by 10°, Sites 4 and 6 by 10°, and Sites 5 and 7 by 14°. Thus, the fitting process has shown that the pairs of centers are related to one another only by fortuitous coincidences which arise from the crystal geometry.

The results in Table II are unambiguous in the sense that they describe the principal axis directions of each of the eight orientationally inequivalent Cu(II) ions within the unit cell. As explained earlier, however, there are two different sets of local tensor axis orientations which could give rise to the sets of direction cosines listed in Table II. (For example, Site 3 in Table II could be the Cu(II) of either the "orange" or "yellow" subunit). Both possible sets are shown in Fig. 7 and are also listed in Table III.

The direction cosines for the $g_x$ axes of the eight Cu(II) in cyanide-treated crystals are listed in Table II. Since axial symmetry was assumed in the fitting of spectra recorded from these crystals, $g_z$ and $g_y$ directions are not meaningful. Fig. 2 shows representative EPR spectra obtained from the cyanide-treated crystals along with the fits obtained using the parameters listed in Table II. Again, we have an ambiguity in the direction of $g_x$ with respect to the atomic coordinates (Table III), and the two possible directions of $g_x$ are shown in Fig. 8. Note that, no matter which of the possible $g_x$ directions is the correct one, the $g_x$ direction for the cyano derivative is different from that of the native protein.

**DISCUSSION**

For Cu(II) liganded to four nitrogen atoms, as in a tetraimidazole complex, molecular orbital calculations (30-33) show that $g_x$ will be perpendicular to the Cu/ligand atom plane provided the unpaired electron resides in the $B_1$, (approximately $d_{x^2-y^2}$) orbital. Under a tetrahedral distortion of the complex (raising one N atom above its original position in the plane), the $g_x$ axis will shift direction slightly, but still remains roughly perpendicular to the original plane. The $g$ tensor will, however, no longer be axially symmetric; one of the new principal axes ($g_x$ or $g_y$) will be directed from the Cu(II) to a point somewhere between the old and new positions of the

![Fig. 7. Stereo view of the "orange" metal binding center of bovine superoxide dismutase observed along the [010] (b) axis, showing the two equivalent orientations of the $g$ tensor. $a$, axis set "4" in Table II (native protein). $b$, axis set "3" in Table II (native protein).](http://www.jbc.org/)
displaced nitrogen atom. The other axis will point toward one of the other nitrogen atoms at a corner of the square adjacent to the displaced atom (25).

In the Cu(II) binding site of the native protein molecule, His 46N' is in a position out of the "plane" formed by His 44N', His 61N', His 118N', and Cu(II). Insofar as the structure resembles a tetrahedrally distorted square, His 46N' may be thought of as the "displaced ligand" in the preceding discussion, with His 61N' and His 118N' being the adjacent atoms.

Stereochemical considerations may be used to determine which of the two possible sets of local magnetic tensor axis orientations is most likely to be correct. For both possible sets of tensor axis directions (Fig. 7, a and b), g, lies roughly along the direction from Cu(II) to His 61N'. For the g tensor axis directions shown in Fig. 7a, g, is along a direction which is roughly in line with His 44N'-Cu(II)-His 46N'. It extends from Cu(II) along a direction somewhere between the Cu(II) → His 46N' direction (in the direction of the "displaced" N atom) and the His 46N' → Cu(II) direction ("undisplaced" direction). For the alternative orientation (Fig. 7b) the g, direction is farther from the His 44N'-Cu(II)-His 46N' plane, and g, would not lie between the "displaced" and "undisplaced" positions of His 46N'.

From the qualitative discussion above, it appears that the correct tensor axis orientation is the one illustrated in Fig. 7a. This choice corresponds to associating Sites 1 and 5 in Table II with the subunit labelled "blue" by Richardson et al. (7, 35), Sites 2 and 6 with the "green" subunit, and Sites 3 and 7 with the "yellow" subunit, and Sites 4 and 8 with the "orange" subunit.

Since no structural information is available from crystallographic studies for the cyanide derivative, it is more difficult to determine which of the two possible g, orientations (Fig. 8) is the correct one for this complex. However, despite this una-
voidable unambiguity, a very interesting conclusion may be obtained by comparing the two possible g directions of the cyano complex with the chosen g direction for the native protein. In Fig. 8a, the g direction is 30 ± 10° from the g direction for the native protein; in Fig. 8b, it is 60 ± 10° from the native g direction. In either case, the shift is significantly greater than the experimental limit of 10° from the g direction.

Such a shift is unlikely unless there is a significant rearrangement of the ligating atoms around the Cu(II). The fact that the principal values of the magnetic tensors found by computer fitting change from rhombic to axially symmetric upon binding CN⁻ provides additional evidence for a structural change in the immediate vicinity of Cu(II). The super-hyperfine structure which changes from anisotropic to nearly isotropic upon cyanide binding also supports this conclusion. More recently, results of the electron spin echo experiments have shown that the Cu(II)-Im⁻⁻Zn(II) bridge is not broken upon binding CN⁻, and combined ENDOR and EPR studies of the cyano complex have been interpreted in terms of the Scheme 2. Such an arrangement of ligating atoms requires that one of the histidine N atoms is either displaced from the coordination shell by cyanide or shifts from an equatorial to an axial bonding position.

Acknowledgments—We thank Dr. Martha L. Ludwig for generous help with concepts and methods in crystallography and for use of her equipment. The help of Dr. W. R. Dunham and W. E. Downer in the construction and maintenance of equipment is also greatly appreciated. We acknowledge valuable discussions with the Richardson's group at Duke University: J. A. Tainer, E. D. Getzoff, K. M. Beem, J. S. Richardson, and D. C. Richardson.

REFERENCES

A study of the electron paramagnetic resonance properties of single monoclinic crystals of bovine superoxide dismutase.
R A Lieberman, R H Sands and J A Fee


Access the most updated version of this article at http://www.jbc.org/content/257/1/336

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/1/336.full.html#ref-list-1