Electron paramagnetic resonance crystallography of ^{17}O -enriched oxycobaltomyoglobin: Stereoelectronic structure of the cobalt dioxygen system

(sperm whale myoglobin/metal-replaced enzymes/ ^{17}O and ^{59}Co hyperfine tensors)

L. CHARLES DICKINSON AND JAMES C. W. CHIEN†

Department of Chemistry, Materials Research Laboratory, University of Massachusetts, Amherst, Massachusetts 01003

Communicated by Richard H. Holm, November 28, 1979

ABSTRACT An electron paramagnetic resonance crystallographic study was made on oxycobaltomyoglobin with the dioxygen ligand enriched to 19.1% in ^{17}O . There are two spectroscopically distinct cobalt dioxygen species. The less abundant species, II (40%), has nonequivalent oxygen atoms with superhyperfine tensors $^{\text{O}}A_{\alpha} = (5, -67.5, 22.4)\text{G}$ and $^{\text{O}}A_{\beta} = (5.4, -83.3, 30.3)\text{G}$. Together with the previously reported ^{59}Co hyperfine tensor [Chien, J. C. W. & Dickinson, L. C. (1972) *Proc. Natl. Acad. Sci. USA* 69, 2783–2787], the orbital spin densities are found to be $O_{\alpha}(p_{\gamma}) = 0.48$, $O_{\alpha}(p_{\beta}) = -0.11$, $O_{\beta}(p_{\gamma}) = 0.74$, $O_{\beta}(p_{\beta}) = -0.16$, $\text{Co}(d_{xz}) = -0.01$, $\text{Co}(d_{yz}) = 0.06$ for a total electron density of 1.01. The O—O axis is directed toward His-E7, suggesting a possible hydrogen bonding interaction which may contribute to the nonequivalency of the oxygen atoms; its projection approximately bisects $\text{N}_1\text{—Fe—N}_2$. The z axis of the $^{\text{Co}}A$ tensor is tilted at an angle of 28° from the heme normal, resulting in a Co—O—O angle of 120° . The more abundant species, I (60%), has equivalent oxygen atoms with $^{\text{O}}A_{\gamma} = (12, -72.5, 20)\text{G}$ and orbital spin densities of $O_{\gamma}(p_{\gamma}) = 0.54$, $O_{\gamma}(p_{\beta}) = -0.05$, $\text{Co}(d_{xz}) = -0.02$, $\text{Co}(d_{yz}) = 0.09$ for a total spin density of 1.10. Although the direction cosines for this molecule cannot be precisely determined, the projection of its O—O axis approximately bisects $\text{N}_2\text{—Fe—N}_3$ and is parallel to the imidazole ring of His-F8. Increase of temperature changes g , $^{\text{Co}}A$, and $^{\text{O}}A$ values, with the largest effect seen with $^{\text{O}}A$. This temperature dependence indicates averaging of the two bond structures which are stabilized at 77 K.

The bonding of dioxygen to heme proteins has long been a topic of interest, extensive research activity, and debate. Various techniques and approaches have been brought to bear on the problem of seeking an accurate geometric and electronic description of the metal–dioxygen bond. Because of high electron density near the heme and ease of autoxidation of the ferrous ion, x-ray crystallography has yet to yield a definitive structure of oxymyoglobin or oxyhemoglobin. Recent development of low-temperature x-ray diffraction techniques in several laboratories may overcome these difficulties. Model oxyheme complexes have been synthesized and found by x-ray crystallography to have an Fe—O—O bond angle of $\approx 120^\circ$ (1). One helpful development was the preparation of the Co analogs of hemoglobin ($^{\text{Co}}\text{Hb}$) and myoglobin ($^{\text{Co}}\text{Mb}$) (2); these bind dioxygen reversibly. In the case of $^{\text{Co}}\text{Hb}$ the binding of dioxygen is cooperative (2–4). We have performed electron paramagnetic resonance (EPR) crystallography on $^{\text{Co}}\text{Mb}$ and $^{\text{Co}}\text{MbO}_2$ and have reported the g and $^{\text{Co}}A$ tensors (5). At -195°C , two oxy species having the same g but different $^{\text{Co}}A$ tensors were observed (5), with one O—O axis turned approximately 90° with respect to the other in the crystal. By assuming the principal axes of the g tensor to be coincident with the molecular axes of the dioxygen ligand, the results were in-

terpreted in favor of a π -bonded structure for $^{\text{Co}}\text{MbO}_2$ for species II.

The present EPR work on $^{\text{Co}}\text{Mb}^{17}\text{O}_2$ single crystals had several objectives. First, we wished to ascertain whether the g and $^{\text{O}}A$ tensors in fact share the same principal axis system as previously assumed (5). Second, Gupta *et al.* (6) had reported powder EPR spectra of $^{\text{Co}}\text{Hb}^{17}\text{O}_2$. Their spectral analysis appears to be somewhat in error (see below) and the deduction of Co—O—O angle based solely upon symmetry arguments of $^{\text{O}}A$ superhyperfine splitting (shfs) is not entirely warranted, as shown by the rhombic $^{\text{O}}A$ tensors observed for O_2^- ions trapped in MgO, ZnO, and KCl matrices (7, 8). However, a preliminary report on the x-ray structure of $^{\text{Co}}\text{MbO}_2$ has appeared (9). The data were taken at -16°C and a Co—O—O angle of near 120° is reported as contrast to the possible π -bond structure (5). The discrepancy in the Co—O—O bond angle must be resolved. Finally, the central purpose of this work was to determine all the hyperfine tensors and relevant eigenvectors in order to arrive at the detailed stereoelectronic structure of $^{\text{Co}}\text{MbO}_2$.

MATERIALS AND METHODS

$^{\text{Co}}\text{Mb}$ was prepared and crystallized as described (5) with the addition of a purification step of adsorption onto a CM-cellulose column at pH 6.6 in 0.01 M phosphate and desorption with 0.1 M phosphate buffer at pH 7.5. There was approximately a 30% loss of non-native material at this step. The protein was crystallized over a pH range of 5.98–6.76 and an $(\text{NH}_4)_2\text{SO}_4$ concentration range of 3.04–3.18 M in the deoxy state in tubes sealed with serum-stoppers and in turn contained in sealed vessels filled with nitrogen. In our previous work (9) the protein was crystallized directly in the oxy state.

Single deoxy crystals were selected rapidly with a polarizing microscope under anaerobic conditions and kept, until EPR study, in the complete absence of oxygen. For conversion of the deoxy $^{\text{Co}}\text{Mb}$ to the ^{17}O -enriched derivative, a few crystals were placed in a small open cell in a 10-ml reaction chamber fitted with stopcocks and a large ground-glass joint. This was connected to a vacuum line equipped with Toeppler pump and a vessel containing 19.1% ^{17}O -enriched oxygen. At this level of enrichment, splitting due to doubly labeled species is effectively absent and signals due to singly labeled molecules are optimized. The isotopic oxygen was purchased from Miles–Yeda (Rehovoth, Israel). Complete oxygenation of the crystal without appreciable autoxidation was carried out at -20°C and required 25 days.

Abbreviations: EPR, electron paramagnetic resonance; $^{\text{Co}}\text{MbO}_2$, oxycobaltomyoglobin; $^{\text{Co}}\text{Mb}^{17}\text{O}_2$, ^{17}O -enriched oxycobaltomyoglobin; $^{\text{Co}}A$, ^{59}Co hyperfine tensor; $^{\text{O}}A$, ^{17}O superhyperfine tensor; $^{\text{Co}}\text{Hb}$, cobaltohemoglobin; hfs, hyperfine splitting; shfs, superhyperfine splitting.

† To whom correspondence should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Crystals were oriented and mounted in the goniometer as described (10, 11). The best-developed face of the $^{59}\text{CoMb}$ crystal corresponds to the (001) plane as identified by the $^{59}\text{CoMb}^{17}\text{O}_2$ EPR spectral variation. It was inconvenient to align the crystals along the crystallographic axes, so an arbitrary (a' , b' , c') orthogonal coordinate system was chosen in these measurements. EPR spectra were obtained at 9.3 GHz and 77 K with a Varian E-9 spectrometer. A sample of solid diphenylpicrylhydrazyl in the dual cavity served as a marker for the magnetic field. Spectra were recorded at 10° angular intervals for more than 180° of rotation at each plane.

RESULTS

Because of the complexity of the data and the number of coordinate systems involved, the following notations, consistent with those contained in the previous paper (5), are used. In the type A crystal of Mb (12, 13) there are two molecules in a unit cell, referred to as sites A and B. For $^{59}\text{CoMbO}_2$, each of these molecules exist in two different chemical species, I and II. The laboratory frame for the crystal is (a' , b' , c'), the crystallographic axes are (a^* , b^* , c^*), the ^{59}CoA tensor system is (x , y , z), the x-ray crystallographic heme normal is N , and the common principal axis system for the g and $^{\text{O}}\text{A}$ tensors are (ξ , η , ζ). The direction cosine is the dot product of the relevant unit vectors. The two nonequivalent oxygen atoms in species II are labeled $\text{O}_\alpha\text{O}_\beta$; the two equivalent oxygen atoms in species I are designated $\text{O}_\gamma\text{O}_\gamma$.

For an arbitrary orientation, a $^{59}\text{CoMb}^{17}\text{O}_2$ EPR spectrum for species II is shown in Fig. 1. The central features are due mainly to $^{59}\text{CoMb}^{16}\text{O}_2$ with a contribution from unresolved $^{59}\text{CoMb}^{17}\text{O}_2$ transitions. Slight irregularities in the spacings between ^{59}CoA hyperfine splitting (hfs) may be due to quadrupole contributions which amount to a few gauss but would not strongly influence the determination of the g value. The satellites in Fig. 1 become resolved at 10-fold higher instrument gain as shown in the *Insets*. These are due to ^{17}O shfs because no satellites were seen in the $^{59}\text{CoMb}^{16}\text{O}_2$ crystals. The nuclear magnetic moments of ^{59}Co and ^{17}O are positive and negative, respectively, whereas their major hyperfine interactions are both negative. Consequently, the high-field satellites in Fig. 1 are transitions from $^{59}\text{CoM}_I = +7/2$ and $^{\text{O}}\text{M}_I = -1/2, -3/2$, and $-5/2$ of two nonequivalent O_α and O_β of species II. The assignment of these satellites to shfs of two nonequivalent ^{17}O atoms is unequivocal. For instance, in the $a'b'$ plane the observed shfs for the two sets of lines maintained a constant ratio of $0.78 \pm$

FIG. 1. Single-crystal EPR spectrum of $^{59}\text{CoMb}^{17}\text{O}_2$ at -195°C for arbitrary orientation, showing ^{17}O shfs of species II.

0.03 over a 110° span of rotation. If, instead, they belonged to two ligands in different sites, this ratio would vary with changes in $g(\theta)$ for the sites.

Complete angular variations of $g(\theta)$ and $^{\text{O}}\text{A}(\theta)$ were obtained for species II (Fig. 2). They have the same turning points. Consequently, the two tensors share the identical principal axes, as previously assumed (5). These data permitted the application of Schonland's equation (11) and previously described procedures for nonlinear least-square fitting of $g_i^2(\theta)$ ($i = a', b', c'$). The resulting tensor was diagonalized to give the principal g_j values (Table 1) in the ($j = \xi, \eta, \zeta$) frame. The results are in close agreement with those obtained earlier (5) for different crystal orientations. The $^{\text{O}}\text{A}_j$ and $^{\text{Co}}\text{A}_k$ ($k = x, y, z$) principal values were obtained by a similar fitting procedure for g_i^2 $^{\text{O}}\text{A}_i^2$ and g_i^2 $^{\text{Co}}\text{A}_i^2$ corrected for second-order effects before the regression analysis.

Turning now to species I, we give another EPR spectrum (Fig. 3) showing the high-field ^{17}O shfs satellites (*Inset*). The

FIG. 2. Angular variation of hfs and g values for $^{59}\text{CoMbO}_2$ species II for the $a'b'$ (A), $b'c'$ (B), and $a'c'$ (C) planes. Experimental data are given for $^{\text{O}}\text{A}_\alpha$ ($\circ$), $^{\text{O}}\text{A}_\beta$ ($\square$), and g value ($\times$).

Table 1. Principal EPR values for $\text{CoMb}^{17}\text{O}_2$

Tensor	Eigenvector	Species I	Species II
g	ξ	2.081 ± 0.002	2.079 ± 0.002
g	ζ	2.000 ± 0.01	2.013 ± 0.002
g	η	1.99 ± 0.01	1.985 ± 0.002
$^{\text{O}}A_{\beta}$	ξ		5.4 ± 5.0
$^{\text{O}}A_{\beta}$	ζ		-83.3 ± 2.0
$^{\text{O}}A_{\beta}$	η		30.3 ± 2.0
$^{\text{O}}A_{\alpha}$	ξ		$\approx 5.0^*$
$^{\text{O}}A_{\alpha}$	ζ		-67.5 ± 2.0
$^{\text{O}}A_{\alpha}$	η		2.4 ± 2.0
$\text{Co}A$	z	-13.1 ± 1.0	-9.33 ± 0.5
$\text{Co}A$	x	-23.5 ± 1.0	-16.7 ± 0.5
$\text{Co}A_{\gamma}$	y	-8.8 ± 1.0	-5.95 ± 0.5
$^{\text{O}}A_{\gamma}$	ξ	$+12^*$	
$^{\text{O}}A_{\gamma}$	ζ	-72.5 ± 2.0	
$^{\text{O}}A_{\gamma}$	η	$+20^*$	

Data are shown as mean $\pm$ SEM.

* Estimated value with unknown precision.

spectra for species I are consistent with equivalent O_{γ} atoms. The *Inset* at the high-field side has the more intense lines due to shfs transitions of $\text{Co}M_I = +\frac{7}{2}$ and $\text{O}M_I = -\frac{1}{2}, -\frac{3}{2},$ and $-\frac{5}{2}$ of singly labeled $\text{CoMb}^{16}\text{O}_{\gamma}^{17}\text{O}_{\gamma}$; the far weaker lines belong to $\text{CoMb}^{17}\text{O}_{\gamma}^{17}\text{O}_{\gamma}$ species. In the $b'c'$ plane it was possible to follow the shfs over $\pi/2$ radians and there was no evidence of unequal splittings. The intensity of these shfs lines is approximately twice that of the satellites in species II, further corroborating equivalency.

It was not possible to obtain similarly precise direction cosines for species I because of limitations imposed by overlap of lines which prevented the complete determination of $g(\theta)$ or $A(\theta)$ for all angles in three perpendicular planes. Instead, the principal values for this species had to be inferred by a less direct method. In the $a'b'$ plane the g value for species I reaches nearly its minimal principal value of 1.989 and $^{\text{O}}A$ attains the maximum of 72.5 G at the same angular orientation of the crystal (i.e., the two tensors have the same turning point). Furthermore, g and $^{\text{O}}A$ share two common turning points in the other planes. In view of the fact that $g_{\min}$ corresponds to $^{\text{O}}A_{\max}$ in species II, if we assume the correspondence to apply also to species I then the observed $^{\text{O}}A_{\max}$ of 72.5 G may be taken as one of the principal values. It follows that the extrema for $^{\text{O}}A$ in the plane perpendicular to the former gave the other principal values for $^{\text{O}}A$. Because of the approximation involved, it was not deemed justified to calculate the direction cosines for the species I $^{\text{O}}A$ tensor which would not have accuracy better than $\pm 20^\circ$. Table 1 contains the principal values of g , $\text{Co}A$, and $^{\text{O}}A_{\gamma}$ for species I as estimated in the above manner.

A $\text{CoMb}^{17}\text{O}_2$ crystal was placed at a fixed orientation in the

FIG. 3. Single-crystal EPR spectrum of $\text{CoMb}^{17}\text{O}_2$ at -195°C for arbitrary orientation showing ^{17}O shfs of species I. DPPH, diphenylpicrylhydrazyl.

FIG. 4. Temperature dependence of ^{17}O hyperfine lines in the EPR spectra of CoMbO_2 . The vertical line at the left marks the resonance line on which the observed ^{17}O splitting is centered.

EPR cavity and the temperature was varied from -120 to -30°C . There was a change in g value from 2.059 to 2.053 and a decrease of cobalt hfs from 17.5 to 13.0 G with this increase in temperature. The temperature effect on ^{17}O shfs was more pronounced. Whereas there were two sets of nonequivalent ^{17}O shfs at -195°C , they become equivalent at -50°C (Fig. 4). These changes are reversible and are not attributable to the effect of temperature on line width, cavity Q , or microwave resonance frequency. The spectra of the reference compound diphenylpicrylhydrazyl displays no corresponding spectral shift.

DISCUSSION

Geometric Considerations. CoMbO_2 exists in two distinct forms having identical g but different $\text{Co}A$ and $^{\text{O}}A$ tensors. At -195°C , integration of EPR signals gives 60% species I and 40% species II. Earlier we (5) assumed the vector $g_{\max}$ to coincide with the $\text{O}-\text{O}$ axis as in O_2^- (8), which is designated as ξ . This assignment is now confirmed by the fact that the $^{\text{O}}A_{\xi}$ is the smallest principal value of the tensor. The other two vectors, η and ζ , are parallel to the axes of the two π^*_g orbitals of dioxygen. The relationship between (ξ, η, ζ) and (x, y, z) of $\text{Co}A$ tensor for species II is depicted in Fig. 5A with $x \parallel \xi$ and η and ζ rotated about the former so that they are 35° from the z and y axes, respectively.

The orientations of g , $\text{Co}A$, and $^{\text{O}}A$ with respect to the "heme" can be deduced from the atomic coordinates of native Mb.[†] In the case of deoxy CoMb , the axis of its $\text{Co}A$ tensor lies within a degree or two of the heme normal N (5) (Table 2), as one would expect. What is surprising is that the z of $\text{Co}A$ in CoMbO_2 is not along the "heme" normal but is $\approx 28^\circ$ from it. Similar but much smaller deviations have been reported for the x-ray structures of two other dioxygen adducts—of $N,N'-(1,1,2,2\text{-tetramethylethylene})\text{bis}(3\text{-fluorosalicylideneiminato})-(1\text{-methylimidazole})\text{-Co(II)}$ (14) and Co(CN)_5^{3-} (15)—in which the $\text{Co}-\text{O}_{\alpha}$ axis is tilted to the same side away from the cobalt complex normal by 3.8° and 5.3° , respectively. As a result of this large deviation from coincidence for the z and N vectors in CoMbO_2 , the angle between the $\text{O}-\text{O}$ axis and the z axis of $\text{Co}A$, which is $\cos^{-1}(\xi \cdot z) = 92^\circ$, cannot be taken to be the $\text{Co}-\text{O}-\text{O}$ bond angle. Instead, the angle is obtained in the following manner. The x-ray atomic coordinates were given in (a, b, c^*) by Kendrew and Watson[‡], in which N has direction cosines of $(0.89, \pm 0.36, 0.29)$. In the present (a^*, b, c) frame the direction cosines are $N = (0.92, \pm 0.342, 0.090)$. For the $\text{O}-\text{O}$ axis, $\xi^{\pm} = (\pm 0.16, 0.98, \pm 0.13)$, where the signs refer to the two molecules in a unit cell. The two dot products of interest are $N^{\pm} \cdot \xi^{\pm} = 0.507$. Therefore, the $\text{Co}-\text{O}-\text{O}$ bond angle is $\approx 120^\circ$ which brings x-ray and EPR results into agreement for species II.

Comparison with a model made from the x-ray crystallographic atomic coordinates reveals the following structural

[†] J. C. Kendrew and H. C. Watson, personal communication.

Table 2. Arc cosine of the dot product of the z axis of CoA in CoMb and CoMbO_2 and heme normal in FeMb

CoMb*	CoMbO ₂ †	FeMb‡
(z·a*) = 23°	(z·a*) = 31°	(N·a*) = 21°
(z·b) = 70°	(z·b) = 80°	(N·b) = 68°
(z·c) = 89°	(z·c) = 60°	(N·c) = 88°

* From ref. 5.

† From ref. 14.

† For species II.

details. The ξ axis in species **II**, and therefore the axis of dioxygen, points into the volume occupied by the N_e atom of His-E7; its projection on the heme plane approximately bisects the N_1 —Fe— N_2 (or N_3 —Fe— N_4) angle [N_i are heme pyrrole nitrogen atoms labeled according to Kendrew[†]]. The ξ axis of species **I**, on the other hand, has projection bisecting the N_2 —Fe— N_3 (or N_1 —Fe— N_4) angle. Thus, the directions for the O—O axis in species **I** and in species **II** are approximately 90° apart with respect to the heme, giving strong evidence that these are two potential wells for binding dioxygen in $^{56}\text{CoMnO}_2$ at -195°C . There are possible chemical origins for the above stereochemistry. For species **II** there is a possibility of hydrogen bonding interaction between dioxygen and the distal histidine. Some support for this comes from the observation (16) that the frozen solution EPR line width of $^{56}\text{CoMnO}_2$ is different in $^2\text{H}_2\text{O}$ than in $^1\text{H}_2\text{O}$. Such interaction should render the two oxygen atoms nonequivalent in species **II**. The orientation of the O—O axis in species **I** would not be in a position for interaction with His-E7 but rather would be approximately parallel to the imidazole ring of His-F8, thus suggesting possible stabilization by $p\pi$ — $d\pi$ — $p\pi^*$ interactions of the respective imidazole (His-F8), Fe, and dioxygen orbitals.

In contrast to the two species found in our EPR work, there is apparently only one type of Co—dioxygen bond in the x-ray study at -16°C (9). The temperature dependence of the g , $^{\text{Co}}A$, and $^{\text{O}}A$ values showed clearly some averaging of different modes of Co—O₂ bonding. This is not unprecedented. For instance, various single crystals of Hb and MetHb derivatives labeled at the $\beta 93$ position with iodoacetamidonitroxide give EPR spectra with specific orientations for the label at room temperature but with multiple orientations at -195°C (17). The molecules average to some common conformations at room temperature. Temperature-dependent EPR spectra have also been found for the single crystal oxy- and deoxy-vitamin B-12r doped into vitamin B-12b (18). The EPR spectra of the $^{17}\text{O}_2$ adduct of Co(bzacen)/py complex (19) was interpreted as having the ligand jumping between two bent structures.

hfs Tensors.

The signs of the principal values of each $^{\infty}\mathbf{A}$ are taken to be negative. This is based on the negative Fermi contact interaction for transition metal ions from core polarization and configurational interaction effects (20). The observed tensor

is comprised of

$$Co_A = Co_a + A_{dip} + Co_T + Co_{T'} \quad [1]$$

in which ${}^{\text{Co}}a$ is the Fermi contact interaction and is the trace of the observed tensor; it has values of -15.1 and -10.7 G for species I and II, respectively. The dipolar tensor $\mathbf{A}_{\text{dip}}$ arises from the interaction of the magnetic dipole of the electron in the π^*_{g} -orbital on the dioxygen with the cobalt nucleus. It is estimated with the assumption of a Co—O bond length of $1.9 \text{ \AA}$ (see below). The magnitude of $\mathbf{A}_{\text{dip}}$ is small and does not change appreciably with reasonable variation in the position of the dioxygen. Table 3 summarizes the principal values of these tensors. None of the ${}^{\text{Co}}T$ and ${}^{\text{Co}}T'$ tensors has a maximum in the z direction. This is taken to mean that unpaired electrons are populated not in the d^2_{xy} orbital as often assumed but are rather in the d_{xz} and d_{yz} orbitals. The hfs principal value for an electron in these orbitals is negative and has a value of -77.9 G (20), from which we obtain a significant positive spin density in the d_{yz} orbital and a small negative spin density in the d_{xz} orbital via polarization. The participation of the d orbitals in dioxygen bonding has also been discussed by Symons (21) in conjunction with oxyferrous Mb electron capture centers. However, we have neglected the small contribution from spin-orbit interactions.

^{17}O shfs has been investigated both empirically and theoretically (6-8, 22). The signs of the principal values of $^{\text{O}}\text{A}$ are chosen in accordance with theory. From semi-empirical grounds the isotropic splitting due to the ^{17}O nucleus is expected to be proportional to the spin density in the π orbital, $a_{\text{O}} = -41\rho_{\pi}$ (19), and the anisotropic tensor would have the form $(51\rho_{\pi}, -102\rho_{\pi}, 51\rho_{\pi})$ in the (ξ, η, ζ) coordinates. The observed $^{\text{O}}\text{A}$ is then the sum of

$${}^0\mathbf{A} = {}^0\mathbf{a} + {}^0\mathbf{T} + {}^0\mathbf{T}' \quad [2]$$

in which the dipolar contribution from electrons in d orbitals of the Co nucleus is too small and may be neglected. The resulting $^{\text{OT}}$ and $^{\text{OT'}}$ tensors are given in Table 4. The spin densities are positive in the π_r -orbital but negative in the π_i -orbital.

Gupta *et al.* (6) analyzed powder EPR spectra of $\text{CoHb}^{17}\text{O}_2$ and obtained principal values of $\text{O}A_\alpha$ and $\text{O}A_\beta$ of 65 and 93 G, respectively. These values are different from those obtained in the present work. Those workers took the total span of the $^{17}\text{O}_2$ satellites to obtain the shfs constant but failed to take into account the contribution of the Co hfs. After they are corrected for this omission, their results are in accordance with ours.

Electronic Structure. The electronic structure of oxygenated Co complexes has been extensively discussed and recently reviewed (23). The favored formalism of Co(III)O_2^- is based on similarity of certain physical properties with those of superoxide ion. Often cited is the O—O bond distances of 1.26–1.30 Å in Co–dioxygen complexes (see ref. 24 for this and related ex-

FIG. 5. (A) Principal axes system for the $^{\text{Co}}\text{A}$ tensor (x, y, z) and the $\mathbf{g}$ and $^{\text{O}}\text{A}$ tensors (ξ, η, ζ) of $^{\text{Co}}\text{MnO}_2$ (species II). (B) Geometry of deoxy- and oxycobaltoporphyrin, depicting effect of oxygenation.

Table 3. Principal values (in gauss) for the Co hfs tensors in the (x, y, z) axes system

	A_{dip}	CoT	$\rho(d_{yz})$	CoT'	$\rho(d_{xz})$
Species I	(-0.7, 1.4, -0.7)	(-7.0, 3.5, 3.5)	0.09	(-0.7, 1.4, -0.7)	-0.02
Species II	(-0.7, 1.4, -0.7)	(-4.8, 2.4, 2.4)	0.06	(-0.5, 1.0, -0.5)	-0.01

amples) compared to 1.28 Å in KO₂ (25). However, recent measurements (26) and rigorous calculations showed a bond distance of 1.34 Å for the gaseous O₂⁻. The g values for ⁵⁵MbO₂ and ⁵⁵HbO₂ are said to be very close to those reported for O₂⁻ trapped in certain matrices, but the latter values are very sensitive to the environment. Another similarity is the O—O stretching frequency in infrared spectra (23). Although the simple formalism of Co(III)O₂⁻ is a useful approximation and has been related to molecular orbital descriptions there still is a lack of knowledge about unpaired spin density distributions. This work offers such data.

The unpaired spin density distributions in species I in the various atomic orbitals are $O_\gamma(p_\eta) = 0.54$, $O_\gamma(p_\zeta) = -0.05$, $Co(d_{xz}) = -0.02$, $Co(d_{yz}) = 0.09$ to give a total electron density of 1.10. The corresponding distributions in species II are $O_\alpha(p_\eta) = 0.48$, $O_\alpha(p_\zeta) = -0.11$, $O_\beta(p_\eta) = 0.74$, $O_\beta(p_\zeta) = -0.16$, $Co(d_{xy}) = -0.01$, $Co(d_{yz}) = 0.06$ for a total of 1.01. These values are probably subject to errors of about 10%. The estimated total unpaired electron density suggests little or no electron delocalization onto the N_ε atom of His-F8 or atoms of the porphyrin ring. An important feature of these results is the spin density values found at the Co nucleus and in the $d\pi$ orbitals, the latter indicating significant participation of excited states in the electronic structure of the Co-porphyrin-dioxygen complex.

The stereoelectronic structure for species II of ⁵⁵MbO₂ is depicted in Fig. 5B, unlikely out-of-plane positioning of the Co atom neglected. For the deoxy state, the Co—N (pyrrole) and Co—N_ε bond distances are taken to be 1.977 and 2.157 Å, respectively, as in Co(II)TPP(1-MeIm) (27). Oxygenation causes a contraction of the Co—N bond distance to an estimated value of 1.926 Å as in Co(III)TPP(Im)₂⁺ (28). The bond lengths for Co—O and O—O are taken to be 1.86 and 1.26 Å, respectively (23). Because EPR gives the direction only, the position of the two oxygen atoms in Fig. 5B along the ξ axis is not known. The observed unpaired spin density distribution and the relative orientations of the η and ζ axes with respect to the porphyrin suggest a structure in which one of the π_g molecular orbitals of dioxygen, arbitrarily taken to be $\pi_g(\eta)$, σ interacts strongly with the d_{z^2} orbital of Co and less strongly, in a pseudo π -type of bonding, with the d_{xz} orbital. The bond formation concomitantly causes some charge transfer into the region of bonding. The other $\pi_g(\zeta)$ molecular orbital of the dioxygen overlaps with d_{yz} orbital of cobalt in a $p\pi$ - $d\pi$ interaction. Both the tilting of the ⁵⁵Co— z axis and the rotation of the ⁵⁵Co— η and ⁵⁵Co— ζ axes are to optimize these various interactions. The large unpaired spin densities on the ligand are not due to charge transfer but simply result from the fact that the dioxygen has two unpaired electrons before ligation and retains most of its second electron unpaired after bonding the Co. Fantucci and Valenti (29) ar-

rived at the same conclusions in their theoretical calculations on Co(acacen)O₂.

In conclusion, the EPR crystallographic study has provided detailed information concerning the stereoelectronic structures of the Co-dioxygen system in ⁵⁵MbO₂. Theoretical treatments up to now have been quite crude, leading to axial hyperfine tensors and no involvement of the metal d orbitals. The above results should encourage more refined calculations including interaction of excited state configurations.

- Collman, J. P., Gagne, R. R., Reed, C. A., Robinson, W. T. & Rodley, G. A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1326–1329.
- Hoffman, B. M. & Petering, D. H. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 637–643.
- Chien, J. C. W. & Dickinson, L. C. (1973) *J. Biol. Chem.* **248**, 5005–5011.
- Snyder, F. W., Jr. & Chien, J. C. W. (1978) *Eur. J. Biochem.* **91**, 83–88.
- Chien, J. C. W. & Dickinson, L. C. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2783–2787.
- Gupta, R. K., Mildvan, A. S., Yonetani, T. & Srivastava, T. S. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1005–1012.
- Melamud, E., Silver, B. L. & Dori, Z. (1974) *J. Am. Chem. Soc.* **96**, 4689–4690.
- Lunsford, J. H. (1973) *Catal. Rev.* **8**, 135–137.
- Petsko, G. A., Rose, D., Tsernoglou, D., Ikeda-Saito, M. & Yonetani, T. (1978) in *Frontiers of Biological Energetics*, eds. Dutton, P. L., Leigh, J. S. V. & Scapa, A., (Academic, New York), pp. 1011–1017.
- Chien, J. C. W. (1969) *J. Chem. Phys.* **51**, 4220–4227.
- Chien, J. C. W. & Dickinson, L. C. (1971) *J. Am. Chem. Soc.* **93**, 5036–5040.
- Kendrew, J. C. & Parrish, R. G. (1957) *Proc. R. Soc. London Ser. A* **238**, 305–327.
- Dickinson, L. C. & Chien, J. C. W. (1971) *Nature (London) New Biol.* **324**, 107.
- Avdeef, A. & Schaefer, W. P. (1976) *J. Am. Chem. Soc.* **98**, 5153–5159.
- Brown, L. D. & Raymond, K. N. (1975) *Inorg. Chem.* **14**, 2595–2599.
- Yonetani, T., Yamamoto, H. & Iizuka, T. (1974) *J. Biol. Chem.* **249**, 2168–2174.
- Chien, J. C. W. (1979) *J. Mol. Biol.* **133**, 385–398.
- Schweiger, A., Jorin, E. & Gunthard, H. H. (1979) *Chem. Phys. Lett.* **61**, 223–227.
- Getz, D., Melamud, E., Silver, B. L. & Dori, Z. (1975) *J. Am. Chem. Soc.* **97**, 3846–3847.
- Goodman, B. A. & Raynor, J. B. (1970) *Adv. Inorg. Chem. Radiochem.* **13**, 136–362.
- Symons, M. C. R. & Peterson, R. L. (1978) *Proc. R. Soc. London Ser. B* **201**, 285–300.
- Melamud, E. & Silver, B. L. (1973) *J. Phys. Chem.* **77**, 1898–1900.
- Summerville, D. A., Jones, R. D., Hoffman, B. M. & Basolo, F. (1979) *J. Chem. Ed.* **56**, 157–163.
- Gall, R. S., Rogers, J. F., Schaefer, W. P. & Christoph, G. G. (1976) *J. Am. Chem. Soc.* **98**, 5135–5144.
- Abraham, S. C. (1956) *Q. Rev. Chem. Soc.* **10**, 407–423.
- Celotta, R. J., Bennett, R. A., Hall, J. R., Siegel, M. W. & Levine, J. (1972) *Phys. Rev. A* **6**, 631–642.
- Scheidt, W. R. (1974) *J. Am. Chem. Soc.* **96**, 84–89.
- Ibers, J. A., Lauer, J. W. & Little, R. G. (1974) *Acta Cryst.* **B30**, 268–272.
- Fantucci, P. & Valenti, V. (1976) *J. Am. Chem. Soc.* **98**, 3832–3838.

Table 4. Principal values (in gauss) for the ¹⁷O shfs tensors in the (ξ , η , ζ) axes system

	Species I	Species II	
	O_γ	O_α	O_β
a	-13.5	-13.3	-15.8
OT	(28, -56, 28)	(24.4, -48.4, 24.2)	(37.9, -75.8, 37.9)
OT'	(-2.5, -3, 5.5)	(-5.7, -5.7, 11.4)	(-16.4, 8.2, 8.2)
$\rho(p_\eta)$	0.54	0.48	0.74
$\rho(p_\zeta)$	-0.05	-0.11	-0.16