\textbf{Communication}

\textbf{1\textsuperscript{H} Electron-Nuclear Double Resonance of Cobalt Hemoglobin\textsuperscript{*}}

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Proton \textsuperscript{1}H electron-nuclear double resonance spectra were obtained from cobalt-substituted hemoglobin \textsuperscript{A}. For oxy cobalt-substituted hemoglobin (\textsuperscript{Co}Hb), two major couplings were found. One is exchangeable by deuterium and has a coupling of \(-5.6 \text{ MHz}\) which is assigned to the N\textsubscript{x}-proton of the distal histidine (E7), interacting with the ligand. The other splitting (\(\approx 2.4-3.2 \text{ MHz}\)) is attributed to a methyl-proton on Val (E11). No coupling of protons on the proximal side is resolved. Deoxy \textsuperscript{Co}Hb exhibits only one prominent interaction (\(-1.3 \text{ MHz}\)) which is assigned to one of the protons on the proximal histidine (F8). The influence of the distal amino acids His (E7) and Val (E11) on the ligand affinity in oxy \textsuperscript{Co}Hb is discussed.

ENDOR\textsuperscript{1} spectroscopy of weakly coupled protons in paramagnetic heme proteins is suitable for probing structural details of the close neighborhood of the heme center. We have applied this method to \textsuperscript{Co}Hb which is known to be a functional analogue of the native ferrous Hb (1, 2) but has the advantage of being paramagnetic in both ligation states of interest, oxy \textsuperscript{Co}Hb and deoxy \textsuperscript{Co}Hb.

Previous ESR studies have indicated the existence of a hydrogen bond between the ligand and the proton attached to the nitrogen N\textsubscript{y} of the distal histidine E7 in oxy \textsuperscript{Co}Hb. First, Yonetani et al. (3) noted a decrease in the line width of the ESR spectra of frozen solutions when using D\textsubscript{2}O as a solvent instead of H\textsubscript{2}O. Subsequently, Gupta et al. (4) employed powder-type spectra of oxy \textsuperscript{Co}Hb with oxygen \textsuperscript{17}O isotope enrichment to evaluate the spin-density distribution over the dioxygen. These authors found a nonequivalence for the two oxygens which was ascribed to an influence of the distal histidine via a hydrogen bond. More detailed stereochemical information about the cobalt-oxygen complex has been obtained from work on single crystals of the myoglobin derivative oxy \textsuperscript{Co}Mb. Using \textsuperscript{17}O enrichment also, Chien and Dickinson (5, 6) have shown the existence of two paramagnetic cobalt-oxygen complexes labeled type I and type II which differ in the distribution of the unpaired spin over the dioxygen and in the directions of the O--O bond projections onto the heme plane. It was argued that this difference reflected the presence or absence of a hydrogen bond between the ligand and the distal histidine nitrogen.

In this report, we present direct spectroscopic evidence of the existence of the hydrogen bond in question and show that both type I and type II cobalt-oxygen complexes, if present, are capable of forming it. Other proton couplings in both oxy \textsuperscript{Co}Hb and deoxy \textsuperscript{Co}Hb are presented and ascribed to protons on the distal side in oxy \textsuperscript{Co}Hb and on the proximal side in deoxy \textsuperscript{Co}Hb.

**EXPERIMENTAL PROCEDURES**

\textsuperscript{Co}Hb was prepared using \textsuperscript{Co}protoporphyrin IX according to the literature prescriptions (5, 7). The exchange of the 0.1 M phosphate, H\textsubscript{2}O buffer system against 0.1 M phosphate, D\textsubscript{2}O was performed via ultrfiltration. Deoxygenation was achieved by several cycles of pumping and N\textsubscript{2} flushing in 50-ml vessels. The hemoglobin concentrations used were between 2.5 and 3 mM/oxygen binding site. All samples were kept at pH 7.0.

ESR spectra were taken on a Bruker ER 420 instrument (X-Band, \(-9.4 \text{ GHz}\)). The ENDOR equipment used was as described previously (8). Data acquisition (1K points/spectrum) typically involved multi-scan averaging controlled by an on-line Dietz 621/8 computer. The temperatures of measurement were between 10 and 15 K using a He-flow cryostat (Thor).

\textbf{RESULTS}

\textbf{Deoxy \textsuperscript{Co}Hb—}Fig. 1 shows the X-band ESR spectrum of deoxy \textsuperscript{Co}Hb (inset) indicating the three g-factors on which the ENDOR spectra were taken. Fig. 1, bottom, gives a typical ENDOR spectrum obtained along \(g_1\) showing the pairs of lines labeled AA' and BB' which are mirrored about the “free proton” frequency \(\nu_N\) as expected from the resonance condition for weakly coupled protons

\begin{equation}
\nu_N = [\nu_x \pm \frac{a_0}{2}]
\end{equation}

in which \(a_0\) is the hyperfine coupling of the proton concerned. The scale beneath the spectrum gives the distance in MHz of the lines from \(\nu_N\) which is set as zero. Table I lists the coupling values extracted from the spectrum together with the values obtained at the other \(g\)-factors indicated in Fig. 1. It is noted that along \(g_2\) only one coupling (1.1 MHz) is discernible, whereas \(g_1\) and \(g_2\) exhibit two pairs which, moreover, are about identical (0.4 and 1.35 MHz).

The axial symmetry of the ESR spectrum produces a single crystal-like ENDOR response along \(g_2\) which contains the interaction of the molecules oriented parallel to the magnetic field with their heme normals. Along \(g_1/g_2\), a powder-type ENDOR spectrum is expected which, to a first crude approximation, contains the information about the remaining two interaction elements in a way comparable to an axially symmetrical ESR spectrum occurring however twice, mirrored about the nuclear Zeeman frequency \(\nu_N\). Thus, the two line pairs along \(g_1/g_2\) can be associated with the one pair observed along \(g_2\) to yield an interaction tensor of one proton with elements of 0.4, 1.35, and 1.1 MHz.

In order to distinguish qualifying candidates of protons, dipole calculations between the cobalt ion and protons in the neighborhood can be performed using the conventional interaction formula for point-dipoles at a distance \(r\)

\begin{equation}
ad = \frac{C}{r^3} \times \rho \times (3 \cos^2 \varphi - 1)
\end{equation}

with \(a_0\) being the coupling value, \(C = g_2 \rho_0 \beta g_2 \beta_0\), and \(\varphi\) the angle between the external field direction and the connection

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\textsuperscript{1}The abbreviations used are: ENDOR, electron-nuclear double resonance; \textsuperscript{Co}Hb, cobalt-substituted hemoglobin.
Proton ENDOR of Cobalt Hemoglobin

A

Deoxy CoHb

g = g_1

pH 7.0

Fig. 1. 'H ENDOR of deoxy CoHb taken along g_1, as indicated on the ESR spectrum in the inset. Magnetic field value and g-value, together with splitting parameters, are given in Table I. Experimental conditions are given under "Experimental Procedures." The ENDOR spectrum took ~5 min of signal averaging corresponding to 16 additions of spectra taken at a rate of 10 MHz/20 s.

<table>
<thead>
<tr>
<th>Table I</th>
<th>'H ENDOR couplings (MHz) of CoHb and oxy CoHb</th>
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<tbody>
<tr>
<td></td>
<td>CoHb</td>
</tr>
<tr>
<td></td>
<td>H_2O</td>
</tr>
<tr>
<td>g_1</td>
<td>2.078</td>
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<tr>
<td>H_0</td>
<td>3.28 kG</td>
</tr>
<tr>
<td>g_2</td>
<td>2.006</td>
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<tr>
<td>H_0</td>
<td>3.40 kG</td>
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<tr>
<td>g_3</td>
<td>1.385</td>
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<tr>
<td>H_0</td>
<td>3.44 kG</td>
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<td>D_2O</td>
</tr>
<tr>
<td>g_1</td>
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-2 -1 0 1 2 3 4 5 MHz

Line of the two dipoles under consideration. The spin-density p on the cobalt ion has to be taken as 100% since in deoxy CoHb the unpaired electron fully resides at the central ion.

Using the crystallographic geometry determined for myoglobin (9), the closest protons on the distal side (H(N~)) on His (E7) and the methyl protons on Val (E11) yield maximum interactions of about 0.5 MHz and are therefore to be excluded from consideration. On the proximal side and in the heme plane, the protons at N_ and C_ of the proximal histidine F8 and the meso-protons should be considered. For both former protons, the calculations yield tensor elements in good agreement with the experimental values (0.4, 1.4, and 1.0 MHz), whereas for the meso-protons about 0.8 MHz is calculated as maximum interaction. We thus tentatively assign the observed interaction to either or both of the protons of the proximal histidine.

Oxy CoHb—The ESR spectrum indicating the different g-positions on which ENDOR data were acquired for oxy CoHb is shown in the inset of Fig. 2. The 'H part of the ENDOR spectra obtained along g_2 both in H_2O and D_2O buffer form the top and bottom part of Fig. 2. Comparison of the H_2O spectrum with that of deoxy CoHb immediately shows that the width of the individual lines in oxy CoHb is enhanced and that, in addition to a multitude of lines around g_2, two larger couplings, CC' and DD', are present, of which DD' is lost when using D_2O in the buffer solution. Another effect of the D_2O buffer is a resolution in the center part yielding a clear EE' pair. The coupling values of these pairs, together with those taken on g_1 and g_3 in H_2O and D_2O containing buffers, are listed in Table I.

In the assignment of the couplings, we have to assume, based on the difference between oxy and deoxy CoHb, that the large couplings CC' and DD' must arise from protons on the distal side of the heme. From 170-substituted CoMb single crystal ESR studies, Chien and Dickinson (5) found that the bulk of the unpaired spin (>90%) in the Co-O_2 complex resides on the dioxygen. Moreover, two different complexes labeled type I and type II were present with type I having two oxygens (O_Y) sharing equal parts of the spin-density. In the type II complex, the outer oxygen (O_D) was found to carry a higher spin-fraction than O_Y, the oxygen bound directly to the cobalt ion. In addition, while the projection of the O-O bond direction onto the heme plane points towards the distal histidine (E7) for type II, this direction is rotated by roughly 90° for the type I complex. Fig. 3B shows a projection of the O-O directions of both complexes onto the heme plane.

Although Gupta et al. (4) found no evidence for two complexes in their work on frozen solutions of oxy CoHb, we shall assume that both types of Co-O_2 complexes may be present and calculate the oxygen positions for both. The direction cosines of g_1, given in Ref. 5 for type II, define the direction of the O-O bond. From the hyperfine tensors of the 17O-interaction in II, the angle formed by the Co-O and the heme normal are known. Additionally, the bond lengths and the fact that the heme normal vector is defined as pointing towards the distal side from crystallographic studies (9), the positions of the oxygen...
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atoms are unequivocally localized. As a result, we find that $O_6$, the outer oxygen atom for the type II complex, actually points away from His (E7), $O_5$ lying basically above the cobalt ion. Chien and Dickinson (5) give no exact direction cosines for the $g$-tensor elements of the type I complex. With their estimate on the relative orientation of the $O-O$ bond projection in relation to type II, and in analogy to $O_6$ pointing away from His (E7), the approximate positions for $O_y$, and $O_3$ for type I could be calculated. The configurations are shown in Fig. 3.

With this information, possible candidates for interaction can be assigned by point-dipole calculations using the known spin-densities on the ligand (5). Considering first the pair $DD'$, this coupling must arise from an exchangeable proton in a hydrogen bond since it is lost in the deuterated buffer. Calculating the possible interaction for the proton on the $N_6$ of His (E7), we find that both complexes, type I and type II, yield the same strong coupling of 5.9 MHz along $g_1$ and 5.6 MHz along $g_2$ using spin-densities of 0.49 and 0.6 on the respective outer oxygen atoms as given in Ref. 4. Along $g_3$, the calculations yield 0.4 MHz in accord with 0.3 MHz arising from the requirement that the trace of the dipolar hyperfine interaction tensor should vanish.

The surprising agreement between experimental and calculated interactions along $g_1$ and $g_2$ leads us to assign pair $DD'$ to the interaction of the distal histidine (E7) proton. The fact that along $g_3$ about the same, large coupling of 5.7 MHz is observed is assigned as arising from simultaneous ENDOR excitations of ESR lines belonging to different $g$-turning points in the ESR spectrum of rhombic symmetry due to the close proximity of $g_2$ and $g_3$. Support for this assumption comes from the fact that for both $g_2$ and $g_3$ the use of D$_2$O buffer brings about the resolution in the center part visible in Fig. 2. We ascribe this to the loss of the 0.4 MHz coupling expected only on $g_3$ from the calculations but observable on both $g_2$ and $g_3$ experimentally.

The second of the larger coupleings yields values of about 2.45-2.5 MHz along $g_1$ and 2.90-3.20 MHz along $g_2/g_3$. It must arise from a nonexchangeable proton located close to the dioxygen ligand. Investigating the crystallographic situation, we find the methyl group of Val (E11) pointing towards the ligand. Assuming a rigid position of the methyl protons at the temperature of observation with one of them being aligned along the shortest connection line between the outer oxygen and the methyl group carbon, the dipole calculations yield 2.5 MHz interacting along $g_1$ and 3.05 MHz along $g_2$ for the type II cobalt-oxygen complex. With the magnetic field parallel to the direction of $g_3$, a value of 0.6 MHz can be calculated which is probably masked experimentally between the 0.45-
1.25-MHz couplings observed in this direction. For the type I complex, due to its different geometry, none of the protons considered gives calculated coupling values comparable to the experimental ones. The close agreement between calculation and experiment for the type II configuration leads us to assign this coupling tensor to one of the Val (E11) methyl protons.

The remaining weak proton couplings seen along $g_2/g_3$, again in the same magnitude due to the nearly axial symmetry of the ESR spectrum (0.4 and 1.25 MHz), cannot be assigned with reasonable certainty. The proximal side protons can, again, be excluded since their distance from the main site of spin-density (the ligand) is too large and since the interaction with the closer cobalt ion is negligible (less than 0.2 MHz) owing to its small spin-density. The calculation for porphyrin-based protons again gives very small values unable to account for the observed ones. It remains to consider the distal side protons for which only Val (E11) can be the origin. Estimates for the other two protons of its methyl group give, for the type II complex again, values between 0.5 and 1.2 MHz which allow a tentative assignment.

DISCUSSION

The proton-ENDOR data of deoxy and oxy CoHb show the interaction of the proximal histidine (F8) with the central cobalt ion in the deoxy state and that of the distal amino acids His (E7) and Val (E11) with the dioxygen ligand in the oxy state of cobalt hemoglobin. For the latter, the existence of a hydrogen bond between the $N_e$-proton and oxygen as discussed previously (3, 4, 6) has been shown directly by H/D exchange. For iron myoglobin ($^{57}$Mbo), the recent neutron diffraction data (10) also prove the formation of this hydrogen bond. Similarly, in our recent ENDOR studies of NO-hemoglobin (8), the same hydrogen bond was found to exist in the $r$-state of $\alpha$-chains and to be lost upon a transition to the $t$-state. It is therefore likely that the presence of the $N_e$-proton bond to the ligand is a universal instrument to stabilize the ligand in the 6th position of both cobalt and iron containing hemoglobins. Interestingly, the recent work of Momenteau and Lavalette (11) on iron-porphyrin model compounds has yielded an enhanced stability of oxygenated derivatives in the presence of an NH-group on the “distal side” of the heme group.

From our frozen solution ESR spectra, we are unable to determine whether oxy CoHb contains a mixture of cobalt-oxygen complexes (type I and type II). The calculations yield, in contrast to previous interpretations by Chien and Dickinson (5), the possibility for the formation of the same $N_e$-proton/O$_2$ interaction. However, a difference in ENDOR data is to be expected for the coupling to one of the Val (E11) methyl protons. The experimental observation leads us to assume that the type II complex is present in the tetramer with the ligand interacting strongly with the methyl proton, whereas type I is either not present or, as suggested by the calculated couplings, has a negligible interaction with the Val (E11) methyl group.

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