Electron Paramagnetic Resonance of Single Crystal Deoxycobaltohemoglobin

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Summary: Single crystals of horse ^{Co}Hb were obtained by reduction of $^{Co}Hb^{+}$ crystals with dithionite. Epr measurements showed that the g and ^{Co}A tensors are both axial and share the same principal axis systems. Of the four subunits, the "heme" normals of C and D subunits lie in the ab plane 29 ± 1° from b; they have the same orientation as the corresponding hemes in methemoglobin. The normals of "hemes" A and B are 47° above the ab plane as compared to 16° in methemoglobin.

Introduction: The preparation of the cobalt derivatives of hemoglobin and myoglobin by Hoffman and Petering (1) opened the way to study both the oxy and deoxy species of the proteins with epr. Single crystal epr of ^{Co}Mb and $^{Co}MbO_2$ (2) revealed a wealth of stereochemical information. The oxygen ligand was found to be π-bonded to the cobalt atom. Equally as important and interesting is the comparison of allosteric properties of the cobalt and the iron proteins which brings out the central role of the metal atom in detail (3). The cobalt species, in comparison to the native enzyme, binds oxygen with one-twentieth of the affinity, 80% of the cooperativity, and 40% of the alkaline Bohr effect. Furthermore, relative to human $^{Fe}Hb_A$, $^{Co}Hb_A$ has greatly reduced CO_2 binding, enhanced affinity for DPG and spatially more open "heme" pocket in both the oxy and deoxy species. These properties can be attributed to conformational differences in the iron and the cobalt proteins. To further delineate

Abbreviations: ^{Co}Hb, deoxycobaltohemoglobin; $^{Co}HbO_2$, oxycobaltohemoglobin; $^{Co}Hb^{+}$, cobaltihemoglobin; ^{Co}Mb, deoxycobaltomyoglobin, $^{Co}MbO_2$, oxycobaltomyoglobin; ^{Fe}Hb, deoxyhemoglobin; $^{Fe}HbO_2$, oxyhemoglobin; $^{Fe}Hb^{+}$, methemoglobin; epr, paramagnetic resonance; $g_{\parallel}$ and $g_{\perp}$, principal values of axial g-tensor; ^{Co}A, cobalt nuclear hyperfine; ^{N}A, nitrogen nuclear hyperfine; G, gauss.

these differences we have undertaken epr and x-ray diffraction studies of cobaltohemoglobins. The epr results on ^{Co}Hb crystals are presented here.

Preparation: ^{Co}Hb was prepared from fresh native horse hemoglobin by the method previously described (3). The crystallization procedure was the same as that used to crystallize $^{Fe}Hb^{+}$ (4). As it was crystallized, the protein was in the oxidized state and had no detectable epr signal. It was subsequently reduced by exposure to mother liquor containing excess sodium dithionite for two days.

The crystal habit of horse ^{Co}Hb is essentially the same as that of horse $^{Fe}Hb^{+}$. The diamond shaped crystal is best developed in the [001] plane. The apical angles are 60° and 118° (±2°) in ^{Co}Hb as compared to 64° and 120° in the native enzyme.

Epr spectra were obtained with a Varian E-9 instrument operating at X-band frequency. The goniometer used and the procedures for data acquisition and analysis have been given previously (2, 5, 6). Spectra were recorded every 10° in three perpendicular planes: $\underset{\sim}{ab}$, $\underset{\sim}{ac}^*$, and $\underset{\sim}{bc}^*$ where $\underset{\sim}{c}^* = \underset{\sim}{a} \times \underset{\sim}{b}$ and $\underset{\sim}{a}$ bisects the larger apical angle of the [001] face.

Results: Whereas, the epr lines are best resolved in the $\underset{\sim}{a}^*\underset{\sim}{b}$ plane in ^{Co}Mb and $^{Co}MbO_2$ (2), the resolution is the best in the $\underset{\sim}{ab}$ plane in ^{Co}Hb. In both cases, because ^{Co}A is larger than the $\underset{\sim}{g}$ anisotropy at X-band frequency, the overlap problems are severe. The situation is worse for ^{Co}Hb since there are four nonequivalent sites at any arbitrary orientation. However, analysis of the data for ^{Co}Hb was facilitated by the knowledge of the epr parameters for ^{Co}Mb (2).

A typical epr spectrum in the $\underset{\sim}{ab}$ plane is shown in Figure 1; the angular variations of the resonance lines in this plane are given in Figure 2. Using the designation for the four hemes of the tetramer by Bennett et al. (7), the eight cobalt hyperfine multiplets for "hemes" $\underset{\sim}{C}$ and $\underset{\sim}{D}$ are clearly resolved for most orientations in the $\underset{\sim}{ab}$ plane. As in the case of ^{Co}Mb, the hyperfine lines have narrower width at lower

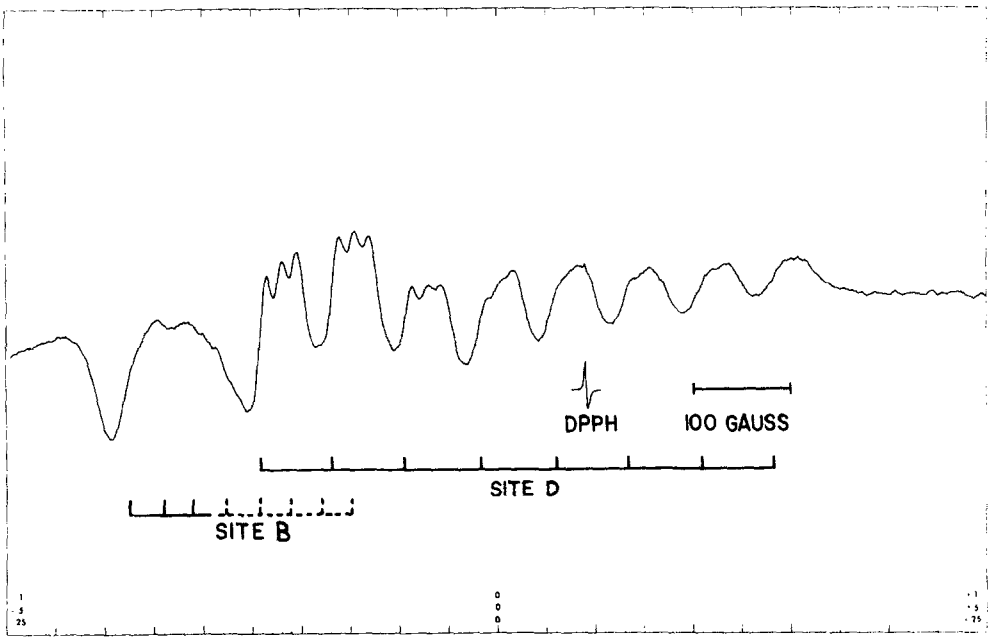

Figure 1. Epr spectrum of ^{Co}Hb single crystal with the dc magnetic field in the ab plane approximately 29° from the a axis.

field with lines broadening to higher field. The narrower lines are further split into triplets undoubtedly due to the ε-nitrogen of the proximal histidine. The maximum value for ^{N}A is observed to be 17.5 G.

Near b-axis the g-value approaches 2.31; the resonance has no resolvable hyperfine structures. Using 2.31 from frozen solution spectra as $g_{\perp}$ for ^{Co}Hb ($g_{\perp}$ = 2.32 for ^{Co}Mb), we concluded that the "heme" normals for C and D lie in the ab plane and make angles of 29 ± 1° with a. In $^{Fe}Hb^{+}$, these normals make angles of 32° with the a axis (7). Therefore, subunits C and D have the same orientations in ^{Co}Hb and $^{Fe}Hb^{+}$.

The minimum g-value was found to be 2.037 at ±29° from a-axis; $g_{\parallel}$ for ^{Co}Mb is 2.02. Therefore, the epr of both ^{Co}Mb and ^{Co}Hb have axial symmetry and the same g-tensor within experimental error. From these principal g-values the expected angular variation of the resonance positions was calculated. This is shown in Figure 2 and is in fairly good agreement with the observed variations. The maximum ^{Co}A is 76 G.

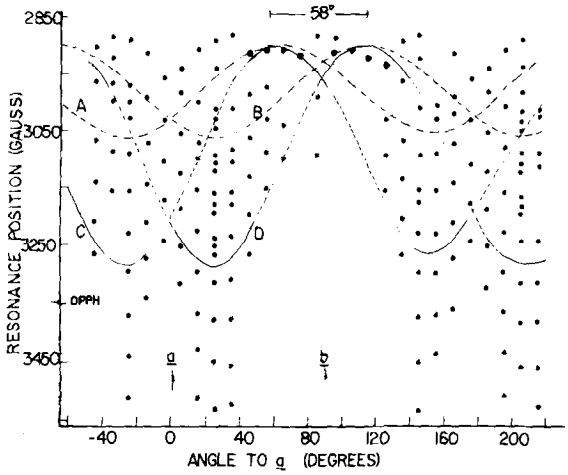

Figure 2. A plot of resonance position of epr transitions in gauss versus angle between the dc magnetic field and the a axis. The filled circles indicate transition positions, the larger circles indicate intense lines with no hyperfine splitting. The lines drawn show calculated g variation using g extrema values as discussed in the text. The solid part of these lines show the region over which the calculated g variation can be observed to follow the observed g variation. Outside the region of solid lines for a given site, the line widths are so large as to make the transition unobservable.

From frozen solution epr spectrum of ^{Co}Hb, Hoffman and Petering (1) reported $^{Co}A \simeq 80$ G. We have obtained frozen solution spectra of improved resolution and found $^{Co}A = 76$ G, and $^{N}A = 17$ G. Since the maximum value of ^{Co}A and minimum value of g were found at the same crystal orientation, the two tensors apparently have a common principal axis system.

At $\pm 29^{\circ}$ from $\underset{\sim}{a}$ in Figure 2 at least ten lines are resolved not counting the nitrogen hyperfine splitting. The two additional lines cannot be due to hyperfine lines of $\underset{\sim}{C}$ superposed on the main spectrum of $\underset{\sim}{D}$ and vice versa. The hyperfine splitting becomes small and unresolvable at 50° from the $\underset{\sim}{g}_{\parallel}$ direction. Instead, these lines can be best attributed to the low-field components of the hyperfine multiplets of the other two subunits $\underset{\sim}{A}$ and $\underset{\sim}{B}$. From the separation between these lines, the g-values can be estimated. Knowing the principal g-values, one can cal-

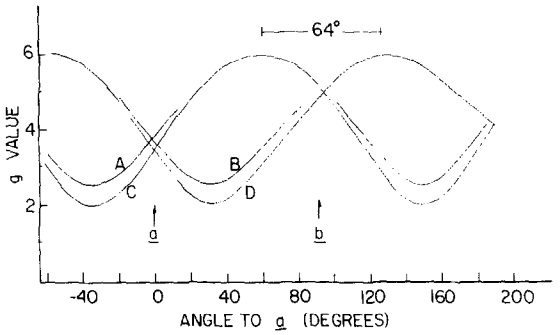

Figure 3. A plot of g value versus the angle between the a axis and the dc magnetic field in the ab plane for Hb^+. These data are replotted directly from reference 7 and are presented for comparison with and clarification of Figure 2.

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culate readily a tilt angle for the "heme" normals of 47° from ab. Even though this angle is probably only accurate to ±5°, it is significantly different from the tilt of 16° for these same subunits in methemoglobin (7).

The line width variation observed for ^{Co}Hb follows the pattern of $^{Fe}Hb^+$ (8, 9) and ^{Co}Mb (2) which have been attributed to imperfect alignment of the g-tensor in hemoprotein single crystals. The lines for subunits C and D are narrowest in both $^{Fe}Hb^+$ and ^{Co}Hb at ±29° from a in the ab plane.

Epr spectra in the ac* and bc* planes contain less detail than the ones in the ab plane. In the ac* plane hyperfine lines about 30 gauss in width were resolved only at g = 2.09 within ±20° of a. In the vicinity of c* only a single line 40 gauss wide at g = 2.31 is observed. The bc* plane shows little g variation and no resolvable hyperfine splitting. Near b the line width is 41 gauss and g is 2.30. The line becomes asymmetric near c with a "width" of 200 gauss centered at g = 2.24.

Attempts to reoxygenate the reduced ^{Co}Hb crystals by flushing with fresh oxygen-free saturated dithionite buffer fail to generate epr signals which could be assigned to $^{Co}HbO_2$. The same treatment was capable of converting ^{Co}Mb to $^{Co}MbO_2$. Most likely ^{Co}Hb became oxidized rather than oxygenated. In the absence of allosteric interactions,

oxygenation of ^{Co}Mb is apparently able to compete with autoxidation. In the case of ^{Co}Hb, allosteric transition, which accompanies oxygenation, probably presents a hindrance factor in the competition against oxidation.

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