Hydrogen Bonds in Proteins

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Direct Evidence for a Hydrogen Bond to Bound Dioxygen in a Myoglobin/Hemoglobin Model System and in Cobalt Myoglobin by Pulse-EPR Spectroscopy**

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In memory of Arthur Schweiger

Discrimination between dioxygen and carbon monoxide binding to the respiratory proteins myoglobin (Mb) and hemoglobin (Hb) is vital for aerobic life. There is still an ongoing debate about the nature and molecular mechanism of this discrimination.[1] It is widely believed that the distal histidine stabilizes bound dioxygen by a hydrogen-bond interaction, although a direct observation and characterization of this proposed hydrogen bond has been difficult and ambiguous.[1,2b] Functional Co^{II} analogues of the natural, Fe^{II}-containing Mb and Hb can be used to study the interactions of bound dioxygen with its surroundings by EPR methods.[2] It has been shown that the dioxygen adducts of Co-Mb and natural Mb adopt very similar geometries.[3] Herein, we present a new Co^{II}-containing model complex **1-Co** for the dioxygen-binding site of Mb and Hb, together with a direct pulse-EPR evidence for a distal hydrogen bond in the corresponding dioxygen adduct **1-Co**-O_2. Furthermore, we extended our study to Co-Mb and provide a comprehensive pulse-EPR study of the distal hydrogen bonding in Co-Mb-O_2 as well.

Compound **1-Co** consists of a Co^{II} porphyrin core with an alkyl-tethered imidazole base to mimic the proximal histidine in Mb and Hb, and an alkyne-appended benzimidazole residue mimicking the distal histidine (Scheme 1). Only the distal hydrogen-bond-donating proton can be exchanged by D_2O in complex **1-Co**.

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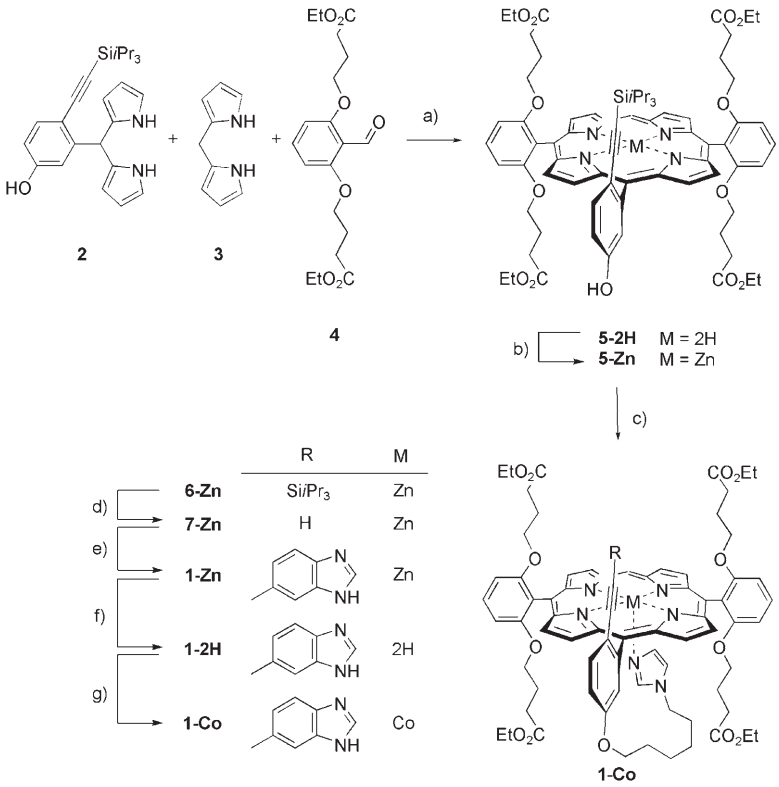
Model compound **1-Co** was synthesized via the corresponding Zn^{II} derivative **1-Zn**, using a classical porphyrin condensation as the key step (Scheme 1).[4] Dipyrromethanes **2**[5] and **3**[6] and aldehyde **4**[7] were subjected to the condensation reaction to yield free-base porphyrin **5-2H**. Subsequent Zn^{II} insertion gave **5-Zn**, to which the axial base was attached using 1-(6-bromohexyl)imidazole,[8] leading to formation of **6-Zn**. Removal of the Si*i*Pr_3 protecting group furnished **7-Zn**, and Sonogashira cross-coupling with 5(6)-iodobenzimidazole[9] provided **1-Zn** with the fully functionalized porphyrin core. After the Zn^{II} ion was removed with TFA, Co^{II} was inserted into the intermediate free-base porphyrin **1-2H** using $CoCl_2$.

X-band (ca. 9.6 GHz) continuous wave (CW) EPR spectra of **1-Co**-O_2 in frozen solution (120 K) were recorded to demonstrate the spectroscopic purity of the obtained samples. The spectra exhibit the common features of a low-spin Co^{II} species with dominating d_{z^2} character in a porphyrin environment (see Supporting Information). Dioxygen adducts were obtained within seconds by exposing samples of **1-Co** to dioxygen at 20 °C. By forming the dioxygen adducts, the spin population is transferred from the central Co^{II} ion to the attached dioxygen nuclei. This transfer results in a different X-band CW EPR spectrum of the frozen solution (120 K), exhibiting narrower features (see Supporting Information).

Proton hyperfine splittings can be resolved by pulse-EPR methods.[10] However, a variety of ESEEM (electron spin echo envelope modulation) and ENDOR (electron nuclear double resonance) methods failed to measure the complete proton hyperfine splittings in **1-Co**-O_2 (see Supporting Information) because of a severe cross suppression effect.[11] The Q-band (ca. 35 GHz) Davies-ENDOR experiment[10d,e] combined with short preparation pulses (which enhance the relative intensity of larger hyperfine splittings[10d]) allowed the complete determination of the hyperfine interactions caused by protons close to bound dioxygen. These experiments were carried out at low temperature (10 K) on a frozen solution of **1-Co**-O_2. Field-swept FID-integral-detected EPR spectra were recorded to determine the magnetic field (observer) positions for the pulse-EPR experiments. Subsequently, proton ENDOR spectra were recorded at twelve different observer positions for the complete detection of the proton splittings. The largest proton hyperfine coupling for complex **1-Co**-O_2—ranging from 6.0 MHz at the single crystal posi-



Scheme 1. Synthesis of **1-Co**. a) TFA, CH_2Cl_2, 20 °C, 16 h, then chloranil, 40 °C, 2 h, 15 %; b) $Zn(OAc)_2{\cdot}2H_2O$, MeOH/$CHCl_3$, 65 °C, 1 h, 96 %; c) 1-(6-bromohexyl)imidazole, Cs_2CO_3, DMF, 20 °C, 14 h, 79 %; d) nBu$_4$NF, THF, 20 °C, 40 min, 93 %; e) 5(6)-iodobenzimidazole, [Pd(PPh$_3$)$_4$], CuI, NEt$_3$, DMF, 100 °C, 4 h, 51 %; f) TFA, $CHCl_3$, 20 °C, 12 min, quant.; g) $CoCl_2$, 2,6-lutidine, THF, 20 °C, 12 h. TFA = trifluoroacetic acid.

tions g_z and g_y (low and high field, respectively) to 14.0 MHz along g_x—disappeared upon D_2O exchange (Figure 1 a). This hyperfine splitting for an exchangeable proton is exceptionally large when compared to all the splittings for related Co^{II} dioxygen adducts reported to date.[2] It clearly demonstrates a hydrogen-bonding interaction in complex **1-Co**-O_2 between the bound dioxygen and a defined and exchangeable nearby proton. Additionally, Davies-ENDOR spectra of **1-Co**-O_2 were recorded in CD_2Cl_2 as solvent, to probe possible solvent interactions (see Supporting Information). The only significant differences to the spectra recorded in CH_2Cl_2 appear at smaller values for the proton hyperfine splittings (up to 6.0 MHz; see Supporting Information). Samples of **1-Co**-O_2 prepared in wet and dry solvent showed the same large hyperfine splittings. Thus, a dominating interaction of solvent molecules or residual solvent water with bound dioxygen in **1-Co**-O_2 can be precluded. To elucidate whether the hyperfine matrix of the exchangeable proton has both positive and negative principal values, the X-band 6-pulse HYSCORE[12] spectrum of **1-Co**-O_2 was measured at the field position corresponding to the largest proton interaction. Although the 6-pulse HYSCORE spectrum did not allow the full exchangeable splitting to be measured, its off anti-diagonal ridge was clearly visible (see Supporting Information). This spectrum demonstrates that both positive and negative principal values exist, which in turn shows that the hyperfine coupling associated with the hydrogen bond is dominated by a through-space contribution with only a small isotropic contribution. The hydrogen bond is thus mainly dipolar in character.[10c]

Simulations of CW EPR and ENDOR spectra of **1-Co**-O_2 were carried out with the main emphasis on deriving the geometry parameters and bonding properties of the exchangeable proton. CW EPR spectra simulations of **1-Co**-O_2 were performed to obtain the Co–O_2 geometry. The g-values were $g = [2.0027 \pm 0.0005, 1.989 \pm 0.001, 2.0723 \pm 0.0005]$, the principal values of the metal hyperfine tensor $A^{Co} = [-54, -28, -28]$ MHz (each ± 2 MHz) and the Euler angles $(\alpha, \beta, \gamma) = (0° \pm 10°, 70° \pm 10°, 0° \pm 20°)$ with respect to the g-frame, that is, the angle between the O–O axis and the heme normal is 70° in complex **1-Co**-O_2 (Figure 2).

Subsequently, Davies-ENDOR simulations were carried out at each observer field position to fit the experimental difference spectra of the exchangeable proton (Figure 1 b) and the solvent protons (see the Supporting Information). We obtained a hyperfine tensor for the exchangeable proton of $A^H = [-6.8, -6.0, 13.5]$ MHz (each ± 0.5 MHz), with Euler angles $(\alpha, \beta, \gamma) = (0°, 105°, 0°) \pm 10°$ (Figure 2). This result indicates a mainly dipolar character of the hydrogen bond, with the strongest proton interaction (A_z^H) directed towards dioxygen. The exchangeable proton is positioned directly above the dioxygen, with an angle between the distal proton and the O–O axis of $\pm 105°$. Although the hyperfine tensor is not entirely axial, and the bulk electron spin population is not localized at one atom but distributed between the two oxygen nuclei, we assumed the spin density to be centered at one point in space.[13] A distance of (2.3 ± 0.2) Å between this spin-density center and the NH proton of the distal benzimidazole was obtained by using the point-dipole approximation with a dipolar contribution $T = 6.5$ MHz.[10c] A preliminary modeling at the semiempirical PM5 level of theory of the Fe^{II} substituted complex **1-Fe**-O_2 (for easier modeling) showed that the distal benzimidazole NH proton is located close to dioxygen at a very similar distance of approximately 2.40 Å (see the Supporting Information). Taken together with the fact that the NH proton of the distal benzimidazole is the only proton that can be exchanged by D_2O, we conclude that the hydrogen bond observed indeed arises from interactions between this distal NH proton and bound dioxygen.

Having found the suitable pulse-EPR method to measure the complete proton hyperfine splittings of porphyrin Co^{II} dioxygen adducts, we completed our study with the examination of the hyperfine splittings in Co-Mb-O_2. Co-Mb-O_2 was prepared in aqueous buffer solution and in deuterated buffer solution using apo-Mb from equine skeletal muscle according to standard methods.[14] An even larger hyperfine splitting, ranging from 10.0 MHz (g_z and g_y at low and high field, respectively) to 19.0 MHz (near g_x), was found in the

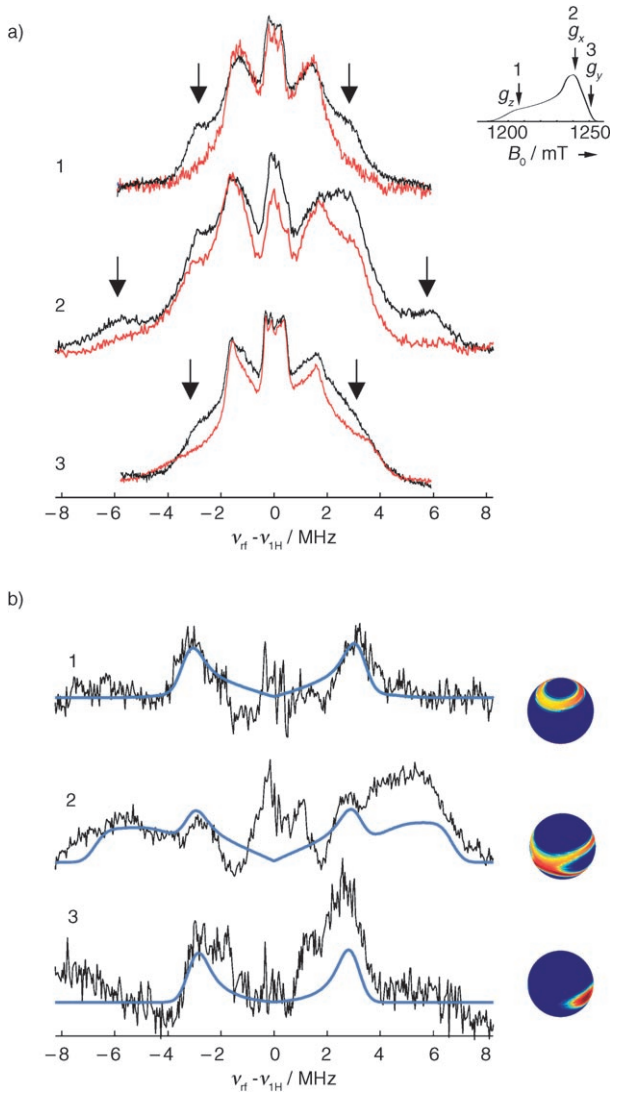


Figure 1. a) Frozen-solution (CH_2Cl_2) Davies-ENDOR spectra recorded at Q-band frequency at 10 K of **1-Co**-O_2 (black), and after D_2O exchange (red). Arrows indicate the largest and exchangeable proton splitting. Observer positions 1–3 are indicated in the inset showing the FID-integral-detected EPR spectra. The preparation pulse length at observer position 1 (close to g_z) and 3 (g_y) is 160 ns, and at observer position 2 (g_x) 80 ns. b) Corresponding difference spectra of **1-Co**-O_2 before and after D_2O exchange at the three different observer positions 1–3 (black) and simulated spectra of the exchangeable proton (blue). The orientations contributing to the experimental spectra are projected on the unit spheres.

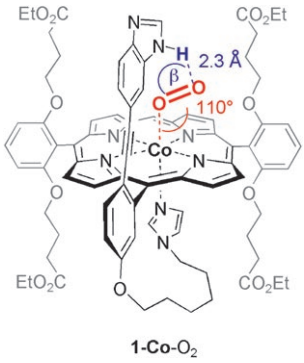


Figure 2. A distal hydrogen bond to bound dioxygen is formed in complex **1-Co**-O_2. The angles and distance shown were obtained from the frozen-solution EPR measurements of **1-Co**-O_2; $\beta = 105°$.

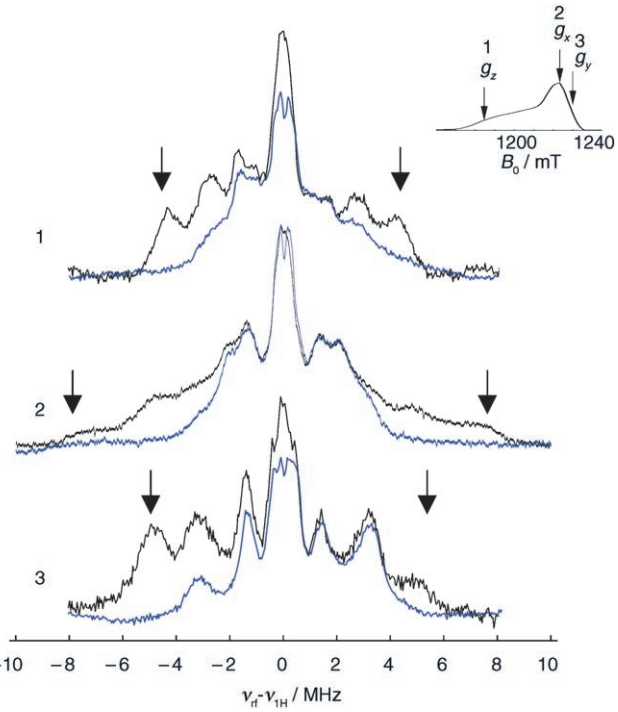


Figure 3. Frozen-solution Davies-ENDOR spectra recorded at Q-band frequency of Co-Mb-O_2 in H_2O buffer (black), and in D_2O buffer (blue) using a 80 ns preparation pulse. Arrows indicate the largest and exchangeable proton hyperfine splitting. Observer positions 1–3 are indicated in the inset showing the FID-integral-detected EPR spectra.

corresponding frozen-solution Q-band Davies-ENDOR spectra of Co-Mb-O_2 (13 K). Upon D_2O exchange of the buffer solution, this large hyperfine splitting disappeared (Figure 3).

In Co-Mb-O_2, as in our model compound, a defined distal hydrogen bond was detected. The evolution of the largest and exchangeable proton hyperfine splitting over the different field positions in Co-Mb-O_2 is the same as in the model complex **1-Co**-O_2, only the magnitude is greater in the protein, which suggests a similar orientation of the two hydrogen bonds but a smaller distance (2.0 ± 0.2 Å) to the interacting proton in Co-Mb-O_2. Also similar to our model complex **1-Co**-O_2 is the character of the hydrogen bond in Co-Mb-O_2, which is mainly dipolar in nature. This was again demonstrated by the X-band 6-pulse HYSCORE spectrum measured at the position of the largest extension of the exchangeable proton hyperfine splitting (see Supporting Information).

Although the proton hyperfine splittings of Co-Mb-O_2 have been studied already, only one field position (corresponding to g_y) was measured and the observed exchangeable proton splitting was considerably smaller (9.0 MHz).[2b] In the light of our results, we suppose that this 9.0 MHz proton hyperfine splitting is actually only the inner part of the complete proton hyperfine splitting at this field position.

As the protein is studied in H_2O as solvent, exchangeable proton splittings are hard to assign to particular protein residues or surrounding H_2O molecules. However, our model-complex study revealed the hydrogen-bonded benzimidazole NH proton to be positioned above dioxygen. As the geometry of the distal hydrogen bond is similar in our model and in Co-Mb-O_2, the interacting proton in the protein must also be located above dioxygen. Only the distal histidine NH proton is able to reach the bound dioxygen in such a position and at a distance of 2.00 Å. H_2O in the distal pocket has a completely different position[15] and thus, it is very unlikely that H_2O is the cause of the largest and exchangeable proton hyperfine splitting in our Davies-ENDOR spectra.

As has been observed earlier in EPR crystal studies,[13b,16] at low temperature ($T < -50\,°C$) there are two different dioxygen species of Co-Mb-O_2 (species 1, species 2), differing by approximately 90° in the orientation of bound dioxygen with respect to the cobalt heme group. We can confirm these findings, and were able to separate the FID-integral-detected EPR spectra of both species using a longitudinal relaxation time filter at Q-band frequency (see Supporting Information). Additionally, by using the same filter in combination with the Davies-ENDOR sequence, we were able to attribute the largest and exchangeable proton hyperfine splitting exclusively to the species 2 (longitudinal relaxation time of ca. 450 μs). Apparently, the dioxygen of species 1 (longitudinal relaxation time of ca. 80 μs) does not strongly interact with an exchangeable proton within 2.70 Å, such an interaction would have been observable in our spectra. As the absolute geometries of dioxygen in the two species have not been assigned as of yet, we can only speculate about the reasons of this finding. Further studies are being pursued to answer the open questions.

In summary, we directly detected distal hydrogen bonding to bound dioxygen in model complex **1-Co**-O_2 and in Co-Mb-O_2 by Q-band Davies-ENDOR and present the complete EPR parameters for this interaction in **1-Co**-O_2 obtained by simulations. The dipolar character of the hydrogen bond as well as its orientation was found to be similar in the model complex **1-Co**-O_2 and in Co-Mb-O_2. Thus, we conclude that complex **1-Co**-O_2 is an excellent functional model for the dioxygen-binding site of Mb and Hb, and reproduces well crucial properties of the natural proteins.

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