EPR STUDY OF ^{17}O NUCLEAR HYPERFINE INTERACTION IN COBALT-OXYHEMOGLOBIN: CONFORMATION OF BOUND OXYGEN

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Received September 23,1975

SUMMARY

The electron spin resonance spectra of cobalt-oxyhemoglobin, with oxygen enriched to 95 atom % with ^{17}O, are broadened due to ^{17}O nuclear hyperfine interaction with the unpaired electron spin and show at X-band and 7°K the presence of several well-resolved ^{17}O satellites at low and high field extremes, all of which are absent in ^{16}O cobalt-oxyhemoglobin. The ^{17}O EPR spectra are interpreted in terms of two nonequivalent hyperfine interaction constants of 65 ± 5 G and 93 ± 5 G for the two oxygen atoms. The observed broadening and hyperfine splittings in the EPR spectra indicate complete transfer of the unpaired spin to oxygen orbitals. The observed difference in the hyperfine coupling constants of the two oxygen atoms establishes an asymmetric linkage of the oxygen molecule to the metal, consistent with the Pauling model of oxyhemoglobin.

INTRODUCTION

The conformation of the oxygen molecule bound to hemoglobin and myoglobin has been a subject of controversy in the literature ever since Pauling and Coryell proposed a linear structure for it (1). Subsequently Pauling (2) proposed a bent structure with nonequivalent oxygens and Griffith (3) a π-bonded structure with equivalent oxygens equidistant from the metal atom. While X-ray crystallographic studies of azido-metmyoglobin have established a bent ligand conformation (4), such studies of the oxygen ligand in oxymyoglobin are not well defined but also suggest a Pauling-type structure (5). Crystallographic as well as spectroscopic studies on reversibly formed model dioxygen complexes indicate both Pauling and Griffith type conformations (6-14).

In a recent communication EPR evidence has been presented supporting the existence of magnetically equivalent oxygen atoms in the mononuclear cobalt complex Co(bzacen)pyO_2 in solution (10). The experimental results consisted of the solution EPR spectrum of the ^{17}O-enriched complex, which was interpreted in terms of two equal isotropic ^{17}O splittings of 21.6 G. The total spread of the EPR spectra in the ^{17}O-labeled complex has proved invaluable in these studies. Frozen solution EPR spectra of 40 atom % ^{17}O-enriched Co(bzacen)pyO_2 complex have also been studied (9). The total hyperfine splitting, A_o, due to the ^{17}O nucleus in the frozen state is a sum of an anisotropic (dipolar), A_d, and an isotropic (contact), A_c, contribution. It has been shown that to a good first approximation $A_d = -103\ \rho_o$ and $A_c = -41\ \rho_o$ (15), where ρ_o is the spin density on the ^{17}O nucleus in question. From the measured separation between the outermost pair of ^{17}O satellites at the low or high field extremes of the first derivative spectrum in frozen solutions of Co(bzacen)pyO_2 of $\sim$ 60 G, which must equal one of the hyperfine splitting constants, and the total spread of the spectrum (five-times the sum of the splitting constants) of $\sim$740 G which implies that the sum of hyperfine splitting constants is $\sim$148 G, Getz et al. concluded that the O_2^- group in frozen solutions is asymmetrical.

Since oxyhemoglobin is diamagnetic, the nature of the linkage of oxygen to heme in oxyhemoglobin cannot be determined by EPR spectroscopy. Replacement of iron by Co^{2+}, however, results in a paramagnetic system and Co^{2+} substituted hemoglobins have recently been prepared and characterized chemically and spectroscopically (16-20). Cobalt-hemoglobins are functional in reversible oxygen binding and show cooperative behavior that is qualitatively similar in all respects to that of hemoglobin. Hence, it is reasonable to assume that the conformation of bound oxygen in cobalt-oxyhemoglobin is identical to its conformation in oxyhemoglobin. Further, there is evidence for at least a partial transfer of the unpaired spin density from low spin cobalt (s = 1/2) to the oxygen orbitals in cobalt-hemoglobin since a free radical type signal around g = 2 is observed (16,17,20). However, the extent of spin-transfer

is unknown. A non-vanishing cobalt nuclear hyperfine interaction due to residual unpaired electron on cobalt is also observed in the EPR spectra (20).

In this paper, we report an EPR study of ^{17}O enriched cobalt-oxyhemoglobin carried out with a view to determine the extent of transfer of unpaired spin to oxygen orbitals and to determine the nature of metal oxygen linkage.

MATERIALS AND METHODS

Cobalt-hemoglobin was prepared by incorporating cobalt protoporphyrin into apohemoglobin by standard procedures (16-20). An 0.3 ml solution (∿1 mM) pH 7.0, contained in an EPR tube (quartz, 5 mm O.D.) was then degassed to a pressure of 500 microns. This resulted in an unavoidable concentrating of the final solution by a factor of ∿3. The sample was then opened to an atmosphere of pressurized 95 atom % ^{17}O-enriched oxygen and equilibrated for ∿2 minutes with mixing. The sample was then frozen and the sample tube sealed. A control sample was prepared following an identical procedure but substituting regular air in place of ^{17}O-enriched oxygen. The samples were then thawed and well mixed to ensure complete formation of oxyhemoglobin.

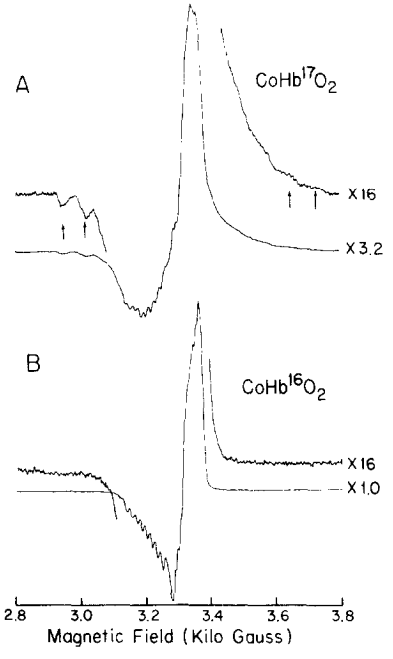

Figure 1: X-band EPR spectra at 7°K of (A) ^{17}O cobalt-oxyhemoglobin (B) ^{16}O cobalt-oxyhemoglobin. The small satellites marked with arrows arise from ^{17}O nuclear hyperfine interaction. The relative instrumental gain settings are as indicated.

X-band EPR spectra were recorded at 7°K and at 77°K on a Varian E-4 spectrometer and on a Varian 4502 EPR spectrometer respectively. The latter instrument was also used for obtaining K-band spectra at 77°K.

RESULTS AND DISCUSSION

The resulting spectra at X-band and 7° K showed best resolution and are presented in Figure 1. The spectra obtained with ^{17}O oxyhemoglobin (Figure 1A) and ^{16}O oxyhemoglobin (Figure 1B) are different; the one with ^{17}O shows considerable broadening of the electron resonance signal compared to ^{16}O. In addition several well-resolved equally intense satellites are present at the extreme low and high field regions of the ^{17}O spectrum. The satellites at the high field end are approximately three-fold broader than the ones at the low field end and indicate the dependence of the line-boradening on the nuclear spin quantum number. All of these features are absent in the ^{16}O spectrum. The spectra obtained at K-band (Figure 2) did not show any better resolution perhaps due to broadening effects of the increased magnetic field. The parallel and perpendicular regions of the EPR spectra are, however, better separated

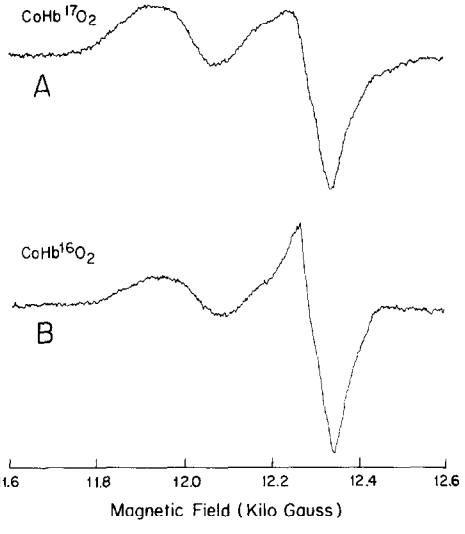

Figure 2: K-band EPR spectra at 77°K of (A) ^{17}O cobalt-oxyhemoglobin (B) ^{16}O cobalt-oxyhemoglobin.

at K-band and the K-band spectra clearly indicate a dominant interaction of the unpaired electron with the ^{17}O nuclei in the perpendicular region. Hence it follows that the observed ^{17}O satellites must arise from the perpendicular region of the EPR spectrum which is most broadened and split by interactions with the ^{17}O oxygen molecule. This analysis is consistent with the observed lack of cobalt nuclear hyperfine splitting of the ^{17}O satellites, such splitting being observable only in the parallel region of the ^{16}O spectrum. The observed differences between ^{17}O and ^{16}O spectra indicate a strong interaction of the unpaired electron with the oxygen nuclei, establishing a substantial transfer of the unpaired spin from the cobalt atom to the oxygen π-orbitals. The presence of satellites at 2945, 3010, 3660 and 3730 G in the ^{17}O spectra at X-band and 7°K and their absence in the ^{16}O spectra indicates that they arise from the ^{17}O nuclear hyperfine interaction. The EPR spectra of oxyhemoglobin with 95 atom % ^{17}O is a superposition of hundreds of lines due to the unlabeled and singly and doubly labeled molecules which are present in the ratios of 0.0025:0.095:0.9025. The following analysis is, however, independent of the actual percentage of the ^{17}O enrichment, the only assumption, based on instrumental sensitivity considerations, being that there are at least 10% doubly labeled molecules of oxygen present. Thus a small dilution of the ^{17}O label in the process of handling will not invalidate our conclusions. This assertion is strengthened by the reproducibility of our ^{17}O spectra under varying handling conditions. The satellites at the high and low field extremes must arise from doubly labeled molecules since their spectra would show maximum spread owing to the ^{17}O-nuclear hyperfine interaction. It can be easily shown theoretically that the observed separation between the extreme high or low field pair of satellites must correspond to the smaller of the two hyperfine interaction constants for the oxygens if they are non-equivalent or the identical coupling constant of both oxygens if the two are equivalent. The observed separation

between the pair of extreme low field satellites is 65 ± 5 G and the separation between the extreme low and high field satellites which measures the total spread of the spectrum is ∿790 G. This value of 790 G for the total spread should equal five-times the sum of the hyperfine splitting constants of the two oxygens. Since one of the coupling constants has the measured value of 65 ± 5 G, the other one must be 93 ± 5 G. This analysis indicates that the electronic structures of the two oxygen atoms in oxyhemoglobin must be very different consistent with the Pauling model.

The observed difference in the hyperfine interaction constants which corresponds to a 0.4:0.6 distribution of the unpaired electron between the two oxygen atoms is similar to that observed in a model system (9). This unequal coupling in model systems arises from a greater spin density on the terminal oxygen atom as shown by selective ^{17}O labeling (21) as may well be true in the present case.

The difference between the hyperfine interaction constants of the two oxygen atoms in cobalt-oxyhemoglobin may be due to the hydrophobic environment and the proximity of distal histidine which could alter the unpaired electron distribution in favor of the terminal oxygen atom. This may be shown by considering the relative contributions of various resonance

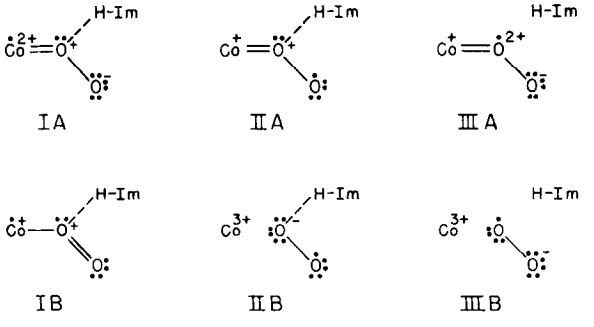

Figure 3: Possible resonance forms contributing to the structure of cobalt-oxyhemoglobin. The formal charges on the metal and oxygen atoms are as indicated.

forms to the overall structure of cobalt-oxyhemoglobin, using Pauling's valence bond notation (Figure 3). Resonance form IIA would contribute more than resonance form IIIA in a hydrophobic environment because of the greater formal charge of the latter. Further, resonance forms IIA and IIB would contribute more than IIIA and IIIB because the former pair are more strongly hydrogen bonded by the distal histidine.

From the magnitude of the coupling constants we estimate the unpaired spin density on the oxygen atoms to be $\frac{65 + 93}{144}$ or 110 $\pm$ 5% suggesting complete transfer of the unpaired electron from the cobalt atom to the oxygen molecule upon oxygenation. Although the difference between the observed unpaired spin of 1.1 and the net unpaired spin of unity is not well beyond our experimental error, it might be interpreted to mean that the residual spin on the cobalt is negative and corresponds to a tenth of an unpaired electron, or -10%. The magnitude of the unpaired spin induced on cobalt is consistent with the calculations of other workers based on the measured cobalt nuclear hyperfine coupling constants in the parallel and perpendicular regions of the EPR spectrum of model systems (11). A negative sign would result if the spin transfer occured by a spin-polarization mechanism (22) rather than by the contribution of resonance forms IA and IB (Fig. 3).

In conclusion, the above findings establish the inequivalence of the two oxygen atoms in oxyhemoglobin and for the first time enable one to make an unequivocal choice of the Pauling over the Griffith model and its variants (23).

ACKNOWLEDGMENTS

We thank Dr. E. Melamud for his generous gift of $^{17}O_2$. This work was supported by National Institutes of Health Grants AM-13351 and HL-14508, National Science Foundation Grant BMS74-03739, by Grants CA-06927 and RR-05539 to the Institute for Cancer Research from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

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