ELUCIDATION OF THE MODE OF BINDING OF OXYGEN TO IRON IN OXYHEMOGLOBIN BY INFRARED SPECTROSCOPY

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Summary. The infrared difference spectrum of packed human erythrocytes treated with $^{16}O_2$ vs $^{18}O_2$ or with $^{16}O_2$ vs CO has a unique band at 1107 cm^{-1} assigned to ^{16}O-^{16}O stretch for bound $^{16}O_2$. The frequency and intensity of this band prove non-linear end-on binding of O_2 to Fe(II) in oxyhemoglobin. An O-O bond order of ca. 1.5 is indicated. This is analagous to the change in bond order when CO, NO, and N_2 are similarly bound to iron. In consequence it seems unnecessary to use a bond description for O_2 bound to iron which is fundamentally different from that used for CO, NO, and N_2. The preferred bonding description is Fe⁼O⁼O. The strong covalent bonding between Fe and O_2 that results upon π-donation from Fe(II) to O_2 represents a quite sufficient reason for dioxygen to dissociate from oxyhemoglobin as O_2 rather than O_2^- and relegates the presence or absence of a nonpolar or hydrophobic environment to a minor role.

Introduction. , Since the finding by Pauling in 1936 that oxyhemoglobin is diamagnetic (1) considerable attention has been directed to the elucidation of the chemical nature of the bonding between oxygen and the heme iron of hemoglobin (2,3). To this date no direct evidence concerning the chemical nature of bound oxygen in any hemeprotein has been reported. We present here results of an infrared study of the O_2 ligand of oxyhemoglobin which resolve much of the controversy surrounding the structural basis for the reversible binding of oxygen to hemoglobin.

Methods and Materials. To obtain D_2O exchanged erythrocytes heparinized human blood was centrifuged at low speed, the plasma removed and the erythrocytes suspended in D_2O-saline (0.9%). After gentle agitation for 6 hours the suspended erythrocytes were again centrifuged and exchanged with D_2O-saline twice more in a similar manner. The use of D_2O allowed the transmission of

more energy through the region of interest, 1150-1000 cm^{-1}, than did H_2O, however both solvents were used for the examination of the region 1450-750 cm^{-1}.

Oxygenated blood was saturated with CO by exposing it to gaseous CO for 30 minutes. Isotopically labeled oxygenated blood was prepared by equilibrating partially deoxygenated blood with a four fold excess of $^{18}O_2$ (90% ^{18}O, Miles Laboratories) for 4 hours. This sample was then partially deoxygenated under vacuum and reequilibrated with $^{18}O_2$ as before. A portion of this sample was then reconverted to $^{16}O_2$ oxygenated blood to ensure that the isotopic differences were not an artifact of the preparation.

Infrared difference spectra were recorded for $^{16}O_2$ vs CO, $^{18}O_2$ vs CO, and $^{16}O_2$ vs $^{18}O_2$ samples of packed erythrocytes and isolated hemoglobin preparations using either CaF_2 or KRS-5 windows with pathlengths of either 0.025 or 0.050 mm. Cell pathlengths and protein concentrations must be accurately matched due to intense protein absorptions throughout the regions studied. Spectra were recorded on a Perkin-Elmer Model 180 infrared spectrophotometer using absorbance mode, expanded ordinate and abscissa, and a resolution of 3 cm^{-1}.

Results. Oxyhemoglobin and carbonyl hemoglobin show a number of spectral differences within the region 1450-750 cm^{-1}. Figure 1A shows the difference between D_2O exchanged oxygenated vs CO saturated packed erythrocytes in the region 1150-1000 cm^{-1}. Figure 1B shows the spectrum of the same sample when the $^{16}O_2$ has been partially exchanged with $^{18}O_2$. The change in the spectrum is immediately apparent. The 1107 cm^{-1} band decreases in intensity while a broader band at 1065 cm^{-1} appears. Upon replacement of the $^{18}O_2$ with $^{16}O_2$ the spectrum in Figure 1A is regenerated. These differences are also illustrated in Figure 1C of the spectrum of $^{16}O_2$ vs $^{18}O_2$ treated blood by the positive band at 1107 cm^{-1} and a negative band at 1065 cm^{-1}. The locations of these bands are reproducible in blood and in isolated hemoglobin and are not affected by changing the solvent from H_2O to D_2O. The narrow band (7.5 cm^{-1} half bandwidth) at 1107 cm^{-1} which is sensitive to changes in oxygen isotope must then be due

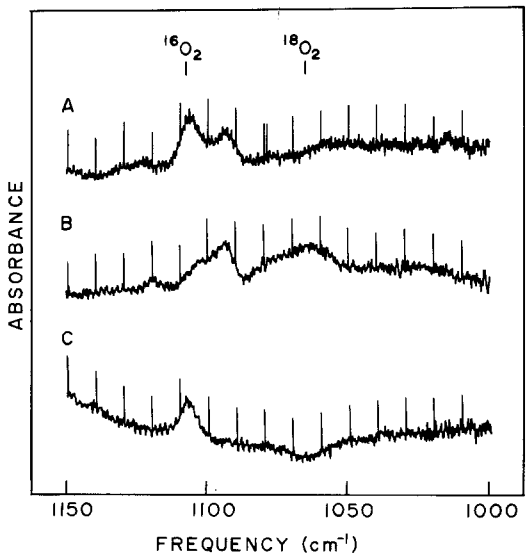
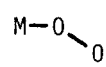


Figure 1 Infrared difference spectra of packed human erythrocytes exchanged with D_2O-saline in 0.025 mm pathlength CaF_2 cells representing: A. $^{16}O_2$ oxyhemoglobin vs carbonyl hemoglobin, B. $^{18}O_2$ oxyhemoglobin vs carbonyl hemoglobin, C. $^{16}O_2$ oxyhemoglobin vs $^{18}O_2$ oxyhemoglobin.

to O_2 bound to oxyhemoglobin.

Discussion. In mononuclear dioxygen complexes of transition metals both oxygen atoms (I) or only a single oxygen atom (II) may be bound to the metal (4).

I



II

Metal dioxygen complexes shown by X-ray (5) and oxygen isotopic infrared studies (6) to have both oxygen atoms bound to the metal as in I have an O-O stretch between 800 and 900 cm^{-1} (5,6) and an O-O bond order of about 1.0. On the other hand cobalt dioxygen complexes shown by X-ray (7) and EPR (8) to have a configuration as in II and an O-O bond order of 1.5 exhibit an O-O stretching frequency between 1100 and 1150 cm^{-1} (8,9). Thus O_2 bound to hemoglobin with an O-O stretching frequency of 1107 cm^{-1} is best represented as nonsymmetrically

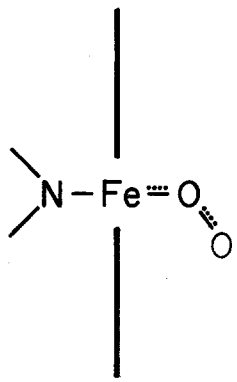


Figure 2 A diagramatic representation of the oxygen binding site of oxyhemoglobin with the heavy line representing the plane of the porphyrin ring. The dotted lines represent nonintegral bond orders which may be explained either by resonance contributions or by π electron delocalization from a filled t_{2g} orbital of the iron into the empty π^* orbital of oxygen. The partial negative charge developed on the distal oxygen may be stabilized by interactions with the imidazole of the distal histidine.

bound as in II with an O-O bond order of 1.5 (Figure 2). This configuration is in accord with Pauling's early formulations (10). The intensity of the O-O band in oxyhemoglobin, about 15% of that of CO bound to carbonyl hemoglobin, is also strong support for nonsymmetrical bonding between the heme iron and oxygen.

The reduction in bond order of about 0.5 for the O-O bond results from the transfer of electron density through d-orbitals of Fe(II) onto the bound O_2 molecule. The direction of the transfer is consistent with the effects of porphyrin ring substituents on ligands in hemes and in reconstituted myoglobins and hemoglobins (2,11,12). This is similar to the reduction in bond order in going from gas to heme ligand in CO [gas, 2143 cm^{-1} (13); pyridine protoheme CO, 1972 cm^{-1} (12), CO HbA, 1951 cm^{-1} (14)], NO [gas, 1876 cm^{-1} (13); pyridine protoheme NO, 1670 cm^{-1} (15); NO HbA, 1615 cm^{-1} (15)], and N_2 [gas, 2331 cm^{-1}

(13); pyridine protoheme N_2, 2101 cm^{-1} (16,17)]. The reduction of bond order on the ligand is accompanied by an increase of bond order or covalency between the heme iron and the ligand. Indeed the greater the electron transfer from iron to ligand the stronger is the iron ligand bond. Polar interactions of ligands with the imidazole of the distal histidine, while not required, could thus stabilize ligand binding. The bonding in these systems will be discussed in detail in subsequent reports.

The ability to detect the O-O stretching band is of particular interest in that it provides the opportunity to explore the properties and reactions of oxyhemeproteins in general as well as other oxymetalloproteins. It is yet to be determined how sensitive the infrared band for bound oxygen is to ligand and medium effects.

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