INFRARED EVIDENCE FOR THE MODE OF BINDING OF OXYGEN TO IRON OF MYOGLOBIN FROM HEART MUSCLE

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Summary. Infrared spectra for oxymyoglobin isolated from bovine heart muscle reveal non-linear end-on binding of O_2 to Fe(II) similar, but not identical, to that found for oxyhemoglobins. Difference spectra for $^{16}O_2$ Mb vs CO Mb and $^{16}O_2$ Mb vs $^{18}O_2$ Mb have a band at 1103 cm^{-1} assigned to bound $^{16}O_2$. Human oxyhemoglobin A and other hemoglobins exhibited an analogous band at 1107 cm^{-1}. A bonding description with strong covalent bonding between Fe(II) and O_2 (i.e., Fe=O-O) thus applies to oxymyoglobin as well as to oxyhemoglobins. CO Mb and CO HbA give ν_{CO} bands at 1944 and 1951 cm^{-1} respectively with about 10-fold greater intensity than the O-O bands.

Introduction. We recently reported finding an infrared band at 1107 cm^{-1} due to oxygen bound to hemoglobin in human erythrocytes (1). Thus infrared spectra provide direct evidence for non-linear end-on binding which we represent as Fe=O-O to indicate strong covalent bonding between iron(II) and O_2. This approach is potentially useful in the study, qualitatively or quantitatively, of O_2 bound to metal proteins in general. Here we report the comparison of oxyhemoglobin (O_2 Hb) and oxymyoglobin (O_2 Mb) by infrared methods.

Methods and Materials. Oxymyoglobin was isolated from bovine heart by a modification of the method of Yamasaki *et al* (2) in which a molecular sieve column as described by Awad *et al* (3) and Gotoh, *et al* (4) was used. All procedures were carried out at 0-4°C. Glass distilled water was used throughout. 750g of fresh bovine heart muscle which had been freed of fat and ligaments was minced in a meat grinder in the presence of 1.5 l water brought to pH 7.5 with 2N NH_4OH. Once thoroughly minced, the mixture was

similarly readjusted to pH 7.5 to retard the rate of myoglobin autoxidation. After 30 min the extract was separated from remaining tissue by squeezing through several layers of cheesecloth, brought to 70% saturation in $(NH_4)_2SO_4$ with the pH kept at 7.5, centrifuged at 14000 xg, and the precipitate (mainly Hb) discarded. The supernatant was saturated with $(NH_4)_2SO_4$, 5g celite was added, and the mixture was stirred for 30 min. The precipitate was collected by vacuum filtration and extracted with minimal (~40 ml) 0.005 M Tris buffer, pH 8.5. The extract applied to a G-100-120 Sephadex column equilibrated with the same buffer yielded a Mb fraction free of both Hb and $(NH_4)_2SO_4$. This fraction was applied to a DEAE cellulose column (4x14 cm) equilibrated with 0.005 M Tris buffer, pH 8.5 to isolate the oxy species. Upon elution with a stepwise gradient (0.005 M - 0.050 M Tris buffer, pH 8.5) the O_2 Mb followed the metMb. Fractions with our absorbance ratio for α to β bands (A_{582}/A_{544}) greater than or equal to 1.06 were pooled and concentrated to 11 mM in heme with an Amicon ultrafiltration system under O_2. The CO Mb was prepared by exposure of O_2 Mb to CO for 45 min.

The infrared cells used were precisely matched in terms of pathlength (0.025 mm) and CaF_2 window thickness. The CaF_2 windows allow both the visible - uv and infrared spectra to be recorded in the same cell. Under these conditions both before and after the infrared spectra were recorded O_2 Mb gave band maxima at 544 nm(β) and 582 nm(α) with A_α/A_β, 1.05 and CO Mb at 541 (β) and 578 (α) with A_α/A_β, 0.87. Infrared spectra were recorded on a Perkin-Elmer Model 180 infrared spectrophotometer in the absorbance mode with expanded ordinate and abscissa and a resolution of 3 cm^{-1}.

Results. Four different types of difference spectra are shown in Figure 1. Spectrum A represents CO Mb in the sample cell vs $^{16}O_2$ Mb in the reference cell. The intense band with a maximum at 1944 cm^{-1} and slight asymmetry on the lower frequency side is similar to earlier findings (5,6). Several other spectral differences are noted but only one appears sensitive to the isotopic composition of bound O_2. This band, as shown in spectra B

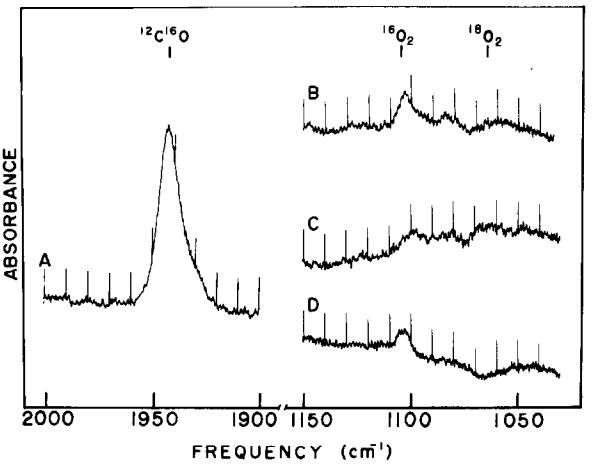

Figure 1. Infrared difference spectra of bovine heart myoglobin in 0.05 M Tris, pH 8.5, representing: A, carbonyl myoglobin vs $^{16}O_2$ oxymyoglobin in the region 2000 - 1900 cm^{-1}, B, $^{16}O_2$ oxymyoglobin vs carbonyl myoglobin in the region 1150 - 1030 cm^{-1}; C, $^{16}O_2$ oxymyoglobin partially exchanged with $^{18}O_2$ vs carbonyl myoglobin to give a lower intensity 1103 cm^{-1} band; D, $^{16}O_2$ oxymyoglobin vs $^{18}O_2$ oxymyoglobin, positive band at 1103 cm^{-1} due to the $^{16}O_2$, negative band at 1065 cm^{-1} due to the $^{18}O_2$.

and D, is the fairly prominent band at 1103 cm^{-1} which can be assigned to $^{16}O_2$ bound to Mb. Somewhat less apparent is the band at 1065 cm^{-1} due to bound $^{18}O_2$ (this band appears as a negative band in spectrum D where $^{16}O_2$ vs $^{18}O_2$ is recorded). The properties of the infrared bands of oxygen and carbon monoxide bound to hemoglobin and myoglobin are compared in Table I.

Discussion. Similarities in frequency, band width, and intensity for the ^{16}O-^{16}O bands in O_2 Mb and O_2 Hb indicate, not unexpectedly, that oxygen bonding has much in common for the two proteins. Indeed much of the reasoning used in the description of O_2 bonding to human erythrocytes based on infrared spectra (1) clearly applies to bovine O_2 Mb as well. However, also not unexpectedly, a significant frequency difference was found. It will be of

TABLE I

Properties of the Infrared Bands of Oxygen and Carbon Monoxide Bound to Hemoglobin and Myoglobin

	Hemoglobin	Myoglobin
Frequency $^{12}C^{16}O$	1951 cm^{-1}	1944 cm^{-1}
Band width at 1/2 height	8 cm^{-1}	12 cm^{-1}

Frequency ^{16}O-^{16}O	1107 cm^{-1}	1103 cm^{-1}
Band width at 1/2 height	9±1 cm^{-1}	8±1 cm^{-1}
Intensity (relative to CO band)	0.1	~0.1

Frequency ^{18}O-^{18}O	1065 cm^{-1}	1065 cm^{-1}

great interest to determine the extent to which this frequency difference is characteristic of the two globin types. More Hbs and Mbs must be examined before this point can be established. Nevertheless there is reason to believe there may be a highly characteristic difference between normal Hbs and normal Mbs as classes of protein in that over a fairly broad range of species a striking tendency for ν_{CO} to group about 1951 cm^{-1} for CO Hbs and about 1944 cm^{-1} for CO Mbs has been seen (7). Information on ν_{O_2} values is far more limited but also consistent with a similar distinction between Hbs and Mbs. Elk and rabbit O_2 Hbs also give a band at 1107 cm^{-1}. Only with more data can a satisfactory interpretation of effects of structure on ν_{O_2} be expected.

Infrared data appear uniquely suited to the determination of oxygen binding in oxygenated heme proteins. The dioxygen ligand is directly measured

and isotopic substitution identifies the vibrations. Neither X-ray nor resonance Raman methods have been successful here. Resonance Raman has been useful for the cases of oxyhemerythrin (8) and oxyhemocyanin (9) where peroxo dioxygen ligands are found. However, in the peroxo frequency region (e.g., 700 - 900 cm^{-1}), there can be ambiguity among μ-peroxo (M-O-O-M), terminal peroxo (M-O-O-H) or cyclic peroxo ($M\langle^{O}_{O}$) forms. Fortunately in the 1100 cm^{-1} region only a bent end-on binding ($M-O_{O}$) is expected (10). This vibrational frequency *per se* does not differentiate between the case of a highly ionic bonding (i.e., an iron(III)-superoxide ion pair) and the case of strong covalent bonding between metal and dioxygen ligand. However, other properties of the O_2 Mb and O_2 Hb systems clearly show the unsuitability of the ion-pair description (11).

Preliminary data for O-O band intensities among O_2 Hbs and the O_2 Mb studied here suggest quantitation of O_2 binding may be quite adequately achieved by infrared spectroscopy. However, the possibility of problems in quantitation may arise with certain proteins is suggested by the unusual character of the bands for ^{18}O-^{18}O observed in O_2 HbA (1) and O_2 Mb. In both cases the ^{18}O-^{18}O bands are significantly broader than the ^{16}O-^{16}O bands and the isotopic shifts in frequency are less than expected (~40 cm^{-1} compared with 63 cm^{-1} (1)). We attribute the greater band width and reduced isotopic shifts to vibronic coupling between the bound $^{18}O_2$ and its environment, a coupling which does not (and need not) occur for $^{16}O_2$. However, the possibility exists in some metal proteins for vibronic coupling at the $^{16}O_2$ frequency thereby affecting $^{16}O_2$ band intensities, widths, frequencies, etc. Of course, if this were the case, then the $^{18}O_2$ region might well be free of such complications. Hopefully, either the $^{16}O_2$ or $^{18}O_2$ region will be free from such vibronic coupling complications.

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