Perturbation of the Fe−O_2 Bond by Nearby Residues in Heme Pocket: Observation of ν_{Fe-O_2} Raman Bands for Oxymyoglobin Mutants

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Elucidation of Fe−O_2 and O_2-protein interactions in oxygenated heme proteins has been the focus of a large number of structural and spectroscopic studies. X-ray crystallographic analysis on oxymyoglobin (O_2Mb) suggested the presence of a hydrogen bond between the distal histidine (His-64) and bound O_2; a proposal was later confirmed in neutron diffraction experiments.[1] Vibrational spectroscopy, including infrared (IR) absorption and resonance Raman (RR) scattering, has provided more detailed information on protein ligand interactions.[2a−c,e,g] Yu and co-workers[2a] suggested that the Fe−CO stretching (ν_{Fe-CO}) frequency depends on the Fe−C−O bond angle and accordingly reflects steric hindrance from nearby residues. More recent work has indicated that the electrostatic field near bound CO exerts a greater influence on the polarization of CO and its ν_{Fe-CO} frequency than does steric hindrance.[2d−g]

The Fe−O_2 complexes of Mb and hemoglobin (Hb) mutants have been much less studied, compared with their CO adducts, because of autoxidation problems. Since the O−O stretching mode (ν_{OO}) couples with internal modes of the trans ligand (histidine),[3] it is difficult to deduce the intrinsic ν_{OO} frequencies from the observed frequencies. The Fe−O_2 stretching (ν_{Fe-O_2}) mode has been identified for several end-on type O_2-bound heme proteins by RR spectroscopy,[4] and its observed frequency directly reflects the strength of the Fe−O_2 bond. We report here the ν_{Fe-O_2} Raman bands for His-64 → Leu (H64L), Leu-29 → Phe (L29F), and Leu-29 → Trp (L29W) mutants of sperm whale Mb and discuss the effects of these residues on the Fe-O_2 vibration.

Preparation of mutant Mbs has been described elsewhere.[2e] The purified protein was dissolved in 50 mM Na-phosphate buffer, pH 7.4. Crystals of L29W MbO_2 were grown in the P6 form using 2.2 to 2.6 M ammonium sulfate, 20 mM Tris-HCl, 1 mM EDTA.[5a] Diffraction data were collected on a Rigaku R-axis IIC imaging plate. The number of unique reflections was 17 658, and the starting model for molecular replacement was L29F MbO_2.[5c] Constrained least-squares refinement was performed by X-PLOR. After nine cycles of refinement and solvent placement, the crystallographic *R*-factor converged to 15.8%, with a final resolution of 1.8A and 77.5% completeness. The coordinates and structure factors are being submitted to the Brookhaven Protein Data Bank.

In order to observe the ν_{Fe-O_2} Raman band of unstable oxy species, we used the oxygenation system originally developed for studies of cytochrome *c* oxidase.[6] About 20 mL of CO-bound Mb (10 μM) was circulated through the system at a flow rate of 20 mL/min. Oxygen ($^{16}O_2$ or $^{18}O_2$) was incorporated into the solution just before the quartz Raman cell (cross section = 0.6 × 0.6 mm^2). Two laser beams, which were obtained from a single Kr^+ laser (Spectra Physics, Model 2016), but separated with a prism, were focused on the flow cell. One laser beam, at the upstream side (406.7 nm, 20 mW), photodissociates CO from COMb. O_2 competitively binds to the photodissociated deoxyMb due to its larger binding rate.[5a,b] Raman scattering from the O_2-bound hemes is excited with the other laser beam (413.1 nm, 2 mW), which is located 100 μm on the downstream side from the 406.7 nm beam. With the flow rates employed, the O_2 adduct is monitored at 0.1 ms after CO-photodissociation. The heme is quickly oxidized after O_2 binding, but its Raman spectrum is observed before autoxidation. The original reduced CO-bound form is restored from the autoxidized Mb during one cycle of the circulation system.[6]

Figure 1(a) shows the RR spectra in the 950 to 350 cm^{-1} region for the $^{16}O_2$ (A) and $^{18}O_2$ (B) adducts of the H64L mutant Mb excited at 413.1 nm. The band at 490 cm^{-1} arises from the ν_{Fe-CO} mode of recombined and unphotolyzed COMb, whose frequency is in agreement with the values reported for the equilibrium form of COMb.[2c,g] Since the ν_{Fe-CO} Raman band is considerably enhanced in intensity near the Soret region, the band appears strong even if its concentration is relatively low. A band at 570 cm^{-1} in spectrum A is shifted to 543 cm^{-1} in spectrum B, and this is unequivocally demonstrated by their difference spectrum (trace C) in which the strong porphyrin band at 675 cm^{-1} is completely canceled. Accordingly, the bands at 570 and 543 cm^{-1} in spectra A and B are assigned to the $^{16}O_2$−Fe and $^{18}O_2$−Fe stretching modes, respectively. We note that these two frequencies are remarkably close to those of the wild-type oxyMb (571/544 cm^{-1}).[4d,h]

Generally the ν_{Fe-CO} and ν_{CO} frequencies are very sensitive to amino acid replacements in the heme pocket.[2c,e,g] For example, ν_{Fe-CO} of H64L Mb (490 cm^{-1}) is significantly lower than that of the wild-type Mb (509 cm^{-1}).[2g] In contrast, the ν_{Fe-O_2} frequency of H64L oxyMb (570 cm^{-1}) is insignificantly different from that of the wild-type oxyMb and native oxyHb (571 cm^{-1}),[4a−d] while the Fe−His stretching frequenceis of H64L (221 cm^{-1}) and native (220 cm^{-1}) deoxyMbs are practically the same.[2g] The same ν_{Fe-O_2} frequency (571 cm^{-1})[4g,h] is seen for cytochrome *c* oxidase (CcO) in which a

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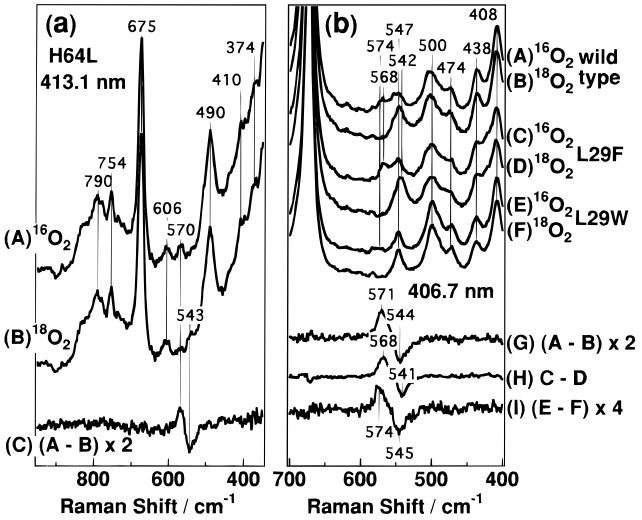

Figure 1. (a) RR spectra in the 950−350 cm^{-1} region for the $^{16}O_2$ (A) and $^{18}O_2$ (B) adducts of Leu-64 mutant Mb and their difference spectrum (C). (b) RR spectra in the 700−400 cm^{-1} region for the $^{16}O_2$ (A, C, and E) and $^{18}O_2$ (B, D, and F) adducts of wild type (A and B), Leu-29 → Phe (C and D) and Leu-29 → Trp (E and F) mutant Mbs, and their difference spectra [$^{16}O_2$ minus $^{18}O_2$; wild type (G), L29F (H), and L29W (I)]. The ordinate scales in all the raw spectra are normalized with the intensity of porphyrin bands. Experimental conditions; (a) probe beam: 413.1 nm, 2 mW; pump beam: 406.7 nm, 20 mW at the sample; (b) excitation: 406.7 nm, 5 mW at the sample; accumulation time: 960 s (a) and 640 s (b) for each spectrum; sample: 10 μM Mb (a) and 40 μM (b) in 50 mM Na-phosphate buffer, pH 7.4.

Cu ion (Cu_B) exists at 4.5 A from the heme iron,[7] whereas the ν_{Fe-CO} frequency of CcO (516 cm^{-1})[8] is significantly higher than those of other heme proteins.[2a,c,g]

Figure 1(b) displays the RR spectra in the 700−400 cm^{-1} region for the $^{16}O_2$ and $^{18}O_2$ adducts of wild-type, L29F, and L29W Mbs excited at 406.7 nm and their difference spectra. The band at 571 cm^{-1} for the $^{16}O_2$-bound wild-type Mb is shifted to 544 cm^{-1} for the $^{18}O_2$ adduct, in agreement with previously reported results.[4d] The ν_{Fe-O_2} band of horse oxyMb was also observed at 571 cm^{-1} under these conditions (data not shown). The corresponding band is at 568 cm^{-1} for L29F Mb and at 574 cm^{-1} for L29W Mb, their $^{18}O_2$-isotopic frequency shifts being more clearly seen in the difference spectra. The frequency shift between L29F and L29W Mb ν_{Fe-O_2} bands is small, ∼6 cm^{-1}. The intensity changes of the ν_{Fe-O_2} band are caused by differences in their O_2 affinities (Table 1).

Comparison of the Fe−O_2 stretching frequencies, F−O−O bond angles, and the rate and equilibrium constants for O_2 binding to wild-type, H64L, L29F, and L29W Mb are shown in Table 1. There is no correlation between O_2 affinity, k'_{O_2}, k_{O_2}, and ν_{Fe-O_2}. Electrostatic interactions with His64 and Phe29 have little effect on the stretching frequency but produce large, 15−50-fold increases in K_{O_2}. There is a correlation between ν_{Fe-O_2} and the Fe−O−O angle observed in the crystal structures of wild-type, L29F, and L29W MbO_2. The L29F mutant shows the expected 120° angle, whereas the angles in the wild-type (118°) and native (116°) proteins appear to be slightly smaller,

Table 1. Comparison of ν_{Fe-O_2} ($^{16}O_2$) and O_2 Binding Parameters for Position 29 Mutants of Recombinant Sperm Whale Myoglobin at 20 °C, pH 7[a]

myoglobin	ν_{Fe-O_2} (cm^{-1})	Fe−O−O angle	k'_{O_2} (μM^{-1} s^{-1})	k_{O2} (s^{-1})	K_{O_2} (μM^{-1})
wild-type	571	118 ± 4	17	15	1.1
H64L	570		98	4100	0.023
L29F	568	120[b]	21	1.4	15
L29W	574	111	0.25	8.5	0.029

[a] The rate and equilibrium constants were taken from ref 5b, and the Fe−O−O angles for wild-type and L29F O_2Mb from ref 5c. [b] From refs 5a and 5d and the estimated errors are ±4°.

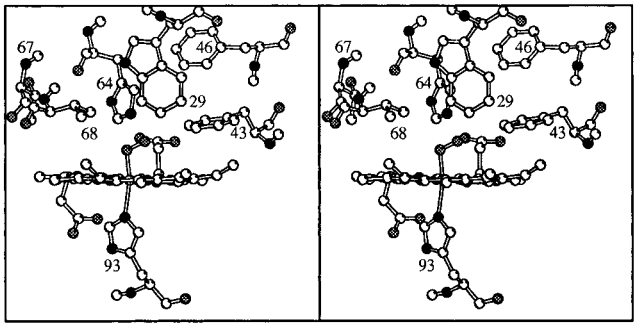

Figure 2. Stereoview of the distal pocket of sperm whale L29W MbO_2.

although the observed differences are within the estimated error of ±4°.[5a,d] The structure of L29W MbO_2 was determined for this work, and its active site is shown in Figure 2. The Fe−O_2 complex is clearly distorted by steric hindrance with the large indole side chain. The Fe−O−O angle is only 111°. The 9° difference between L29F and L29W MbO_2 corresponds with a 6 cm^{-1} increase in ν_{Fe-O_2}; however, the changes in Fe−O−O angle are at the limits detectable by X-ray crystallography (Table 1). The weak inverse relationship between ν_{Fe-O_2} and the Fe−O−O angle is probably due to kinematic effects on the molecular vibrations. In marked contrast to ν_{Fe-CO}, ν_{Fe-O_2} shows no dependence on the electrostatic field adjacent to the bound ligand. The H64L mutation removes the positive field due to N_ϵ−H of the distal imidazole, whereas the L29F mutation adds positive field due to the edge of the phenyl multipole. There is an ∼40 cm^{-1} difference between the ν_{Fe-CO} values for these two mutants,[2g] whereas only a 2 cm^{-1} difference is observed between the corresponding ν_{Fe-O_2} values. The $Fe^{\delta+}$−O−$O^{\delta-}$ system is highly polar, but the bond orders appear to be fixed with no alternative resonance structures. In contrast, the Fe−C−O system is apolar existing as an admixture of two alternative resonance structures involving net changes in bond order and alteration in the charge at the O atom: $Fe^{\delta-}$−C≡$O^{\delta+}$ and $Fe^{\delta+}$=C=$O^{\delta-}$. Our results point out these important chemical differences between the Fe−O_2 and Fe−CO adducts and the differential effects of globin residues on the bond strengths and vibrational properties of these complexes.

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