Evidence for Hydrogen Bonding Effects in the Iron Ligand Vibrations of Carbonmonoxy Myoglobin

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The Fe−CO unit in heme proteins has been used as a sensitive structural probe of the heme active site. Measurement of C−O stretching (ν_{C-O}), Fe−CO stretching (ν_{Fe-CO}), and Fe−C−O bending (δ_{FeCO}) frequencies by vibrational spectroscopies has provided detailed information about protein−ligand interactions.[1] Recent resonance Raman (RR) and infrared (IR) studies of mutant myoglobin (Mb) and model heme compounds have demonstrated that the ν_{Fe-CO} and ν_{C-O} frequencies are mainly determined by electrostatic interactions in the distal pocket,[1,2a] while variations in FeCO geometry are not significant.[2b] The open conformation of MbCO (A_0 conformer), where the distal histidine (His64) is displaced out of the heme pocket and the bound CO is in a less polar environment, shows the ν_{Fe-CO} and ν_{C-O} at 490 and 1965 cm^{-1}, respectively.[3,4] The closed conformation (A_1 and A_3 conformers), where His64 is present in the heme pocket adjacent to the bound ligand and the CO is in a more polar environment, shows the ν_{Fe-CO} and ν_{C-O} at 510−520 and 1945−1935 cm^{-1}, respectively. Replacement of His64 by aliphatic or aromatic amino acids produces ν_{Fe-CO} and ν_{C-O} bands whose frequencies are similar to the corresponding values when the distal histidine is in the open conformation in native MbCO.[1] In this communication we report RR spectroscopic evidence that suggests a hydrogen bond in the A_1 conformation of MbCO between the iron-bound CO and the distal histidine. This implies that a proton exists at the N_ϵ position in the A_1 conformer of MbCO and contributes to the increased ν_{Fe-CO} and decreased ν_{C-O} compared to the A_0 conformation.

We examined[5] the D_2O effects on the resonance Raman spectra of native and five distal histidine mutants (His64 → Ala, Val, Leu, Ile, and Phe) of sperm whale MbCO. Figure 1A shows the low-frequency resonance Raman spectra of native MbCO in buffered H_2O, D_2O, and $H_2{}^{18}O$ solutions, as well as $D_2O - H_2O$ and $H_2{}^{18}O - H_2O$ difference spectra. The $D_2O - H_2O$ difference spectrum (trace d) shows changes around 510 and 580 cm^{-1}. The shift of ν_{Fe-CO} at 510 cm^{-1} is approximately +1 cm^{-1}. When we use ^{13}CO as a ligand, the derivative pattern in the difference spectrum is downshifted by 4 cm^{-1} (trace d → e), demonstrating that the observed D_2O-induced shift originates from the Fe−CO stretching mode.[6]

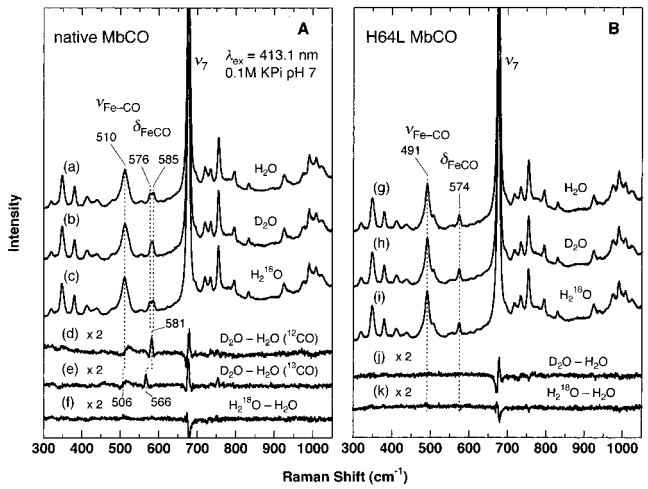

Figure 1. Low-frequency region of resonance Raman and difference spectra of native (A) and the His64 → Leu mutant (B) of sperm whale myoglobins in 0.1 M potassium phosphate buffer, pH or pD 7.0: (a, g) 100% H_2O; (b, h) 90% D_2O/10% H_2O; (c, i) 90% $H_2{}^{18}O$/10% H_2O; (d, j) $D_2O - H_2O$ for $Mb^{12}CO$; (e) $D_2O - H_2O$ for $Mb^{13}CO$; (f) $H_2{}^{18}O - H_2O$.

The D_2O-induced spectral change in the vicinity of the doublet bands near 580 cm^{-1} involves both an upshift and an intensity increase of the 576 cm^{-1} mode, leading to a loss of the doublet structure (trace b) and a prominent difference feature (trace d). Upon ^{13}CO substitution, the doublet feature at 580 cm^{-1} appears as a singlet at 562 cm^{-1} (suggestive of a Fermi resonance), and the H/D exchange again upshifts the frequency (∼2 cm^{-1}) and increases the intensity so that the difference spectrum yields a feature at 566 cm^{-1} (trace e, see also the figure in the Supporting Information). In contrast to Hirota et al.,[7] we agree with the previous assignments[8] for the band at 576 cm^{-1} as the Fe−C−O bending mode having a doublet feature that arises from a Fermi resonance with a nearby porphyrin mode.

Analogous experimental data for the His64 → Leu MbCO (Figure 1B) do not exhibit any detectable H/D isotope substitution effects, nor do the other His64 mutants examined in this study. This demonstrates that His64 contributes to the observed D_2O effect for the native MbCO. The negligible changes due to $H_2{}^{18}O$ in the native and mutant samples serve as an additional control that probes for potential effects associated with water in the distal pocket.

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(5) Resonance Raman spectra were obtained on a Spex 1870B spectrometer equipped with a liquid nitrogen cooled CCD detector (Princeton Instruments, Inc.) and a notch filter (Kaiser Optical Systems, Inc.). Samples were excited with the 413.1 nm line available from a Coherent Innova 300 krypton ion laser. All spectra were taken at room temperature, and the laser power was about 1 mW. The measurements were made on samples contained in a quartz spinning cell to minimize the contribution of photodissociated MbCO. Native Mb was obtained from Sigma Chemical Co. and used without further purification. The ^{13}CO at 99% enrichment (∼10% ^{18}O) was from Cambridge Isotope Laboratories, Inc. Preparation of mutant Mb has been described elsewhere.[1b]

(6) Hirota et al.[7] examined the H/D substitution effects on the ν_{Fe-CO} and δ_{FeCO} Raman bands for horse Mb and human Hb. Although a change at the stretching region can be discerned, their signal-to-noise level did not allow them to recognize it as a real shift.

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Prior infrared studies have searched for deuterium isotope effects on ν_{C-O}. For example, no effect of H/D exchange on the ν_{C-O} mode in Hb was observed in an early experiment.[9] The CO adducts of horseradish peroxidase[10] and cytochrome *c* peroxidase[11] show a 2.0−2.5 cm^{-1} downshift in ν_{C-O} when the sample is prepared in D_2O. This effect was attributed to either a hydrogen bonding interaction between the CO and a distal residue or protein conformational changes in D_2O. In the following, we will discuss possible explanations for the D_2O effects on the ν_{Fe-CO} and δ_{FeCO} modes of MbCO.

Deuterium substitution of exchangeable protons might alter protein tertiary structure. The 1.4 cm^{-1} downshift of the Fe−His stretching mode in deoxy Mb upon deuterium substitution is significantly larger than the value expected on the basis of a simple mass effect (0.7 cm^{-1}),[12] suggesting that deuteration of labile protons could alter the Fe−His bond.[13] While such a change on the proximal side could affect the vibrational character of the FeCO unit, it fails to explain the lack of H/D exchange effects on mutants of MbCO whose His64 is replaced with aliphatic or aromatic residues (Figure 1B). We therefore suggest that a weak hydrogen bond between His64 and the iron-bound CO makes the Fe−CO stretching and Fe−C−O bending modes sensitive to the H/D exchange. This explanation accounts for the lack of effects on the ν_{Fe-CO} and δ_{FeCO} modes of His64 mutants MbCO. Kinetic studies on mutant Mbs have shown that the replacement of His64 increases the CO dissociation rate by a factor of 2−3,[1b,15a,b] while replacement of His64 with aliphatic residues results in an ∼1000-fold increase in the O_2 dissociation rate.[15a,16] These results suggest that hydrogen bonding to His64 stabilizes bound CO by only ∼2 kJ mol^{-1} whereas bound O_2 is stabilized by ∼17 kJ mol^{-1}.[15b,16,17a]

The proposed hydrogen bond between N_ϵ of His64 and the carbonyl oxygen conflicts with the neutron crystal structure,[18] where no proton was found on N_ϵ. However, recent spectroscopic, mutagenesis, and theoretical studies suggest a positive polar interaction between His64 and bound CO,[1,17] consistent with a protonated N_ϵ.[1,15,17] Li and Spiro[19b] have interpreted the inverse correlation between the ν_{C-O} and ν_{Fe-CO} frequencies[1a,b,19] in terms of back-donation from the iron atom. A positive polar interaction, such as hydrogen bonding, increases the back-bonding donation from the Fe d_π to the CO π^* orbital, thereby strengthening the Fe−CO bond (i.e., increasing the frequency) and weakening the C−O bond (i.e., decreasing the frequency). In the absence of such interactions (e.g., in His64 mutants or the A_0 conformation of native MbCO), the ν_{Fe-CO} peak appears at 495 cm^{-1} whereas the peak for ν_{C-O} is found at 1965 cm^{-1}. Thus, the ν_{Fe-CO} and ν_{C-O} peaks at 510 and 1945 cm^{-1} for the A_1 conformation suggest an interaction with a positive electrostatic field, presumably due in part to the protonated N_ϵ of His64. This interpretation is supported by infrared crystallographic determination of the C−O orientation in sperm whale MbCO crystals, which places the carbonyl oxygen 2.6−2.9 Å from N_ϵ, a distance consistent with hydrogen bonding.[20]

The effects of H/D exchange on native Mb shown in Figure 1 provide spectroscopic evidence for a hydrogen bond between His64 and iron bound CO. However, the specific mechanism by which D_2O perturbs the Fe−CO stretching and Fe−C−O bending modes remains uncertain. The H/D isotope effect is not a simple mass effect, since a D_2O-induced upshift of ν_{Fe-CO} and δ_{FeCO} was observed. On the other hand, the reduction in the zero-point energy of the His64 N_ϵ−D bond can stabilize the deuterium bond compared to the hydrogen bond. An important mechanism for this stabilization arises from the reduced mean square displacement of the N_ϵ−D "wagging" motion.[21] The resultant strengthening of the CO···D−N_ϵ interaction could "stiffen" the Fe−CO bending and stretching potentials (with concomitant increases in those frequencies). This scenario does not rely on π back-bonding arguments to explain the H/D isotope shifts and is consistent with the weak H/D perturbations of the C−O mode observed in related infrared experiments.[22] On a more global scale, small alterations in charge distribution, due to changes in the zero-point width and position of the His64 N_ϵ deuterium, could weakly perturb the electric fields and forces felt in the distal pocket, leading to small changes in the orientation of the imidazole side chain and/or the bound ligand that affect the ν_{Fe-CO} and δ_{FeCO} frequencies. The histidine specific nature of the perturbation is suggested by the absence of a H/D exchange effect in the Gln64 MbCO,[23] where the presence of a hydrogen bond is suggested in the oxy form.[15] To examine these possibilities, further RR and IR studies are currently in progress.

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Supporting Information Available: Figure showing the Fe−CO stretching and Fe−C−O bending mode region of the resonance Raman spectra of native and His64 → Leu mutant carbonmonoxy myoglobin (1 page, print/PDF). See any current masthead page for ordering information and Web access instructions.

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