Raman Spectroscopic Observation of a Conformational Change at the Heme−Protein Linkage in Myoglobin at High Pressure

A. Schulte,* S. Buchter, O. Galkin, and C. Williams

Department of Physics and Center for Research and Education in Optics and Lasers, University of Central Florida, Orlando, Florida 32816

Received May 2, 1995

Pressure is a fundamental thermodynamic variable affecting both the equilibrium constant and the rate of a chemical reaction. In proteins, pressure alters conformational states, and it is crucial to the understanding of physical forces which stabilize native structures.[1−3] While the reaction kinetics and the volume of activation for prototype processes such as binding of small ligands to heme proteins have recently received considerable interest,[3−6] surprisingly little is known about conformational contributions and their molecular basis. Ambient pressure Raman studies of heme proteins from different species have shown that the ligand-free ferrous heme is sensitive to variations in the protein structure[7] and that a major part of the binding barrier and the control mechanism predominantly involve the proximal side.[8] Therefore, we chose Raman spectroscopy to gain insight into pressure-induced changes of the heme geometry and the linkage between the iron and the proximal histidine. Up to now, only low-resolution (10 cm^{-1}) Raman measurements of hemoglobin in the frequency range 1350−1700 cm^{-1} have been reported.[9] These were performed with a diamond anvil cell above 2.6 kbar and showed an iron spin-state change at 3.4 kbar, as well as precipitation.[9] However, experiments probing the low-frequency modes as well as the functionally important heme−protein linkage are absent.[10] In the following we demonstrate that a particularly intriguing marker, the iron−proximal histidine vibration ν_{Fe-His}, shifts with pressure. The observed shift to higher frequency is interpreted as a conformational change in the protein, which decreases the tilt of the proximal histidine from the heme normal and the out-of-plane iron position. This suggests that at high pressure, the heme environment is altered toward the bound state and contributes to the rate increase for CO bond formation.

Figure 1 displays the resonance Raman spectra of horse deoxymyoglobin (Mb) in the frequency range from 150 to 550 cm^{-1} as a function of pressure. To avoid irreversible effects and protein denaturation[1,2] and to allow for continuous pressure variation below 200 MPa, we employ a hydrostatic cell using nitrogen gas as a the pressurizing medium. The cell also affords sufficient sample volume (~20 μL) to ensure solution conditions. The lines in the low-frequency spectrum can be classified

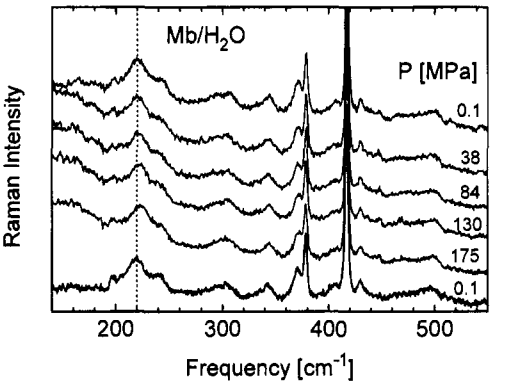

Figure 1. Pressure dependence of the resonance Raman spectrum of (horse) myoglobin in the low-frequency region. Raman scattering was excited with the frequency-doubled output of a CW Ti:sapphire laser (~3 mW) at 441 nm. The sharp lines at 417 and 378 cm^{-1} are due to the sapphire windows and serve as frequency standards. The spectra are shifted along the vertical axis for clarity, but no smoothing or background subtraction was performed. The backscattered Raman light is detected with a thinned back-illuminated charge-coupled device (Princeton Instruments) mounted on a single-grating spectrograph (Instruments SA, HR 640). Efficient Rayleigh line rejection was achieved with a holographic Bragg diffraction filter. Individual Raman spectra were collected in 200 s with a spectral resolution of 2 cm^{-1}. A beryllium−copper cell with axial sapphire windows was employed in high-pressure measurements, using nitrogen gas as the pressure-transmitting medium. Samples were sandwiched between two sapphire windows with a Teflon spacer or contained in a short capillary closed with a membrane. Horse myoglobin was obtained in lyophilized form (Sigma Chemical Co.) and dissolved at ~4 mM concentration in aqueous potassium phosphate buffer, pH 7. Myoglobin solutions were deoxygenated using N_2 gas and reduced with an excess of sodium dithionite.

according to motions involving the iron atom, peripheral substituents on the porphyrin macrocycle, and pyrole out-of-plane modes.[11−13] The band at 220 cm^{-1} at atmospheric pressure (0.1 MPa) has been assigned to the iron−histidine (Fe−His) stretching mode.[14] As the pressure is raised from 0.1 to 175 MPa, the peak position of ν_{Fe-His} shifts to higher frequency, and the intensity relative to the band near 240 cm^{-1} increases. Upon releasing the pressure, the initial spectrum at 0.1 MPa is recovered. A linear least-squares fit to the pressure dependence of ν_{Fe-His} yields a slope of 1.6 ± 0.2 cm^{-1}/(100 MPa), corresponding to a shift of 2.8 ± 0.4 cm^{-1} at 175 MPa.

Our experiments reveal another important feature of the pressure dependence that lies in the fact that there are no significant shifts (>1 cm^{-1}) in the bands due to the porphyrin ring modes, indicating no radical perturbation of the heme structure. This is reinforced by the high-frequency spectra (Figure 2), where the prominent lines are the heme electron density marker (ν_4, 1357 cm^{-1}), the porphyrin core size-sensitive ν_2 (1562 cm^{-1}), and a mode due to a vinyl group (1616 cm^{-1}).[14] The spectra are indicative of a five-coordinate high-spin heme. We do not observe a significant shift (>1 cm^{-1}) of the core size markers over the pressure range of 180 MPa. We have checked this result by performing the same experiment with 457.9 nm excitation from a single line Ar ion laser. Thus, changes in the core size appear to be <0.003 Å based on correlations with skeletal mode frequencies (e.g., 330 cm^{-1}/Å for ν_2) in porphyrins.[16] Our finding may be attributed to the

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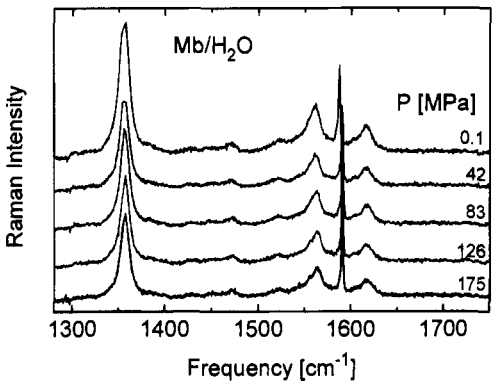

Figure 2. Resonance Raman spectrum of deoxy-Mb in the high-frequency region at various pressures using the same parameters as in Figure 1. The sharp feature near 1590 cm^{-1} is due to a plasma emission from the Ar laser.

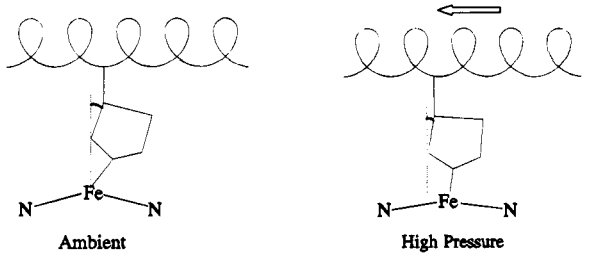

Figure 3. Schematic representation of the pressure-induced change of the iron–histidine conformation.

rather rigid bonds in the porphyrin, indicating predominant control by the protein. In addition, the core size appears to be determined by the size of the central Fe atom, which depends on the spin state.[16]

The most straightforward explanation for a shift in ν_{Fe-His} involves motion of the protein. The geometry of the proximal histidine influences both the frequency ν_{Fe-His} and the intensity of the Fe–His stretch band.[14] Systematic variation in ν_{Fe-His} of myoglobin from various species has been attributed to a F-helix-induced tilt of the proximal histidine with respect to the heme plane.[15] The X-ray structures show that, in unligated myoglobin, the iron is displaced from the heme plane by about 0.35 Å,[18] while upon CO binding, it moves to a position in the heme plane.[19,20] Since the proximal histidine is linked to the iron and also part of the polypeptide backbone, the position of the F-helix changes as well.[13] One possibility is that sliding of the F-helix alters the tilt angle of the proximal histidine with respect to the heme plane as well as the force constant or the length of the iron–histidine bond (Figure 3). Larger tilt angles appear to be correlated with a greater iron displacement q_0.[7] Neglecting the simultaneous tilt of the imidazole, recent semiempirical calculations suggest a relation between the iron out-of-plane displacement and ν_{Fe-His}.[17]

$$\nu_{Fe-His}\ [cm^{-1}] = 209.4 - 184.0(q_0 - 0.4)\ [Å]$$

Following the above model, the observed frequency shift of 2.8 cm^{-1} would correspond to a change in the iron displacement of ~0.015 Å. Thus, the shift to higher frequency of ν_{Fe-His} is consistent with a motion of the iron into a more planar position with respect to the heme plane and to a decrease in the tilt angle between the heme normal and the proximal histidine. Absorption studies of band III near 760 nm[21] can provide additional support for iron movement. We are performing high-pressure experiments now, and preliminary results indicate that there is a 1.2 nm (21 cm^{-1}) red shift of band III at 165 MPa. The spectral shifts observed at high pressure are reminiscent of those measured in MbCO relative to deoxy-Mb. Here the 5 cm^{-1} higher iron–histidine frequency,[14] as well as the red shift of band III by 116 cm^{-1},[22] correlates with changes in the tilt angle (4.9° [20]) and the iron position (0.05 Å [20]). Since the core size expansion appears to be too small to cause significant frequency changes, the structural rearrangement must be driven by the protein and involve the imidazole and the Fe–N bond. A high-pressure (15 MPa) X-ray crystallographic study is available at 2 Å resolution for metmyoglobin, where alternate conformations are found for three internal residues (Leu-135, Phe-138, and Ile-142), implying a repacking of the protein interior.[23] However, these measurements may not resolve the small differences detected by spectroscopy, and there are uncertainties in the interpretation of additional electron density appearing in the proximal pocket.

An important factor affecting the global protein conformation, the heme pocket structure, and the iron–histidine mode is water activity.[24] The altered water activity in a glycerol/H_2O mixture causes a 2.6 cm^{-1} downshift of ν_{Fe-His}[24] as compared to aqueous solution. Apparently, glycerol influences the protein–water interaction with a possible release of bound water molecules from the surface,[24] though the perturbation of the Fe–His frequency is opposite to that from pressure. Molecular dynamics simulations have, indeed, found large pressure-induced changes in the hydration layer, causing the solvation shell water to adopt a more ordered structure.[25] In preliminary experiments with a 75% glycerol/H_2O solvent, we have observed a shift of ν_{Fe-His} due to pressure in the same direction as in aqueous solutions.

Transient absorption measurements of CO binding to myoglobin show an increase of the rebinding rate with pressure.[3–6] For instance, the overall CO association rate at room temperature speeds up by a factor of 3 when the pressure is raised from 0.1 to 200 MPa.[6] Low-temperature experiments of the geminate rebinding indicate that the main effect is on the iron–ligand bond formation step.[3] However, these measurements monitor only the reaction coordinate, whereas Raman bands can be assigned to vibrations of specific structural degrees of freedom. The spectral shifts observed at high pressure are in the same direction (i.e., lower barrier height) as those measured in MbCO with respect to deoxy-Mb.[14,22] The motion of the iron closer to the heme plane with increasing pressure and an overall protein conformational change toward the conformation of the ligated species thus provide a structural mechanism for the rate increase. The results reported here illustrate the potential of combining an external perturbation such as pressure with a structurally specific probe to connect structure and reactivity. They demonstrate the importance of conformational contributions to the reactivity of heme proteins.

Acknowledgment. We would like to acknowledge G. Mizell and Virgo Optics for supplying the $KNbO_3$ crystals used in the frequency doubler, coatings, and a temperature control system. The authors also thank A. Tabirian for help with the sample preparation and NIR absorption measurements. This work was supported by Grant MCB-9305711 from NSF.

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