Pressure Effects on the Proximal Heme Pocket in Myoglobin Probed by Raman and Near-Infrared Absorption Spectroscopy

Oleg Galkin, Scott Buchter, Anna Tabirian, and Alfons Schulte
Department of Physics and Center for Research and Education in Optics and Lasers, University of Central Florida, Orlando, Florida 32816-2385 USA

ABSTRACT The influence of high pressure on the heme protein conformation of myoglobin in different ligation states is studied using Raman spectroscopy over the temperature range from 30 to 295 K. Photostationary experiments monitoring the oxidation state marker bands demonstrate the change of rebinding rate with pressure. While frequency changes of vibrational modes associated with rigid bonds of the porphyrin ring are <1 cm^{-1}, we investigate a significant shift of the iron-histidine mode to higher frequency with increasing pressure (≈ 3 cm^{-1} for $\Delta P = 190$ MPa in Mb). The observed frequency shift is interpreted structurally as a conformational change affecting the tilt angle between the heme plane and the proximal histidine and the out-of-plane iron position. Independent evidence for iron motion comes from measurements of the redshift of band III in the near-infrared with pressure. This suggests that at high pressure the proximal heme pocket and the protein are altered toward the bound state conformation, which contributes to the rate increase for CO binding. Raman spectra of Mb and photodissociated MbCO measured at low temperature and variable pressure further support changes in protein conformation and are consistent with glasslike properties of myoglobin below 160 K.

INTRODUCTION

The problem of connecting the dynamic structure of proteins with their biological function is one of the most intriguing in biophysics. In vivo, functional properties of proteins are affected by environmental parameters such as viscosity, pH, temperature, and pressure. For instance, sea animals survive over a wide range of pressure, from sea level to extreme depths. On the other hand, pressure can deactivate enzymes and kill bacteria (Weber and Drickamer, 1983; Jannasch et al., 1987). As Kauzmann (1987) has pointed out, an understanding of the physical forces that stabilize native protein structures must be considered quite incomplete, "until more searching is done in the darkness of high pressure studies." Pressure is a fundamental thermodynamic variable and its influence is seen at different levels, from changing the equilibrium coefficient and rate of a chemical reaction (Winter and Jonas, 1993) to altering conformational states of proteins (Frauenfelder et al., 1990).

Myoglobin has served as a model system for extensive experimental and theoretical studies of protein dynamics. During the process of reversibly binding small ligands such as O_2, CO, or NO to the heme iron, both the chromophore and the protein undergo conformational changes. The bond between the iron and the proximal histidine imidazole nitrogen is the only covalent linkage between the heme group and protein. Resonance Raman studies at ambient pressure support the view that modulation of the iron by the protein through the proximal histidine exerts control at the level of reactivity (Friedman, 1985; Bangcharoenpaurpong et al., 1984; Ahmed et al., 1991; Traylor et al., 1990). X-ray structural data show that ligand binding to myoglobin is accompanied by a ≈ 0.4 Å motion of the iron atom to a position close the heme plane (Phillips, 1980; Kuriyan et al., 1986; Schlichting et al., 1994). Therefore, the subangstrom displacement of the iron atom with respect to the heme plane is one of the most important factors in the process of ligand binding. Movement of the proximal histidine can also provide a mechanism to allow a nonzero nonadiabatic electronic spin transition of the iron by lowering the site symmetry (Sungar and Gerstman, 1992; Jortner and Ulstrup, 1979). The motions of the iron and the proximal histidine have been probed at ambient pressure with Raman and near-infrared absorption spectroscopy, thus linking structural changes near the heme with function (Ahmed et al., 1991; Lim et al., 1993; Steinbach et al., 1991; Sage and Champion, 1997).

In heme proteins, pressure in the 800 MPa (8 kbar) range can denature metmyoglobin at physiological pH (Zipp and Kauzmann, 1973), but lower pressure can cause more subtle structural changes in the heme cavity and affect the binding kinetics of CO or O_2 to the heme (Jonas and Jonas, 1994). Fersht and Winter (1992) have suggested that the activity of enzymes can be altered by several orders of magnitude with a 1 Å change in the active site. Though there are a number of studies concerning pressure effects on the reaction rate (Frauenfelder et al., 1990; Hasinoff, 1974; Jannasch et al., 1987; Heremans, 1982; Morild, 1981; Weber and Drickamer, 1983; Jonas and Jonas, 1994; Adachi and Morishima, 1989; Projahn et al., 1990; Taube et al., 1990) most of them have been performed over a narrow temperature range close to 290 K. Since they monitor broad absorption bands, these experiments lack the structural sensitivity to follow conformational changes occurring simultaneously with the reaction.

Received for publication 11 April 1997 and in final form 28 July 1997.

Address reprint requests to Dr. Alfons Schulte, Dept. of Physics, University of Central Florida, Orlando, FL 32816-2385. Tel.: 407-823-5196; Fax: 407-823-5112; E-mail: afs@phys.physics.ucf.edu.

0006-3495/97/11/2752/12 $2.00

Resonance Raman spectroscopy is one of the few techniques that can probe the local environment of the active site inside a large biological system. Raman spectra of heme proteins reveal a number of bands that have been well characterized and yield information on the spin state, coordination, and environment of the heme (Spiro, 1983). However, high-pressure Raman measurements over a wide temperature range are challenging due to the constraint that the pressure cell must be contained in the vacuum chamber of a refrigerator while maintaining optical access and a good f-number to detect the weak signals (compared to fluorescence or absorption). Previous high-pressure Raman studies are limited to a low resolution measurement (10 cm^{-1}) of hemoglobin over the frequency range 1350–1700 cm^{-1} (Swanson et al., 1986) and our recent brief report on the room temperature spectrum of deoxy myoglobin (Schulte et al., 1995; Galkin and Schulte, 1996). We have shown that the iron-histidine mode presents a pressure-sensitive marker for the heme environment.

In an extension of this work we use the iron-histidine mode as well as the absorption band III in the near-infrared to investigate pressure-induced changes of the proximal heme pocket and iron motion in different solvents over a large temperature range. We determine the pressure dependence of the photostationary equilibrium between the deligated and bound state of MbCO from the Raman spectrum. Significant changes occurring in the Raman active iron-histidine mode and the absorption of band III are investigated as a function of pressure and temperature. The observed spectral changes suggest that high pressure alters the iron out-of-plane position and the proximal histidine conformation toward the bound state. This contributes for the rate increase of geminate ligand binding with pressure. Low temperature is employed to investigate metastable photoproducts and the relation between conformational changes and glasslike properties of myoglobin.

MATERIALS AND METHODS

Samples were prepared from lyophilized powder of horse myoglobin (Sigma Chemical Co., St. Louis, MO), dissolved into degassed, deionized water. The protein solution was centrifuged at 30,000 rpm for 20 min to remove impurities. Before each experiment the mixture was rendered anaerobic by stirring in a nitrogen atmosphere for a few hours. Reduction of the sample from met Mb to deoxy Mb was accomplished by adding an excess of freshly prepared anaerobic solutions of sodium dithionite. The sample was then stirred for 30 min under a CO atmosphere. Final concentrations between 2 and 4 mM were used in the experiments. The sample, along with a 1-mm annular Teflon spacer, was sandwiched between two sapphire windows inside a beryllium-copper sample holder and placed inside the pressure cell for Raman spectroscopy measurements. Raman spectra of our samples at ambient pressure are in agreement with literature data (Rousseau and Friedman, 1988) and show the quality of the sample preparation procedure.

The experimental setup is similar to that described previously (Schulte and Bradley, 1995). The high pressure cell is used for Raman and absorption measurements as well. The pressure cell is constructed of beryllium-copper that combines the ability to resist high pressure (up to 400 MPa) with good thermal conductivity. Sapphire windows allow measurements from the near UV to the near-infrared region. High pressure was generated by an air-operated diaphragm-type compressor obtained from Newport Scientific (Jessup, MD), which is rated for a maximum operating pressure of 200 MPa. Nitrogen or helium gas is used as the pressurizing medium. With the present setup the pressure could be raised from 0.1 MPa (1 atm) to 170 MPa within 100 s. For cryogenic measurements the pressure cell is mounted to the cold head of a closed-cycle refrigerator (Model 22, CTI-Cryogenics, Waltham, MA). To minimize the heat leakage at the pressure tubing/cold head interface a cold head bell adapter is used. The sample temperature is measured with a Si diode on the high pressure cell and is stabilized to 0.1 K with a temperature controller (RC-93C, Lakeshore, Westerville, Ohio).

Resonance Raman scattering was excited with the frequency-doubled output of a continuous wave Ti:sapphire laser tunable from 441 to 425 nm (Buchter et al., 1995) or by the 457.9-nm line of an Ar ion laser. Detection of the backscattered Raman radiation is accomplished using a thin back-illuminated charge-coupled device (Princeton Instruments, Trenton, NJ) in conjunction with a single-grating spectrograph (HR 640, Instruments SA, Edison, NJ). Rayleigh line rejection was performed using a holographic Bragg diffraction filter (Kaiser, Ann Arbor, MI). The spectra were not corrected for the instrument profile, nor was any smoothing or background subtraction performed. Calibration of frequency is made using spectra of naphthalene and carbon tetrachloride as a reference. The spectral resolution was better than 2 cm^{-1} employing a 2400 groove/mm grating with dispersion of 0.6 nm/mm at the focal plane. The acquisition time for individual spectra was on the order of 200 s. In absorption measurements a tungsten lamp was used as the monitor light source. Neutral density, band pass, and heat-absorbing filters were used to avoid sample photolyzation and heating. Because the absorption lines are quite wide, a 300 grooves/mm grating was used to cover the spectral range from 700 to 820 nm at a resolution of 0.3 nm. The signal accumulation time was several seconds. Photolysis for near-infrared absorption measurements of the MbCO photoproduct was achieved with a 20-ns pulse (10 mJ energy focused to a 2-mm beam diameter) from a frequency-doubled Nd:YAG laser (532 nm).

The regular protocol for experiment was as follows. Raman as well as absorption spectra were measured with both increasing and decreasing pressure to monitor the reversibility of spectral changes. After the desired pressure was achieved the sample was allowed to equilibrate for 30 s before taking the spectrum to ensure equilibrium conditions of the sample. The pressure variation was generally performed at 295 K, which is much higher than the glass temperature (185 K) of the protein-solvent system. In experiments where the temperature was varied at constant pressure the waiting time at the target temperature was 20 min. This defines the experimental time scale and the state of the protein in the glass transition region. Above ~210 K the protein has reached the equilibrium defined by temperature and pressure within 20 min. On the other hand, below ~160 K the time scale for relaxations is long compared to the measurement time. We did not observe changes in the Raman bands within 20 min though the protein is in a metastable state. In experiments performed at low temperature the sample was cooled in the dark to eliminate possible light-induced relaxation (Ahmed et al., 1991; Nienhaus et al., 1994) and we address this further in the last section of the paper.

RESULTS AND DISCUSSION

Pressure Effects on Photostationary States

In Fig. 1 we present the pressure dependence of the resonance Raman spectrum of MbCO over the frequency range from 1300 to 1700 cm^{-1}. Individual spectra were acquired in 100 s with 425 nm excitation at low laser power (<3 mW). The photolysis of ligated myoglobin by the laser beam during the acquisition of a Raman spectrum creates a stationary mixture of bound and photolyzed molecules. This is evident from the oxidation state marker bands (ν_4) which appear at 1354 cm^{-1} for Mb and 1372 cm^{-1} for MbCO.

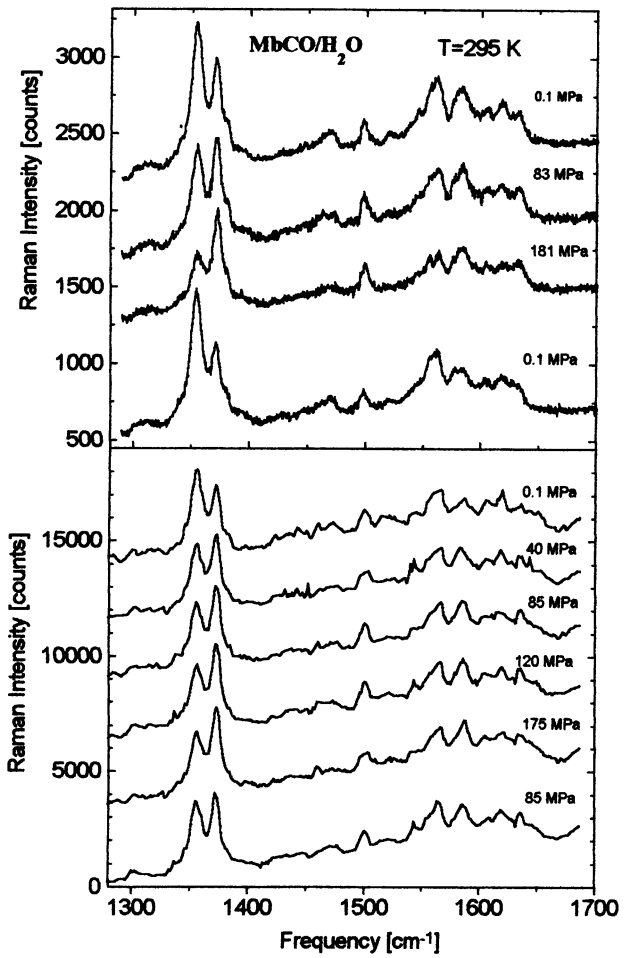

FIGURE 1 Resonance Raman spectra of MbCO in aqueous solution as a function of pressure at 295 K. The excitation wavelength is 458 nm. The sample is in photostationary equilibrium. As the pressure is increased the intensity of the ν_2 band of MbCO (1374 cm^{-1}) increases relative to that of Mb (1356 cm^{-1}). Shown are raw data for two different samples. Spectra are shifted by a constant along the vertical axis for clarity. The spectra shown in top part were measured with a 2400 grooves/mm grating, the ones at the bottom with a 1200 grooves/mm grating.

Here the intensity of the probe beam was chosen to obtain comparable populations of ligated and photodissociated species. The ambient pressure Raman spectra of heme proteins in this spectral region have been well characterized (Spiro, 1988; Yu, 1986; Rousseau and Friedman, 1988) and yield information on the spin and oxidation state of the iron. Upon raising the pressure from 0.1 to 180 MPa (keeping all other parameters fixed) the most noticeable change in the spectra is a significant increase of the peak at 1372 cm^{-1} relative to that at 1354 cm^{-1}. In Fig. 1 the measured intensities of the Raman scattered light for two different samples are plotted, and the spectra show that there is no substantial change in total area. Therefore, the most straightforward explanation is that conversion from the unbound to the bound state occurs, rather than the population of the unbound state just disappearing. Similar changes in relative intensities corresponding to a change in population between bound and unbound states are seen in the core size marker band ν_2 (1560 and 1582 cm^{-1}) and the vinyl modes (1618 and 1632 cm^{-1}). The relative population of Mb* and MbCO is proportional to the ratio of the oxidation state marker bands at 1354 and 1372 cm^{-1} weighted with the scattering cross sections. Apart from the mentioned intensity changes, the spectra in Fig. 1 and data presented previously (Schulte et al., 1995) show no significant shifts (>1 cm^{-1}) with pressure in the positions of the bands in this spectral region. This may be a consequence of the rather rigid bonds in the porphyrin.

Fig. 2 displays the logarithm of the peak ratio of the oxidation state marker lines at 1354 and 1372 cm^{-1} versus pressure for the photostationary equilibrium of photolyzed and bound MbCO. Our experimental data clearly show that application of high pressure alters the rebinding in such a manner that the population of the bound state increases with pressure. The population increase of the bound state with pressure is due to a speedup of the overall rebinding, consistent with the results of transient absorption measurements (Hasinoff, 1974; Taube et al., 1990; Adachi and Morishima, 1989; Frauenfelder et al., 1990). For a more quantitative analysis we use a simplified model in which we omit details of the multi-step kinetics. We consider averaged rates that comprise steps coupled to bond formation and ligand migration and escape through the protein matrix. Under these assumptions, the population ratio between bound and deligated states in photostationary equilibrium is given by

$$\frac{[\mathrm{MbCO}]}{[\mathrm{Mb}][\mathrm{CO}]} = \frac{k}{k_L} = K \tag{1}$$

Here k_L and k denote the photolysis and the overall rebinding rate, respectively; since the photolysis rate is kept constant, the ratio of the rate constants at different pressure can be written as

$$\frac{k(P_2)/k_L}{k(P_1)/k_L} = \frac{K(P_2)}{K(P_1)} = \exp\left[-V^{\ddagger}\frac{P_2 - P_1}{RT}\right] \tag{2}$$

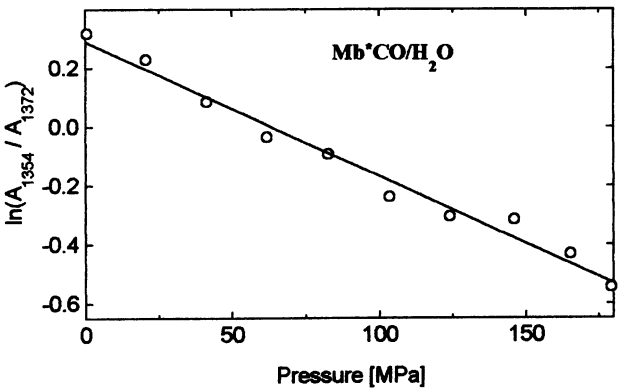

FIGURE 2 Ratio of the oxidation state marker bands intensities for a photostationary mixture of MbCO and Mb* as a function of pressure. The excitation wavelength is 458 nm.

In this expression $K(P_2)/K(P_1)$ is proportional to the intensity ratios since the scattering cross sections cancel and their pressure dependence is negligible. The activation volume of the reaction is obtained from

$$V^{\ddagger} = -\frac{RT}{P_2 - P_1} \ln\left[\frac{K(P_2)}{K(P_1)}\right] \approx \ln\left[\frac{(I_{1354}/I_{1372})(P_2)}{(I_{1354}/I_{1372})(P_1)}\right] \quad (3)$$

Fits of the data in Fig. 2 to Eq. 3 yield an activation volume of -11 cm^3/mol (standard deviation from least squares fit 0.6 cm^3/mol). The activation volume is close to the values of -8.9 cm^3/mol (Hasinoff, 1974) and -10 cm^3/mol (Taube et al., 1990) reported in absorption experiments. The negative activation volume suggests that for MbCO bond formation is the rate-limiting step, however a detailed investigation including rebinding from the solvent and the protein matrix requires time-resolved measurements. Our oversimplified model merely serves as a comparison between population changes in the Raman spectrum and transient absorption data at room temperature. In addition, there might be a change in the preexponential due to pressure effects on the activation entropy. To elucidate this point, kinetic measurements at variable pressure and temperature will be needed.

Pressure-induced conformational changes at the heme-protein linkage probed by the iron-histidine mode and band III

To understand the observed pressure effects on protein reactivity, it is necessary to elucidate the associated structural changes. Since the difference in iron position with respect to the heme plane between ligated and deoxy myoglobin is ≈0.4 Å, functionally relevant motions occur at the subangstrom level. Here we employ the iron-histidine mode in the resonance Raman spectrum and the absorption band III in the near-infrared as high-resolution spectroscopic markers to probe the heme environment. Deoxymyoglobin is used as a reference structure since the reaction process is absent, and pressure-induced changes of the conformation can be separated from those along the reaction coordinate.

The resonance Raman spectra of horse deoxy myoglobin (Mb) and photodissociated carbonmonoxy myoglobin (Mb*) in the frequency range from 150 to 550 cm^{-1} are shown for ambient and high pressure in Fig. 3. The spectrum at atmospheric pressure (0.1 MPa) agrees with literature data (Rousseau and Friedman, 1988; Kitagawa, 1988; Bangcharoenpaurpong et al., 1984). The band at 220 cm^{-1} has been assigned to the iron-histidine (Fe-His) stretching mode (Kitagawa, 1988; Rousseau and Friedman, 1988). The other lines have been classified as follows (Sassaroli et al., 1986). The band near 241 cm^{-1} is a pyrrole ring tilting mode. The modes from 250 to 420 cm^{-1} all involve peripheral substituents, and those from 420 to 520 cm^{-1} are attributed to out-of-plane distortions of the pyrrole rings. (Sassaroli et al., 1986; Spiro, 1988; Choi and Spiro, 1983).

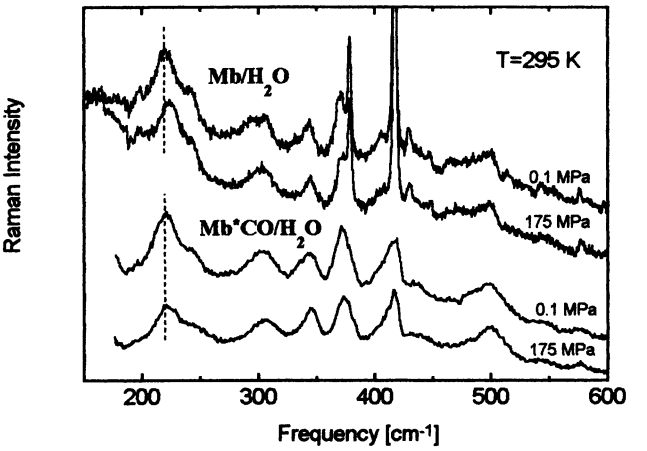

FIGURE 3 Low frequency region of the Raman spectra of deoxygenated and photodissociated myoglobin at ambient and high pressure. Samples are in aqueous solution at pH 7. The sharp lines at 417 and 378 cm^{-1} are due to the sapphire windows.

The bands near 500 cm^{-1} in the spectrum of Mb* are due to the Fe-CO stretching mode of unphotolyzed MbCO.

The most significant spectral change with pressure is a shift of the peak frequency $\nu_{\text{Fe-His}}$ of the iron histidine mode. $\nu_{\text{Fe-His}}$ shifts to higher wavenumber by ≈3 cm^{-1} between 0.1 and 175 MPa in both Mb and photodissociated carbonmonoxy myoglobin (Mb*CO). To probe the iron histidine mode of the photoproduct, the laser intensity was increased so that ~80% of the MbCO is photodissociated. The peak position of $\nu_{\text{Fe-His}}$ as a function of pressure is plotted in Fig. 4. The error bars correspond to a precision of ±0.6 cm^{-1}. To verify these results we also have performed the same experiment in different solvents (75% gly/H_2O) and on Mb from sperm whale. In all studied cases we have observed the shift of Fe-His mode. A smaller shift (close to the resolution of our current setup) is apparent in the band near 343 cm^{-1}. Choi and Spiro (1983) have assigned this band to out-of-plane modes of propionate porphyrin macrocycle substituent groups. Since the hydrogen bonding

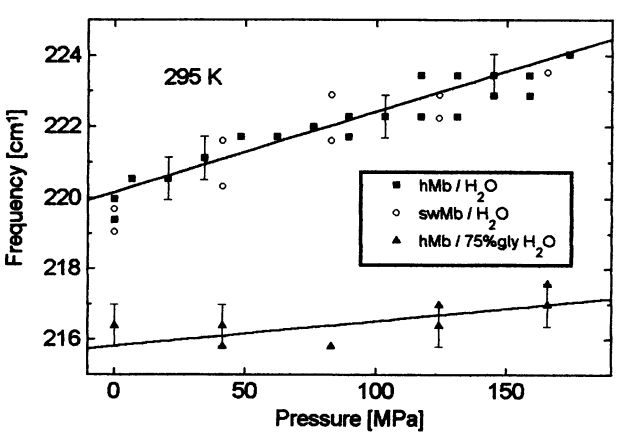

FIGURE 4 Pressure dependence of the peak frequency of the iron-histidine mode in myoglobin in aqueous solution and 75% gly/H_2O.

partner of the carboxyl group of the propionic acid attached to the D pyrrole (Schlichting et al., 1994) is Arg-45 (CD3), changes in the 343 cm^{-1} band may indicate motion of the protein helices.

We have previously reported (Schulte et al., 1995) the absence of a significant shift (>1 cm^{-1}) of the core size marker bands. This indicates that there is no radical perturbation of the heme structure by pressure and that the changes in $\nu_{Fe\text{-}His}$ are connected to the iron-protein linkage rather than to the bonds between the iron and the pyrrole nitrogens. Based on correlations (Spiro and Li, 1988) with skeletal mode frequencies (e.g. 330/cm/Å for ν_2) in porphyrins, the changes in core size appear to be less than 0.003 Å. Since the core size is sensitive to the spin state of the iron (Spiro and Li, 1988), the absence of spectral changes implies no spin transitions in this pressure range, although in NMR experiments it has been observed that above ≈200 MPa the low spin state of the heme iron is favored (Morishima, 1987).

Another key marker for relating changes in protein structure to reactivity is a weak near-infrared absorption band with a center frequency near 13120 cm^{-1} (762 nm) at room temperature. This band has been widely used to characterize the structure and relaxation of the photoproducts of MbCO and MbO_2 at cryogenic and room temperature (Iizuka et al., 1974; Ansari et al., 1985; Campbell et al., 1987; Sassaroli and Rousseau, 1987; Cupane et al., 1988; Ansari, 1988; Chavez et al., 1990; Srajer and Champion, 1991; Ahmed et al., 1991; Nienhaus et al., 1992; Lim et al., 1993). Eaton and Hofrichter (1981) assigned band III to a porphyrin-to-iron charge transfer transition $a_{2u} \rightarrow d_{yz}$. Since both porphyrin π and iron d-orbitals are involved in this transition, the center frequency of band III is sensitive to the position of the iron relative to the plane of the heme. Comparison of picosecond measurements of band III (Lim et al., 1993) with molecular dynamics simulation (Kuczera et al., 1993) suggests that there is a linear relation between the iron out-of-plane position and the peak frequency of band III, although some sensitivity to other structural parameters, such as the proximal histidine orientation to the heme, cannot be excluded (Sage and Champion, 1997). The first results on the pressure dependence of band III in deoxy myoglobin are shown in Fig. 5. The peak frequencies were determined from a nonlinear least squares fit to a Gaussian line profile with cubic background and are plotted in Fig. 6. With increasing pressure a shift of the peak frequency to longer wavelength is observed, and the band area increases approximately linearly with pressure. This can be explained with motion of the iron toward the heme plane, thereby increasing the overlap between the orbitals of the iron and the porphyrin.

The main conclusion from the combined shifts of the iron-histidine mode and band III is that pressure causes global conformational changes in the protein as well as rearrangements of the active site. Characteristic features (Table 1) of deligated Mb are a domed heme, a tilt of the proximal histidine with respect to the heme normal, and high spin. The X-ray structures show that in unligated

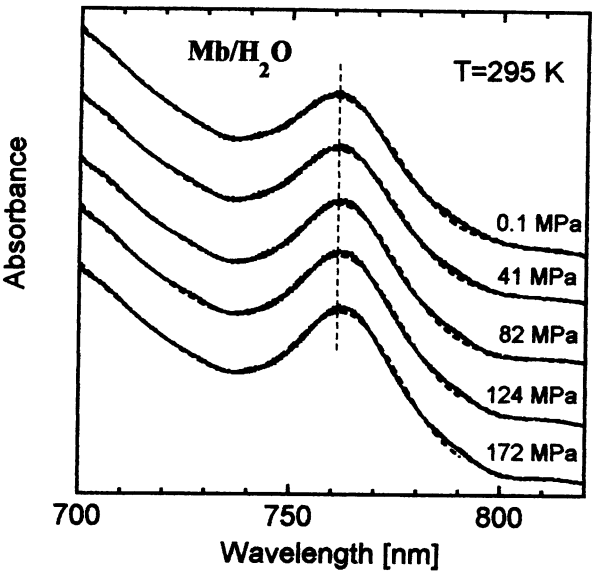

FIGURE 5 Pressure dependence of band III in deoxy myoglobin obtained in aqueous solution in pH 7. The spectra are offset vertically to avoid overlap. The dashed lines are fits to the sum of a Gaussian line shape and a cubic baseline.

myoglobin, the iron is displaced from the heme plane by ~0.35 Å (Phillips, 1980) while upon CO binding it moves to a position in the heme plane (Kuriyan et al., 1986; Schlichting et al., 1994). At the same time the azimuthal angle measured between the proximal histidine plane and the line connecting the pyrrole nitrogens N_1 and N_3 is the same in both bound and unbound myoglobin at ambient temperature. Apart from the electronic causes (Stavrov, 1993), steric repulsion between the imidazole side chain of the proximal histidine and the heme is responsible for the displacement of the iron. The dominant steric repulsion is believed to arise from the contact between the His C_ϵ proton of the His F8 imidazole side chain and the pyrrole nitrogen

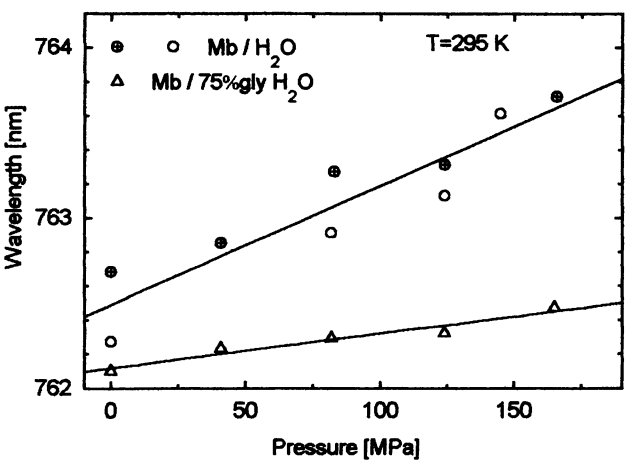

FIGURE 6 Peak frequency of band III as a function of pressure. Data are shown for myoglobin in aqueous solution and in 75% gly/H_2O. Different symbols denote different samples.

TABLE 1 Structural characteristics of deoxy Mb under pressure compared to photodissociated and deoxy Mb at ambient pressure

Marker	Mb (165 MPa) − Mb	Mb*CO − Mb
$\nu_{Fe\text{-}His}$	2.8 cm^{-1}	5 cm^{-1} (Rousseau and Friedman, 1988)
Peak band III	1.2 nm	7 nm (Steinbach et al., 1991)
Fe out-of-plane		0.05 Å (Schlichting et al., 1994)
His F8 tilt angle		4.9° (Schlichting et al., 1994)

N_1. There is also a small shift of the F-helix across the heme face when comparing Mb and MbCO (Sassaroli et al., 1986). The geometry of the proximal histidine influences both the frequency $\nu_{Fe\text{-}His}$ and the intensity of the Fe-His stretch band (Rousseau and Friedman, 1988). Systematic variation in $\nu_{Fe\text{-}His}$ of myoglobin from various species has been attributed to a F-helix-induced tilt of the proximal histidine with respect to the heme plane (Friedman et al., 1982). Larger tilt angles appear to be correlated with a greater iron displacement q_o (Friedman et al., 1990).

Based on semiempirical calculations, Stavrov (1993) has proposed that $\nu_{Fe\text{-}His}$ depends mainly on the value of the iron out-of-plane displacement, the imidazole tilt being able to modulate this dependence indirectly, changing the iron displacement. Using structural and spectroscopic parameters from imidazole complexes of ferroporphyrins and neglecting the simultaneous tilt of the imidazole, Stavrov (1993) finds a correlation between the iron out-of-plane displacement q_o and $\nu_{Fe\text{-}His}$:

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

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 $\nu_{Fe\text{-}His}$ 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. The iron displacement calculated from the Raman data is in good agreement with that calculated from the shift of band III. Assuming a linear correlation between peak wavelength of band III and q_o, we estimate that a 1.2 nm (21 cm^{-1}) shift corresponds to a change in the iron displacement of 0.01 Å.

Due to linkage of the proximal histidine both to the heme iron and the polypeptide backbone, the tilting of the proximal histidine should be accompanied by movement of the F-helix. The X-ray structures show displacement of the F-helix toward its junction with the E-helix in deligated myoglobin compared to the ligand bound structures (Phillips, 1980) and the unrelaxed photoproduct (Schlichting et al., 1994). Neutron diffraction studies of MbCO (Cheng and Schoenborn, 1991) have shown that the propionate acid group attached to one of the pyrrole rings folds back into the proximal histidine side of the heme to form two hydrogen bonds with Ser-92 (F7) and His-97 (FG3). The change in the 343 cm^{-1} band is consistent with alteration of the hydrogen bonding due to changes in the position of the F-helix. Sliding of the F-helix will alter the tilt angle and will change the length and/or force constant of the Fe-His bond. Movement of the F-helix may also provide a mechanism to release the strain and compression of the iron-proximal histidine bond observed in the crystal structure of a photolyzed sperm whale MbCO mutant at 20 K (Schlichting et al., 1994).

Additional structural information from high pressure (15 MPa) X-ray crystallographic studies is only available at 2 Å resolution for metmyoglobin, where alternate conformations are observed for three internal residues (Leu-135, Phe-138, and Ile-142) implying a repacking of the protein interior (Tilton and Petsko, 1988). However, these measurements may not resolve the small differences detected by spectroscopy, and uncertainties in the interpretation of additional electron density appearing in the proximal pocket during the 8-day exposure of the metmyoglobin crystals to N_2 at 145 atmospheres make explicit conclusions with respect to deoxy Mb solutions difficult.

Molecular dynamics computations suggest a strong coupling between protein fluctuations and the hydration shell in myoglobin (Loncharich and Brooks, 1990; Steinbach and Brooks, 1993). In a glycerol/H_2O mixture, the iron-histidine mode is shifted to lower wavenumber by 2.6 cm^{-1} (Sage et al., 1995) compared to aqueous solution (Fig. 3). Glycerol is important as a cosolvent used to stabilize proteins in their native state. The stabilizing effect results from preferential interactions which exclude the cosolvent molecules from the protein domain (Timasheff, 1993). The enrichment of water molecules bound to the protein may perturb the hydrogen-bond network and the overall protein conformation. Champion and coworkers have recently compared the solvent-induced shift in native Mb and a mutant where the distal histidine (His-64) was replaced by leucine making the heme pocket more hydrophobic. The smaller shift of $\nu_{Fe\text{-}His}$ in the mutant may indicate that dehydration of the heme pocket contributes to the glycerol induced shift observed in native Mb (Christian et al., 1996). On the other hand, we observe that the direction of the shift of the $\nu_{Fe\text{-}His}$ due to pressure is in the same direction as in 75% glycerol/H_2O as well as in aqueous solutions. Molecular dynamics simulation have indeed found large pressure-induced changes in the hydration layer causing the solvation shell water to adopt a more ordered structure (Kitchen et al., 1992).

Relation of conformational changes to ligand binding

The above data on the structural changes associated with high pressure can be used to provide a connection with the pressure dependence of the ligand binding kinetics. Transient absorption measurements of CO binding to myoglobin show an increase of the rebinding rate with pressure (Hasinoff, 1974; Adachi and Morishima, 1989; Frauenfelder et al., 1990; Taube et al., 1990). Low temperature experiments

measuring geminate rebinding indicate that the main effect is on the iron-ligand bond formation step (Frauenfelder et al., 1990).

Several studies indicate that control of the bond-forming step and relaxation involve predominantly the proximal side (Srajer et al., 1988; Ahmed et al., 1991). The iron is linked to the F-helix through the proximal histidine (His F8). To bind CO, the iron shifts from a position 0.35 Å away from the heme plane into the heme plane, and the histidine tilt angle moves toward the normal (Phillips, 1980). The analysis of the high-pressure data on Mb suggests that the protein adopts a conformation that more closely resembles that of a ligated protein. Important structural differences between ambient and high-pressure Mb on one hand, and Mb and the unrelaxed low temperature photoproduct Mb*, are summarized in Table 1. The iron histidine mode in the cryogenically stabilized photoproduct is shifted by ≈5 cm^{-1} (Rousseau and Friedman, 1988) to higher frequency. Band III in photodissociated carbonmonoxy myoglobin (Mb*CO) is red-shifted by 116 cm^{-1} relative to deoxy Mb (Steinbach et al., 1991), and a recent low-temperature crystallographic structure shows that the iron atom in Mb*CO is 0.05 Å closer to the heme plane (Schlichting et al., 1994). On the other hand, the peak frequency of band III shifts by 1.2 nm (21 cm^{-1}) toward longer wavelength as the pressure is raised to 165 MPa. Based on a linear interpolation a frequency shift of 21 cm^{-1} would correspond to a displacement of 0.01 Å. 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. The spectral shifts observed at high pressure are in the same direction as those measured in Mb*CO relative to deoxy Mb and correlate with changes in the tilt angle (≈4.9°) and the iron position [≈0.05 Å, Schlichting et al. (1994)]. When compared with unrelaxed Mb*CO, unligated Mb conformations (Schlichting et al., 1994) show an increase in the iron out-of-plane distance, a decrease in the Fe-proximal histidine bond length, a tilting of the proximal histidine, and a decompression and motion of the F-helix toward its junction with the E-helix. If we assume that the iron-ligand bond can occur only when the ligand and heme are closely spaced (proximate geminate pair, encounter complex), then a likely process of going from the encounter complex to the bound heme-ligand is described as follows. The ligand can only form a bond from within the encounter complex when the iron is either in-plane or less out-of-plane compared to the average displacement associated with the starting heme species. In this case, the barrier for rebinding is related to the probability that during the lifetime of the encounter complex, the heme undergoes a fluctuation that creates a transient planar structure that exists long enough for the bond to form. Our data indicate that when the protein is pressurized, the heme assumes a more planar form, thus lowering the barrier for rebinding and increasing the rebinding rate.

The significance of the conformational changes at high pressure is that a substantial part of the enthalpic barrier for ligand binding from the pocket is associated with iron out-of-plane displacement. From kinetic holeburning studies an approximately linear relation between the enthalpic barrier H for the binding step at the heme and the position ν of band III has been proposed (Ansari, 1988; Steinbach et al., 1991) yielding a slope ΔH [kJ/mol] = 0.11 $\Delta\nu$ [cm^{-1}]. Under these assumptions we estimate that a displacement of the iron by 0.01 Å corresponds to a frequency shift of 21 cm^{-1} and a decrease in barrier height by 2.2 kJ/mol. This can be compared with the rate increase observed for the CO rebinding kinetics (Hasinoff, 1974; Adachi and Morishima, 1989; Frauenfelder et al., 1990; Taube et al., 1990). 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 (Adachi and Morishima, 1989). Assuming a linear relation between barrier height and protein coordinate (Agmon and Hopfield, 1983; Young and Bowne, 1984; Steinbach et al., 1991) and using the transition state expression for the innermost barrier $k = A_o \cdot \exp(-H/RT)$ we note that an increase in barrier height by 2.2 kJ/mol corresponds to a rate increase of $\exp(\Delta H/R \times T) = \exp(2200/8.31 \times 295) = 2.5$. Therefore the change in rebinding rate follows directly from the relation of the iron position to the enthalpic barrier on one hand and the pressure-induced conformational change on the other. In other words, rearrangement of the proximal heme environment and iron motion can provide a structural basis for the activation volume. In addition, there may be changes in the preexponential A_o, which will have to be determined from measurements of the rebinding kinetics over wide ranges of time, temperature, and pressure. A correlation of the band III position with the CO association rate has also been proposed by Kiger et al. (1995) who examined several hemoproteins and heme complexes and observed that a blue shift of band III is related to a lower Fe-His Raman frequency. Thus an iron further out-of-plane (or higher heme distortion) is associated with a lower CO binding rate.

The rebinding kinetics at intermediate temperatures shows at least three different phases. The kinetics in the time regime between geminate rebinding and rebinding from the solvent has been attributed to the relaxation of a structural coordinate (Agmon and Hopfield, 1983; Srajer et al., 1988; Steinbach et al., 1991; Balasubramanian et al., 1993) such as the movement of the iron atom from the heme plane to the deoxy position. The relaxation causes a time-dependent barrier for rebinding. If pressure forces the iron atom closer to the heme plane the decrease of the final barrier should cause a speed-up of the kinetics with pressure in the time range of structural relaxation. Preliminary experiments provide support for this interpretation (Galkin et al., 1996).

Pressure effects on metastable photoproducts

Protein function involves a number of intermediate states and a large amount of motions with different energies and

time scales (Frauenfelder et al., 1991), which poses the challenge of structurally characterizing the intermediates. Low-temperature experiments can discriminate between these motions by slowing down the relaxation toward the equilibrium deligated structure. Thus unrelaxed state(s) of photodissociated myoglobin can be studied in detail providing information on heme-protein host interaction. The energy landscape of myoglobin consists of a huge number of minima with nearly equal energy. To explore the properties of the energy surface one can use additional degrees of freedom, namely pressure, in conjunction with temperature. An important feature of proteins lies in the fact that by decreasing temperature below 160 K they are brought to a glasslike state (Austin et al., 1975; Frauenfelder et al., 1988; Angell, 1995). The final state below the transition temperature is dependent on pathway. Pressure allows investigation of metastable states by choosing different pathways in the pressure-temperature plane.

We have performed both Raman and absorption experiments for Mb and Mb* to investigate structurally unrelaxed intermediates with respect to the deoxy state. The Raman spectra of Mb and Mb* obtained at low temperature for ambient (0.1 MPa) and high (175 MPa) pressure are depicted in Fig. 7. Lowering the temperature from 295 to 60 K at 0.1 MPa causes some changes in the deoxy spectrum (Figs. 3 and 8), most notably a shift of $\nu_{\text{Fe-His}}$ from 220 to 229 cm^{-1}. A new line appears near 266 cm^{-1} and the band at 343 cm^{-1} shifts slightly to higher frequency. These findings are in agreement with results published in the literature (Sassaroli et al., 1986; Ahmed et al., 1991; Sage et al., 1995).

At high pressure there are significant changes in positions and intensities of Raman bands. The most important result is that the pressure-induced shift of the iron-histidine observed at room temperature persists while the sample is cooling down. Pressure also affects the intensity of $\nu_{\text{Fe-His}}$ in such a way that it is smaller at high pressure than at ambient. When varying temperature and pressure in a metastable system it is necessary to define the exact path in the temperature-pressure (TP) plane for the experimental protocol. Data for two different pathways are shown in Fig. 7. In the first case, the sample was cooled down at 0.1 MPa and then pressurized (FP). On the second path the sample was pressurized to 175 MPa at room temperature and then cooled (PF). The dependence of the spectral bands on the path in the temperature-pressure plane is reminiscent to that observed in other spectral markers, such as the Soret and the A-states (Frauenfelder et al., 1990). We found that once low temperature ($\leq$60 K) was achieved, pressurizing the sample in the FP state or releasing the pressure in the PF state did not influence the Raman spectra significantly. This shows that at low temperature the protein is frozen and no conformational shifts occur in the Raman spectrum. Elastic shifts are too small (<1 cm^{-1}) to be resolved here.

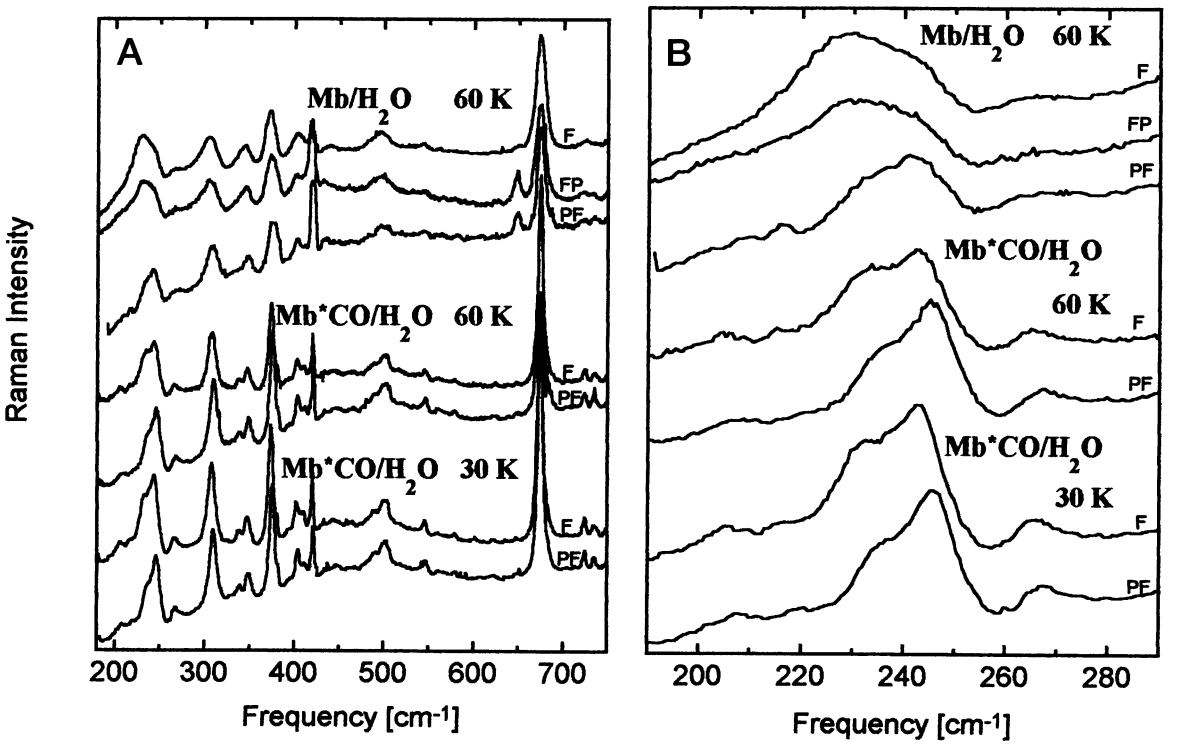

FIGURE 7 Comparison of deligated Mb and photodissociated carbonmonoxy myoglobin at ambient and high pressure. The protein sample is either cooled at ambient (F) or at high (PF) pressure. A spectrum taken along another pathway (pressurized at low temperature) is indicated by FP (see text). Resonance Raman spectra were measured at 60 and 30 K in frozen aqueous solutions. (*a*) Low-frequency region; (*b*) spectral range near the iron-histidine band.

The spectra of photolyzed MbCO exhibit features similar to that of deoxy Mb (Fig. 7), although there are some significant differences consistent with ambient pressure data (Sassaroli et al., 1986; Rousseau and Friedman, 1988). The iron-histidine is shifted to higher frequency in the 60 K photoproduct, namely 4.9 cm^{-1} at 0.1 MPa and 4.3 cm^{-1} at 175 MPa with respect to the Mb spectrum at the same pressure. In both deoxy Mb and Mb* the intensity of the Fe-His mode relative to the low frequency heme bands is smaller for the PF path compared with FP. It is evident from Fig. 7*b*, which enlarges the spectral region between 190 and 290 cm^{-1}, that the shift in the low-frequency bands of the photoproduct is amplified at high pressure. This supports the interpretation of Rousseau and coworkers (Sassaroli et al., 1986) that protein relaxation is constrained in the low-temperature photoproduct inhibiting the tilt of the imidazole away from the heme normal and causing the iron-histidine mode to increase its frequency. When the protein is cooled under pressure the differences between deoxy and photodissociated myoglobin become more pronounced. As observed previously in ambient pressure studies (Sassaroli et al., 1986) the shoulder at 338 cm^{-1} becomes better resolved.

Fig. 8 shows the peak position of the iron-histidine mode versus temperature for the FF and PF pathways corresponding to pressures of 0.1 and 175 MPa. For both paths the dependences show similar features, namely 1) the Fe-His frequency shifts monotonically to higher values as the temperature goes down, and 2) after 60 K there is almost no change in the position of the lines. These features are common both for deoxy Mb and Mb*CO, although in the last case additional considerations should be made. As the probe beam can photolyze the sample, two different possibilities exist when the sample is cooling down. First, if the probe beam illuminates the sample while measuring a spectrum during the cooling process, at a certain temperature the MbCO sample is trapped in the photolyzed state, and after that the Mb* actually is cooling down. In contrast to this case, if the sample is cooled in the dark and then probed by a laser beam, the photolyzed state of the unperturbed ligated Mb is monitored. In order to examine the effects of illumination during cooling we used a second protocol in which the temperature was raised to 160 K after measuring a spectrum [data denoted by *d* in Fig. 8]. During cooling and sample equilibration at the target temperature, the laser beam was blocked. This ensures that the protein has completely rebounded the ligands, and that cooling of the sample starts from the same initial state. On the other hand, when following the first protocol, the sample was not thermally cycled, though in both cases the laser beam was only illuminating the sample during data acquisition.

It is interesting to note that the differences between the two protocols are apparent only for the PF path while there is no difference for cooling at ambient pressure (*F*). For the second protocol the data for the Mb*CO sample are similar to those for the deoxy state, although the shift of Fe-His mode is larger. This fact suggests that pressurized deoxy Mb has the structure that is intermediate between Mb and MbCO at atmospheric pressure.

Fig. 9 shows the dependence of band III on different pathways in the temperature-pressure plane. In comparison to the Raman data, the dependence is somewhat more complicated for band III. Very little difference is observed for deoxy Mb. However, in case of photolyzed MbCO at 30 K there is a small shift in the corresponding spectra (768 nm at 0.1 MPa compared to 769.0 nm at 175 MPa). This may be

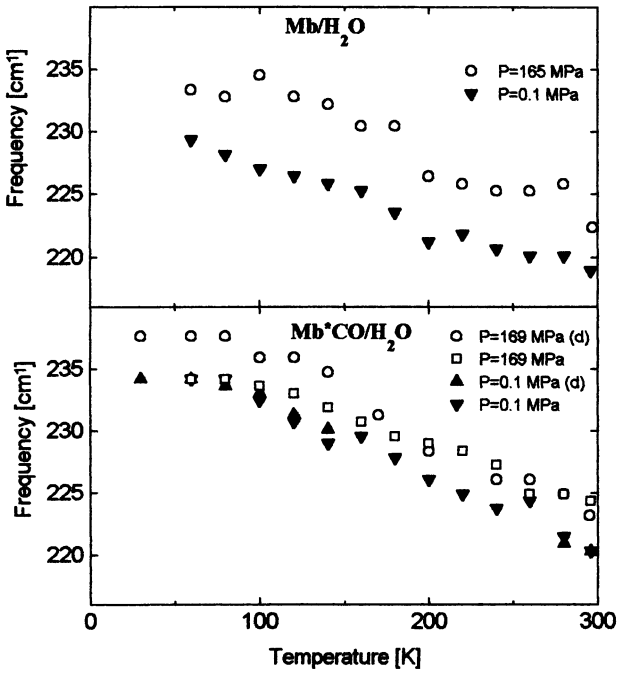

FIGURE 8 Temperature dependence of the peak frequency of the iron-histidine mode in deoxy Mb and photodissociated carbonmonoxy myoglobin. Data are shown at ambient and high pressure. Samples cooled and cycled in the dark are denoted by *d* (see text).

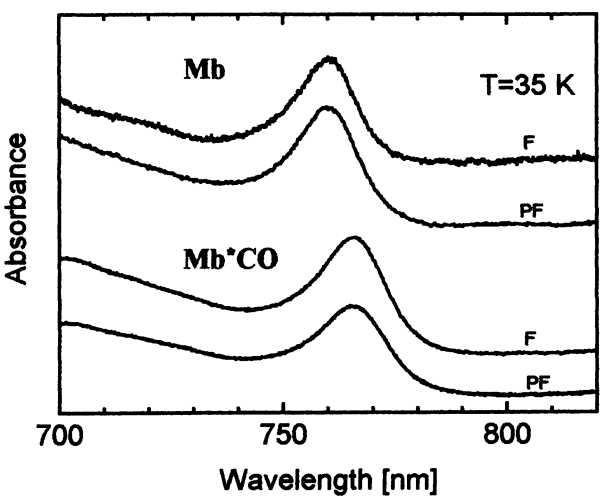

FIGURE 9 Near-infrared absorption spectra of myoglobin and photodissociated MbCO at ambient and high pressure. Spectra were measured at 35 K in 75% gly/H_2O solution.

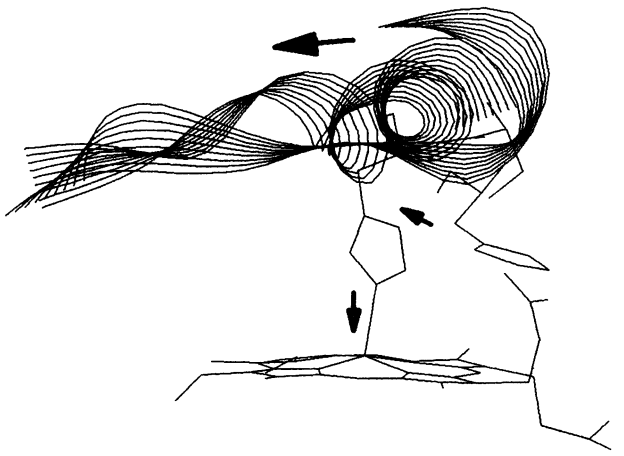

FIGURE 10 Schematic representation of pressure-induced changes in the proximal heme pocket. Arrows indicate motion of the heme iron, proximal histidine tilt angle, and F-helix.

attributed to the fact that Raman and absorption experiments probe slightly different characteristics of the Mb molecule.

The data obtained from Raman and absorption experiments allow us to picture the global changes that occur in the structure of the protein while applying high pressure (Fig. 10) and low temperature. The low-temperature data show that pressure alters the spectral parameters of the photoproducts as probed by the iron-histidine line and band III. As stated earlier, the iron-histidine mode is associated with the tilt angle of the histidine with respect to the normal to the heme plane. A small tilt angle between the proximal histidine and the normal to the heme plane is indicated by the increase in frequency at low temperatures. The inability of the tilt angle to change to the larger value associated with the unbound protein is due to an unrelaxed F helix (Sassaroli et al., 1986). This structure is not free to move at low temperatures, thereby trapping the heme and surrounding structures in a conformation closely resembling that of the bound protein. Due to our protocol and the low laser intensity used, we can rule out light-induced relaxation of Mb* as a major source for the temperature-dependent structural changes. While the changes in frequency seen in the other modes may be attributed to small changes in peripheral interactions due to lattice contraction upon freezing, the rather large shift in $\nu_{\text{Fe-His}}$ may indicate spontaneous relaxation of Mb* or kinetic holeburning due to ligand recombination (Ahmed et al., 1991). The pressure dependence and the results of Ahmed et al. suggest that the peak position of band III and $\nu_{\text{Fe-His}}$ differ in their dependence on the iron displacement.

To describe the energy hypersurface of myoglobin a hierarchical model is widely used. In this model the conformational substates are arranged in tiers (Ansari et al., 1985, 1987; Frauenfelder et al., 1991). In the highest tier, the barriers between minima are largest, and taxonomic substates (denoted by A_0, A_1, and A_3 in sperm whale MbCO) can be distinguished. The rebinding kinetics in the individual A-states (Ansari et al., 1987), Mössbauer spectroscopy (Parak and Nienhaus, 1991), incoherent neutron scattering (Doster et al., 1989), inhomogeneities in the spectral band shapes (Zollfrank et al., 1992), experiments employing optical line narrowing techniques (Thorn Leeson and Wiersma, 1995), as well as computer simulations (Elber and Karplus, 1987) show that there are substates with even lower barriers. The question then arises whether the protein structure monitored in our experiments could be subdivided further to introduce several conformations that are distinguishable by Raman spectroscopy. It has been recently suggested (Gilch et al., 1993, 1995) that the iron-histidine mode can be deconvoluted into three sublines corresponding to substates of a lower tier. In attempts to analyze our data using convolution analysis we were not able to confirm unambiguously the existence of a specific number of sublines in the Fe-His band. While pressure-temperature cycling experiments show the contribution of conformational substates to the iron histidine mode, it is not clear why there should be discrete sublines as opposed to a continuous distribution; rather, the shift in peak frequency might indicate a redistribution in the conformational substates. Sage et al. (1995) have recently shown that perturbation of the vibrational modes by an asymmetric potential caused by nonbonded interactions between the imidazole group and pyrrole nitrogens of the heme can provide a reasonable fit to the observed temperature dependence of $\nu_{\text{Fe-His}}$.

Friedman and coworkers (Ahmed et al., 1991) have found in Raman studies at ambient pressure that the temperature dependence of the iron-histidine mode and kinetic holeburning in band III are consistent with two tiers of structure associated with the proximal heme pocket. They suggest that the first population of conformational substates (CS) might be characterized by different $\nu_{\text{Fe-His}}$ frequencies with barriers large enough that interconversion does not occur below the glass transition. The second level has smaller barriers as reflected in the intensity of the Fe-His mode. Changes in either tier can contribute to the wavelength of band III, where kinetic holeburning and optical pumping may be related to tier 2 and relaxation near the glass transition (Nienhaus et al., 1992) to tier 1. The dependence of the iron-histidine stretch frequency on the path in the pressure-temperature plane may be attributed to global conformational changes induced by pressure.

Our high-pressure data clearly demonstrate another important feature of the glassy state of proteins at low temperature, namely metastability. This is inherently related to the inability of the kinetically arrested protein to globally explore the rugged energy landscape below the glass transition. Metastability implies that the state of the system below the glass temperature depends on its history and that the system is trapped in a substate with an energy above the lowest energy. In our experiments the dependence on history means that below the glass transition the states at the end of the PF and FP pathways could be different. The Raman data indeed exhibit a pathway dependence (Fig. 7). At each temperature the points for the PF-path lie higher

than for the FF-path (Fig. 8). While metastability has been demonstrated before using the Soret absorption and the ligand bands of MbCO in glycerol-water solutions (Frauenfelder et al., 1990), our data directly show that these features can be connected to structural heterogeneities of the heme protein linkage.

CONCLUSIONS

Resonance Raman spectroscopy and NIR absorption spectroscopy have been used as complementary tools to study the influence of high pressure on myoglobin. The Raman spectra show that high pressure shifts the equilibrium of the ligand binding reaction toward the CO bound state. A pressure-induced shift of the iron-histidine mode and band III probing the functionally important heme-protein linkage is investigated at ambient and low temperatures. The results are interpreted as structural changes that involve both the heme and the protein suggesting that at high pressure the structure of deligated Mb is closer to that of the liganded species, thus decreasing the enthalpy barrier for CO binding. Combined application of temperature and pressure also demonstrated metastability of the protein at low temperature as spectroscopically probed by the iron-histidine mode.

The authors thank G. Mizell and VLOC for supplying $KNbO_3$ crystals for frequency doubling of the Ti:sapphire laser.

This work was supported by National Science Foundation Grant MCB-9305711, which is gratefully acknowledged.

REFERENCES

Adachi, S., and I. Morishima. 1989. The effects of pressure on oxygen and carbon monoxide binding kinetics for myoglobin. *J. Biol. Chem.* 264: 18896–18901.

Ahmed, A. M., B. F. Campbell, D. Caruso, M. R. Chance, M. D. Chavez, S. H. Courtney, J. M. Friedman, I. E. T. Iben, M. R. Ondrias, and M. Yang. 1991. Evidence for proximal control of ligand specificity in hemeproteins: absorption and Raman studies of cryogenically trapped photoproducts of ligand bound myoglobins. *Chem. Phys.* 158:329–352.

Agmon, N., and J. J. Hopfield. 1983. CO binding to heme proteins: a model for barrier height distributions and slow conformational changes. *J. Chem. Phys.* 79:2042–2053.

Angell, C. A. 1995. Formation of glasses from liquids and biopolymers. *Science.* 267:1924–1935.

Ansari, A. 1988. Conformational relaxation and kinetic hole-burning in sperm whale myoglobin. Ph.D. Thesis, University of Illinois at Urbana-Champaign.

Ansari, A., J. B. Berendzen, S. F. Bowne, H. Frauenfelder, I. E. T. Iben, T. B. Sauke, E. Shyamsunder, and R. D. Young. 1985. Protein states and proteinquakes. *Proc. Natl. Acad. Sci. USA.* 82:5000–5004.

Ansari, A., J. B. Berendzen, D. Braunstein, B. R. Cowen, H. Frauenfelder, M. K. Hong, I. E. T. Iben, J. B. Johnson, P. Ormos, T. B. Sauke, R. Scholl, A. Schulte, P. J. Steinbach, J. Vittitow, and R. D. Young. 1987. Rebinding and relaxation in the myoglobin pocket. *Biophys. Chem.* 26:337–355.

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of Ligand binding to myoglobin. *Biochemistry.* 14:5355–5372.

Balasubramanian, S., D. G. Lambright, M. C. Marden, and S. G. Boxer. 1993. CO recombination to human myoglobin mutants in glycerol-water solutions. *Biochemistry.* 32:2202–2212.

Bangcharoenpaurpong, O., K. T. Schomaker, and P. M. Champion. 1984. A resonance Raman investigation of myoglobin. *J. Am. Chem. Soc.* 108:1163–1167.

Buchter, S., C. Williams, A. Schulte, T. Alekel III, G. Mizell, and W. Fay. 1995. Intracavity frequency doubling of a continuous wave Ti:Al_2O_3 ring laser and application in resonance Raman spectroscopy of heme protein dynamics. *SPIE Conf. Proc.* 2380:127–136.

Campbell, B. F., M. R. Chance, and J. M. Friedman. 1987. Ligand binding channels reflected in the resonance Raman spectra of cryogenically trapped species of myoglobin. *J. Biol. Chem.* 262:14885–14890.

Chavez, M. D., S. H. Courtney, M. R. Chance, D. Kiula, J. Nocek, B. M. Hoffman, J. M. Friedman, and M. R. Ondrias. 1990. Structural and functional significance of inhomogeneous line broadening of band III in hemoglobin and Fe-Mn hybrid hemoglobins. *Biochemistry.* 29: 4844–4852.

Cheng, X., and B. P. Schoenborn. 1991. Neutron diffraction study of carbonmonoxymyoglobin. *J. Mol. Biol.* 220:381–399.

Choi, S., and T. G. Spiro. 1983. Out-of-plane deformation modes in the resonance Raman spectra of metalloporphyrins and heme proteins. *J. Am. Chem. Soc.* 105:3683–3692.

Christian, J. F., M. Unno, J. T. Sage, S. G. Sligar, E. Chien, and P. M. Champion. 1996. Water in the heme pocket: its effect on the Fe-His stretch in the resonance Raman spectra of deoxymyoglobin. *Biophys. J.* 70:336*a*. (Abstr.).

Cupane, A., M. Leone, E. Vitrano, and L. Cordone. 1988. Structural and dynamic properties of the heme pocket in myoglobin probed by optical spectroscopy. *Biopolymers.* 27:1977–1997.

Doster, W., S. Cusack, and W. Petry. 1989. Dynamic transition of myoglobin revealed by inelastic neutron scattering. *Nature.* 337:754–756.

Eaton, W. A., and J. Hofrichter. 1981. Polarized absorption and linear dichroism spectroscopy of hemoglobin. *Methods Enzymol.* 76:175–261.

Elber, R., and M. Karplus. 1987. Multiple conformational states of proteins: a molecular dynamics analysis of myoglobin. *Science.* 235: 318–321.

Fersht, A., and G. Winter. 1992. Protein engineering. *Trends Biochem. Sci.* 17:292–299.

Frauenfelder, H., N. Alberding, A. Ansari, D. Braunstein, B. R. Cowen, M. K. Hong, I. E. T. Iben, J. B. Johnson, S. Luck, J. R. Mourant, P. Ormos, L. Reinisch, R. Scholl, A. Schulte, E. Shyamsunder, L. B. Sorensen, P. J. Steinbach, A. H. Xie, R. D. Young, and K. T. Yue. 1990. Proteins and pressure. *J. Phys. Chem.* 94:1024–1037.

Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substates in proteins. *Ann. Rev. Biophys. Biophys. Chem.* 17:451–479.

Frauenfelder, H., S. Sligar, and P. G. Wolynes. 1991. The energy landscapes and motions of proteins. *Science.* 254:1598–1603.

Friedman, J. M. 1985. Structure, dynamics, and reactivity in hemoglobin. *Science.* 228:1274–1280.

Friedman, J. M., B. F. Campbell, and R. W. Noble. 1990. A possible new control mechanism suggested by resonance Raman spectra from a deep ocean fish hemoglobin. *Biophys. Chem.* 37:43–59.

Friedman, J., D. L. Rousseau, and M. R. Ondrias. 1982. Time-resolved resonance Raman studies of hemoglobin. *Annu. Rev. Phys. Chem.* 33: 471–491.

Galkin, O., and A. Schulte. 1996. High pressure Raman spectroscopy of the heme-protein linkage in myoglobin. *Proc. XVth Intl. Conf. Raman Spectrosc.* S. A. Asher and P. Stein, editors. John Wiley & Sons, New York. 460–461.

Galkin, O., A. Schulte, and A. Tabirian. 1996. Pressure effects on ligand binding to myoglobin and protein structure. *Bull. Am. Phys. Soc.* 41:620 (Abstr.).

Gilch, H., W. Dreybrodt, and R. Schweitzer-Stenner. 1995. Thermal fluctuations between conformational substates of the Fe^{2+}-His^{F8} linkage in deoxymyoglobin probed by the Raman active Fe-Ne (His^{F8}) stretching vibration. *Biophys. J.* 69:214–227.

Gilch, H., R. Schweitzer-Stenner, and W. Dreybrodt. 1993. Structural heterogeneity of the Fe^{2+}-Ne (His^{F8}) bond in various hemoglobin and myoglobin derivatives probed by the Raman-active iron histidine stretching mode. *Biophys. J.* 65:1470–1485.

Hasinoff, B. B. 1974. Kinetic activation volumes of the binding of oxygen and carbon monoxide to hemoglobin and myoglobin studied on a high-pressure laser flash photolysis apparatus. *Biochemistry.* 13:3111–3117.

Heremans, K. A. H. 1982. High pressure effects upon proteins and other biomolecules. *Ann. Rev. Biophys. Bioenerg.* 11:1–21.

Iizuka, T., H. Yamamoto, M. Kotani, and T. Yonetani. 1974. Low temperature photodissociation of hemoproteins: carbon monoxide complex of myoglobin and hemoglobin. *Biochim. Biophys. Acta.* 371:126–139.

Jannasch, H. W., R. E. Marquis, and A. M. Zimmerman, editors. 1987. *Current Perspectives in High Pressure Biology.* Academic, New York.

Jonas, J., and A. Jonas. 1994. High-pressure NMR spectroscopy of proteins and membranes. *Annu. Rev. Biophys. Biomol. Struct.* 213:287–318.

Jortner, J., and J. Ulstrup. 1979. Dynamics of nonadiabatic atom transfer in biological systems. Carbon monoxide binding to hemoglobin. *J. Am. Chem. Soc.* 101:3744–3754.

Kauzmann, W. 1987. Thermodynamics of unfolding. *Nature.* 325: 763–764.

Kiger, L., F. Stetzkowski-Marden, C. Poyart, and M. C. Marden. 1995. Correlation of carbon monoxide association rates and the position of absorption band III in hemoproteins. *Eur. J. Biochem.* 228:665–668.

Kitagawa, T. 1988. Heme protein structure and the iron histidine stretching mode. *In* Biological Applications of Raman Spectroscopy. Vol. 3. T. G. Spiro, editor. Wiley, New York. 97–132.

Kitchen, D. B., L. H. Reed, and R. M. Levy. 1992. Molecular dynamics simulation of solvated protein at high pressure. *Biochemistry.* 31: 10083–10093.

Kuczera, K., J.-C. Lambry, J.-L. Martin, and M. Karplus. 1993. Nonexponential relaxation after ligand dissociation from myoglobin: a molecular dynamics simulation. *Proc. Natl. Acad. Sci. USA.* 90:5805–5807.

Kuriyan, J., S. Wilz, M. Karplus, and G. Petsko. 1986. X-ray structure and refinement of carbon-monoxy (Fe-II) myoglobin. *J. Mol. Biol.* 192: 133–154.

Lim, M., T. A. Jackson, and P. A. Anfinrud. 1993. Nonexponential protein relaxation: dynamics of conformational change in myoglobin. *Proc. Natl. Acad. Sci. USA.* 90:5801–5804.

Loncharich, R. J., and B. R. Brooks. 1990. Temperature dependence of dynamics of hydrated myoglobin: comparison of force field calculations with neutron scattering data. *J. Mol. Biol.* 215:439–455.

Morild, E. 1981. Theory of pressure effects on enzymes. *Adv. Protein Chem.* 34:93–166.

Morishima, I. 1987. High pressure NMR studies of hemoproteins. *In* Current Perspectives in High Pressure Biology. H. W. Jannasch, R. E. Marquis, and A. M. Zimmerman, editors. Academic, New York. 315–332.

Nienhaus, G. U., J. Mourant, and H. Frauenfelder. 1992. Spectroscopic evidence for conformational relaxation in myoglobin. *Proc. Natl. Acad. Sci. USA.* 89:2902–2906.

Nienhaus, G. U., J. R. Mourant, K. Chu, and H. Frauenfelder. 1994. Ligand binding to heme proteins: the effect of light on ligand binding in myoglobin. *Biochemistry.* 33:13413–13430.

Parak, F., and G. U. Nienhaus. 1991. Glass-like behavior of proteins as seen by Mössbauer spectroscopy. *J. Non-Cryst. Solids.* 131–133: 362–368.

Phillips, S. E. V. 1980. Structure and refinement of oxymyoglobin at Å resolution. *J. Mol. Biol.* 142:531–554.

Projahn, H.-D., C. Dreher, and R. van Eldik. 1990. Effect of pressure on the formation and deoxygenation kinetics of oxymyoglobin. Mechanistic information from a volume profile analysis. *J. Am. Chem. Soc.* 112:17–22.

Rousseau, D. L., and J. M. Friedman. 1988. Transient and cryogenic studies of photodissociated hemoglobin and myoglobin. *In* Biological Applications of Raman Spectroscopy. Vol. 3. T. G. Spiro, editor. John Wiley & Sons, New York. 133–216.

Sage, J., and P. M. Champion. 1997. Small substrate recognition in heme proteins. *In* Supramolecular Chemistry. K. S. Suslick, editor. Elsevier, Amsterdam.

Sage, J. T., K. T. Schomacker, and P. M. Champion. 1995. Solvent-dependent structure and dynamics in myoglobin. *J. Phys. Chem.* 99: 3394–3405.

Sassaroli, M., S. Dasgupta, and D. L. Rousseau. 1986. Cryogenic stabilization of myoglobin photoproducts. *J. Biol. Chem.* 261:13704–13713.

Sassaroli, M., and D. L. Rousseau. 1987. Time dependence of near infrared spectra of photodissociated hemoglobin and myoglobin. *Biochemistry.* 26:3092–3098.

Schlichting, I., J. Berendzen, G. N. Phillips, Jr., and R. M. Sweet. 1994. Crystal structure of photolysed carbonmonoxymyoglobin. *Nature.* 371: 808–812.

Schulte, A., and L. Bradley II. 1995. High-pressure near-infrared Raman spectroscopy of bacteriorhodopsin light to dark adaptation. *Biophys. J.* 69:1554–1562.

Schulte, A., S. Buchter, O. Galkin, and C. Williams. 1995. Raman spectroscopic observation of a conformational change at the heme-protein linkage in myoglobin at high pressure. *J. Am. Chem. Soc.* 117: 10149–10150.

Spiro, T. G. 1983. The resonance Raman spectroscopy of metalloporphyrins and heme proteins. *In* Iron Porphyrins. Vol. 2. A. B. P. Lever and H. B. Gray, editors. Addison-Wesley, Reading. 89–159.

Spiro, T. G., editor. 1988. Biological Applications of Raman Spectroscopy. John Wiley & Sons, New York.

Spiro, T. G., and X. Y. Li. 1988. *In* Biological Applications of Raman Spectroscopy. Vol. 3. T. G. Spiro, editor. John Wiley & Sons, New York. 1–37.

Srajer, V., and P. M. Champion. 1991. Investigations of optical line shapes and kinetic hole burning in myoglobin. *Biochemistry.* 30:7390–7402.

Srajer, V., L. Reinisch, and P. M. Champion. 1988. Protein fluctuations, distributed coupling, and the binding of ligands to heme proteins. *J. Am. Chem. Soc.* 110:6656–6670.

Stavrov, S. S. 1993. The effect of iron displacement out of the polyphyrin plane on the resonance Raman spectra of heme proteins and iron porphyrins. *Biophys. J.* 65:1942–1950.

Steinbach, P. J., A. Ansari, J. Berendzen, D. Braunstein, K. Chu, B. R. Cowen, D. Ehrenstein, H. Frauenfelder, J. B. Johnson, D. C. Lamb, S. Luck, J. R. Mourant, G. U. Nienhaus, P. Ormos, R. Philipp, A. Xie, and R. D. Young. 1991. Ligand binding to heme proteins: connection between dynamics and function. *Biochemistry.* 30:3988–4001.

Steinbach, P. J., and B. R. Brooks. 1993. Protein hydration elucidated by molecular dynamics simulation. *Proc. Natl. Acad. Sci. USA.* 90: 9135–9139.

Sungar, N., and B. S. Gerstman. 1992. Nonadiabatic electronic spin transition in ligand-heme protein binding kinetics and the influence of the heme Fe molecular environment. *J. Chem. Phys.* 96:387–397.

Swanson, B. I., S. F. Agnew, M. R. Ondrias, and R. G. Alden. 1986. Optical spectroscopic studies of heme proteins at high pressure. *J. Am. Chem. Soc.* 108:341–343.

Taube, D. J., H.-D. Projahn, R. van Eldik, D. Magde, and T. G. Traylor. 1990. Mechanism of ligand binding to hemes and hemoproteins. A high pressure study. *J. Am. Chem. Soc.* 112:6880–6886.

Thorn Leeson, D., and D. A. Wiersma. 1995. Looking into the energy landscape of myoglobin. *Nature Struct. Biol.* 2:848–851.

Tilton, R., and G. Petsko. 1988. A structure of sperm whale myoglobin at a nitrogen gas pressure of 145 atmospheres. *Biochemistry.* 27: 6374–6382.

Timasheff, S. N. 1993. The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Ann. Rev. Biophys. Biomol. Struct.* 22:67–97.

Traylor, T. G., D. J. Taube, K. A. Jongeward, and D. Magde. 1990. Steric effects on geminate recombination. *J. Am. Chem. Soc.* 112:6875–6880.

Weber, G., and H. G. Drickamer. 1983. The effect of high pressure upon proteins and other biomolecules. *Quart. Rev. Biophys.* 16:89–1122.

Winter, R., and J. Jonas, editors. 1993. High Pressure Chemistry, Biochemistry and Materials Science. Kluwer Academic Publishers, Dordrecht, Boston and London.

Young, R. D., and S. F. Bowne. 1984. Conformational substates and barrier height distributions in ligand binding to heme proteins. *J. Chem. Phys.* 81:3730–3737.

Yu, N.-T. 1986. Resonance Raman studies of ligand binding. *Methods Enzymol.* 130:350–409.

Zipp, A., and W. Kauzmann. 1973. Pressure denaturation of metmyoglobin. *Biochemistry.* 12:4217–4228.

Zollfrank, J., J. Friedrich, and F. Parak. 1992. Spectral hole burning study of protoporphyrin IX substituted myoglobin. *Biophys. J.* 61:716–724.