Determination of Fe–CO Geometry in the Subunits of Carbonmonoxy Hemoglobin M Boston Using Femtosecond Infrared Spectroscopy†

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ABSTRACT: We have undertaken ultrafast infrared (IR) spectroscopic studies in order to elucidate the geometry of bound CO in the α and β subunits of hemoglobin (Hb) M Boston ^{13}CO. Hb M Boston is a mutant human Hb in which the distal histidine in the α subunits is replaced by a tyrosine. The IR absorptions of bound ^{13}CO fall at 1925 cm^{-1} for the α subunits and 1907 cm^{-1} for the β subunits. Despite a difference of nearly 20 cm^{-1} in these peaks, the measured anisotropies of the bound ^{13}CO depletions following 30% photolysis are nearly identical, with values of -0.142 ± 0.002 obtained for the α subunits and -0.140 ± 0.003 obtained for the β subunits. These translate to values of $20° \pm 1°$ and $21° \pm 1°$ for the values of the average angles between the CO bond and the normal to the heme planes in the α and β subunits, respectively. Our present results and the work of previous investigators [Nagai, M., Yoneyama, Y., & Kitagawa, T. (1991) *Biochemistry 30*, 6495–6503] suggest that a change in the polar interactions of the bound CO with the heme pocket environment upon substitution of tyrosine for the distal histidine and a less bent structure for the Fe–C–O unit in the α subunits are responsible for the difference in the bound CO absorption frequencies in the α and β subunits. A spectrum of the depletion of the bound ^{13}CO peaks following photolysis indicates that both subunits photodissociate CO with the same quantum yield and neither subunit exhibits significant recombination within 1 ns.

In hemoglobin (Hb) the amino acids in the heme pocket play crucial roles in preventing the oxidation of the heme iron, assuring cooperative binding, and determining the affinities of the protein for different ligands. A class of mutant hemoglobins (Hbs) known as the Hbs M are characterized by amino acid substitutions in either the α or β subunits which lead to the iron porphyrin in these subunits being readily oxidized to the ferric state by oxygen. Hb M Boston and Hb M Saskatoon have the distal histidine replaced by a tyrosine in the α and β subunits, respectively. Two others, Hb M Iwate and Hb M Hyde Park, have the proximal histidine replaced by tyrosine in the α and β subunits, respectively, while the fifth, Hb M Milwaukee, has an amino acid substitution of glutamic acid for valine normally found at position E11 in the β subunits.

Our present interest in Hb M Boston CO arises from recent resonance Raman studies by Nagai et al. (1991). They used resonance Raman spectroscopy to study the geometry of CO bound to Hb M Boston. From the frequency shifts of the Fe–C and CO stretching modes upon isotopic substitution of CO, they determined that the Fe–C–O angle in the mutant α subunits was approximately 170° while in the β subunits the angle was ~160°. They also noted that upon continuous illumination the Fe–C Raman stretch in the β subunits disappeared, indicating complete photodissociation with little rebinding of the CO prior to Raman scattering. In contrast, the Fe–C stretch of the α subunits was still present, indicating that either the CO does not photodissociate in these subunits or that if it does, recombination occurs much more rapidly than in the normal β subunits. Recent time-resolved resonance Raman studies (Y. Sakan, M. Nagai, T. Ogura, and T. Kitagawa, 1992, unpublished experiment) indicate that CO does photodissociate from the α subunits and undergoes more extensive geminate recombination and a more rapid bimolecular recombination for CO which has escaped from the protein than the normal β subunits. Geminate recombination is rebinding of a photodissociated ligand which has not left the protein and occurs on a picosecond to nanosecond time scale for CO adducts of heme proteins.

The IR absorption of bound CO in the abnormal α subunits of Hb M Boston CO falls at 1972 cm^{-1}, while in the β subunits the bound CO absorption falls at 1952 cm^{-1}. The range of absorptions for the CO stretch of heme–CO adducts falls from 1980 cm^{-1} to 1906 cm^{-1} (Li & Spiro, 1988). A number of factors affect the frequency of the bound CO absorption, including the electron-donating ability of the ligand trans to the CO, polar interactions of the surrounding environment with the CO, and the geometry of the bound CO (Augspurger et al., 1991; Li & Spiro, 1988). From their results on the shift of the Raman peaks of the Fe–C and CO stretching frequencies upon isotopic substitution of CO in Hb M Boston CO, Nagai et al. (1991) concluded that in the α subunits CO is nearly perpendicular to the heme plane while in the β subunits the CO is bent from the heme plane normal as found in previous studies of human Hb A CO (Moore et al., 1988; Locke et al., 1991). Ultrafast IR spectroscopy provides an ideal method for determining the geometry of the bound CO in heme proteins. Anisotropy measurements of the depletion of the bound CO absorption following photolysis can provide information regarding the angle of the bound CO to the heme plane normal (Moore et al., 1988), while with our current laser system recombination of photodissociated CO can be observed on a time scale of femtoseconds to 1 ns. We define the heme plane as the plane containing the degenerate $\pi \rightarrow$

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π^* transitions of the porphyrin (Locke et al., 1991). We report here the results of ultrafast IR experiments designed to further study the geometry and rate of geminate recombination of CO in the α and β subunits of Hb M Boston CO. The earliest-time anisotropy monitored at the maxima of the bound CO absorptions was nearly identical for both subunits and indicated that the CO is bent by nearly the same angle from the normal to the heme plane in both subunits. We suggest that the reason for the difference in the bound IR absorption frequencies for the subunits in Hb M Boston CO is due to a change in the polar interactions affecting CO upon replacement of the distal histidine with a tyrosine in the α subunits. Both subunits exhibited the same quantum yield of photodissociation and neither subunit showed appreciable geminate recombination within 1 ns. The relationship of our results to those of Nagai et al. (1991) is discussed.

MATERIALS AND METHODS

Sample Preparation. Purification of Hb M Boston was carried out as described previously (Nagai et al., 1980). In a typical sample preparation, 0.10 mL of a 1 mM (heme concentration) Hb M Boston sample in D_2O (0.05 M phosphate, pD = 7) under a nitrogen atmosphere was reduced by the addition of 0.02 mL of a 0.035 M $Na_2S_2O_4$ solution in D_2O. ^{13}CO was added, and the solution was stored under approximately 1 atm ^{13}CO overnight at room temperature. Five or six drops of sample were placed between CaF_2 windows with a 0.1-mm spacer. FTIR and visible spectra indicated neglible sample decomposition during the course of an experiment.

Laser System. Time-resolved absorption measurements were obtained by means of a femtosecond laser system which has been described in detail elsewhere (Anfinrud et al., 1988). Absorption intensities were obtained by unconversion of a continuous wave (cw) IR probe. The upconverted signal, which is detected by means of a photomultiplier, is dependent on the transmitted IR intensity during a slice of time defined by the gating pulse. The optical pulses are generated in a cavity-dumped dye laser, which is synchronously pumped with the output of a frequency-dumped mode-locked Nd-YAG laser. These pulses are compressed in an optical fiber grating compressor and amplified in a multipass dye cell that is pumped by a frequency-doubled Q-switched Nd-YAG laser. The amplified output (580 nm, 5 μJ, 350 fs FWHM, 1 kHz) is split to derive the pump and gate pulses. For the current experiments, the pump intensity was chosen to photolyze $\sim$30% of the sample. The cw IR from a diode laser (Laser Photonics, Bedford, MA) is focused to 100 μm in a sample cell composed of two CaF_2 windows with a 0.1-mm spacer. The pump beam is delivered to the sample colinearly with the cw IR, whose transmitted field is upconverted by the gating pulse in a $LiIO_3$ crystal. Absorption measurements at different times are achieved by means of a delay line that varies the time after photolysis in which the IR is upconverted. The time resolution of the system is determined by the widths of the pumping and gating pulses. The diode laser was tunable from 1900 to 1970 cm^{-1} with a multimode bandwidth of 6 cm^{-1} within this range.

The average and differential (pumped minus unpumped) transmitted IR intensities are recovered with lock-in detection. The computed differential sample absorbance is averaged until a predetermined level of statistical significance. Data accumulated from different scans of the delay line were averaged with appropriate statistical weights. The data shown in Figures 1 and 2 represent 30 min of acquisition time per data point,

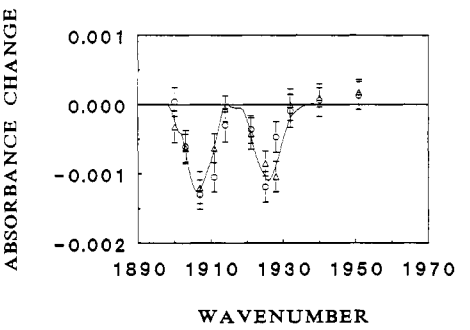

FIGURE 1: Infrared difference spectrum of photodissociated Hb M Boston ^{13}CO. The solid line represents a 30% depletion of the bound ^{13}CO absorptions of the sample utilized. The triangles are data taken at a delay time of 5 ps, while the circles are data taken at a delay time of 1 ns.

and the error bars are one standard deviation calculated by standard methods.

The anisotropy was calculated from the formula

$$r(t) = (\Delta A_{\parallel} - \Delta A_{\perp})/(\Delta A_{\parallel} + 2\Delta A_{\perp}) \quad (1)$$

where $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are the changes in absorption intensities for the IR beam polarized parallel and perpendicular to the photolyzing pulse, respectively. The polarization changes were made by rotating a half-wave plate in the photolysis beam.

The sample cell was moved vertically up and down at a rate of 0.5 cm^{-1} s^{-1} in order to prevent sample burning and excessive heating. Each pulse photolyzed 30% of the sample. At this level of photolysis, the majority of the photolyzed sample will remain in the R quaternary state and rebinding of the dissociated CO will be complete within 1 ms (Hofrichter et al., 1983).

RESULTS AND DISCUSSION

Figure 1 shows the spectrum of photolyzed Hb M Boston ^{13}CO at delay times of 5 ps and 1 ns. We utilized ^{13}CO since this isotope brought the absorbance of the bound ^{13}CO in the α subunits within the range of our IR diode laser. Detailed calculations indicate that the change in the IR absorption frequency for bound CO in heme–CO systems upon isotopic substitution in CO depends not only on the masses of the isotopes as expected for harmonic oscillators (Silverstein et al., 1991) but also on the geometry of the Fe–C–O unit (Nagai et al., 1991). In the α subunits the bound CO IR absorbance changes from 1972 cm^{-1} for ^{12}CO to 1925 cm^{-1} for ^{13}CO, while in the β subunits the absorbance changes from 1952 cm^{-1} for ^{12}CO to 1907 cm^{-1} for ^{13}CO. The ratio of the intensities of the depletions in Figure 1 at 1907 cm^{-1} and 1925^{-1} mirrors the ratio of intensities in the unphotolyzed sample, indicating that the quantum yield of photodissociation is the same for both subunits. The intensity of the depletion signal is nearly the same for both times, indicating that little recombination occurs within 1 ns for either subunit. Figure 2 displays the recombination kinetics of ^{13}CO observed at 1925 cm^{-1} and 1907 cm^{-1} for delay times of 1 ps to 1 ns. Within our signal to noise, there appears to be little recombination within 1 ns.

Recently, Nagai et al. (1991) noted that for a sample of Hb M Boston CO under weak continuous laser illumination the resonance Raman Fe–C peak at 490 cm^{-1} indicative of CO bound to the α hemes was still present while the 505-cm^{-1} band due to CO bound to the β hemes was absent. These results were taken to indicate that either the CO does not photodissociate in the α subunits or, if dissociation does occur, rebinding is much more rapid than in the β subunits. The

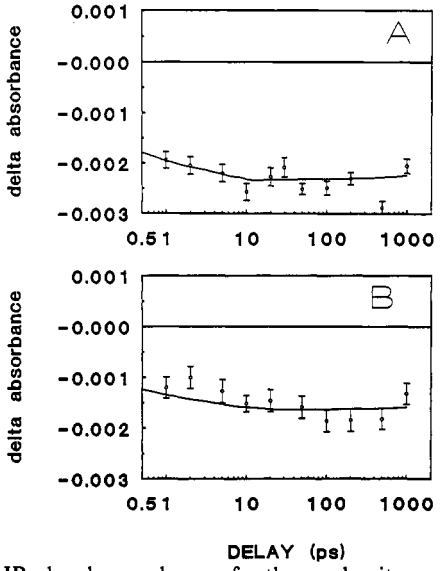

FIGURE 2: IR absorbance changes for the α subunits monitored at 1925 cm^{-1} (panel A) and the β subunits monitored at 1907 cm^{-1} (panel B) following excitation of the heme with a 580-nm pulse. The solid lines are fits of the experimental data with a model that uses the second-order autocorrelation as the instrument response function and assumes 60% geminate recombination in the α subunits and 40% geminate recombination in the β subunits. The geminate recombination is assumed to follow exponential kinetics with a $1/e$ time of 40 ns as found for human Hb A CO (Hofrichter et al., 1983). The 15% bleach after 1 ps is due to a pedestal of the pulse which extends out to 10 ps.

rate at which the photodissociated CO must rebind in order to observe primarily bound heme–CO signals from Raman scattering can be estimated as follows. The rate of excitation of heme–CO units is given by

$$k = \sigma I/(h\upsilon A) \quad (2)$$

where σ is the heme–CO absorption cross-section, I is the beam power, υ is the excitation frequency, and A is the area of the beam. In their experiment, Nagai et al. (1991) used the 406.7-nm line from a Kr laser at 10 mW of power to observe the Raman scattering. The cross-section for heme–CO absorption is 2×10^{-16} cm^2 at this wavelength. With a focus radius of 100 μm for their excitation beam, a heme–CO near the front of the cell (where the beam power is close to 10 mW) will be excited once every 80 μs. This represents an upper limit to the rate of excitation since the intensity of the incident beam will be decreased by absorption further from the cell surface. Hofrichter et al. (1983) measured the rebinding rates of CO photodissociated from human Hb A CO. Geminate recombination was fit to an exponential rate and occurred with a $1/e$ time of 40 ns. The bimolecular recombination for CO which escaped from the protein was exponential and had a $1/e$ time of 200 μs with a heme–CO concentration similar to the one used in the Raman studies. If no CO escapes from the protein and all the CO rebinds on a nanosecond time scale, then for most of the time between absorptions of a photon by the heme CO would be bound and any photon scattered would depict a bound CO. When CO escapes from the protein, however, recombination occurs on a microsecond to millisecond time scale and photons scattered from the heme are likely to find the heme in an unbound state. Szabo (1978) has calculated the diffusion-controlled rate at which a ligand will bind to the heme from the solvent. In this model, the protein is treated as a shell with an opening of radius 1 Å larger than the radius of the ligand. The ligand diffuses through the opening and immediately reacts once inside the protein. With this model a bimolecular rate constant of 5×10^8 M^{-1} s^{-1} is calculated for ligands with a diffusion constant similar to that of oxygen. The solubility of CO in water is ~1 mM, and assuming that the concentration of CO is much greater than the concentration of photodissociated hemes, then any CO which escapes from the protein will rebind exponentially with a time constant of 2 μs according to this model. In Hb and Mb the rate of recombination for photodissociated CO which has escaped from the protein is much slower than this due to the fact that the protein has no opening large enough for a ligand to diffuse through and protein fluctuations which open a gate for the ligand to enter are thought to be involved in the binding process.

Recent time-resolved resonance Raman studies (Y. Sakan, M. Nagai, T. Ogura, and T. Kitagawa, 1992, unpublished experiment) of the rebinding of photodissociated CO within Hb M Boston CO indicate that within the α subunits ~60% of the photodissociated CO rebinds within 100 ns. The extent of geminate recombination is greater for the mutant α subunits of Hb M Boston CO than the normal β subunits or normal human Hb A CO, both of which undergo ~40% recombination within 100 ns (Y. Sakan, M. Nagai, T. Ogura, and T. Kitagawa, 1992, unpublished experiment; Hofrichter et al., 1983). The kinetics of the α subunit rebinding shown in Figure 2 (panel A) are fit assuming 60% exponential geminate recombination with a $1/e$ time of 40 ns, the same rate observed by Hofrichter et al. (1983) for Hb A CO. The small extent of geminate recombination within 1 ns and our current signal to noise does not allow us to accurately determine the geminate recombination in the α subunits, and the difference in rates between the α and β subunits is not precisely known. The bimolecular recombination rate for CO which has escaped from the protein is faster for the α subunits than the β subunits. In the experiments of Sakan et al. (Y. Sakan, M. Nagai, T. Ogura, T. Kitagawa, 1992, unpublished experiment), ~96% of the photodissociated CO had rebound in the α subunits within 10 μs while ~75% had rebound in the β subunits. It appears that a more extensive geminate recombination and a faster bimolecular recombination of CO in the α subunits of Hb M Boston CO can account for the observation of Nagai et al. (1991) that upon weak continuous illumination of Hb M Boston CO the Fe–C Raman band in the β subunits dissappears while the Fe–C Raman band in the α subunits remains.

The faster bimolecular recombination rate of the α subunits of Hb M Boston than that of the β subunits indicates that CO can enter the heme pocket more easily in the mutant α subunits. It is generally believed that the distal histidine must swing out of the way to allow a ligand to enter the heme pocket and the distal histidine and valine E11 provide the primary hindrance to entry of a ligand into the heme pocket (Nagai et al., 1987; Carver et al., 1990; Egeberg et al., 1990). A number of factors could account for the increase in geminate recombination found in the α subunits of Hb M Boston CO. Steric hindrance, strain on the porphyrin, and the energy required to move the proximal histidine into the heme plane during binding have all been shown to affect the association rate of CO binding to hemes (Traylor, 1981; Traylor et al., 1985; Kim et al., 1990; Perutz, 1989). In deoxy-Hb the distal histidine is in van der Waals contact with the heme (Perutz, 1989), and replacement of the distal histidine by a tyrosine could alter the heme position within the pocket and affect any of these factors which help determine the association rate. Without more knowledge about the structure of the heme pocket of the α subunits of Hb M Boston in solution, a more detailed description of the reasons for these subunits increased geminate

recombination and faster bimolecular recombination rate during the rebinding of CO cannot be given.

From shifts of the Fe–C and C–O stretching modes in Raman spectra of Hb M Boston CO upon isotopic substitution of CO, Nagai et al. (1991) concluded that the Fe–C–O linkage was more linear in the α subunits than in the β subunits. From their measurements, however, it was not possible to determine the extent of tilting of the CO bond from the heme plane normal. It has generally been believed that the wave number of the CO IR absorption when bound to iron porphyrins depends upon the geometry of the bound CO, with higher wave numbers for the absorption corresponding to the CO being more perpendicular to the heme plane. This assumption has been verified by low and ambient temperature anisotropy studies of sperm whale myoglobin (Moore et al., 1988; Ormos et al., 1988), which show that the 1944-cm^{-1} absorption corresponds to the CO lying 20° from the heme plane normal while the 1933-cm^{-1} band has the CO lying 35° from the heme plane normal. As discussed by Li and Spiro (1988), however, a number of other factors including polar interactions with the bound CO and the extent of CO tilting, bending, and porphyrin buckling affect the bound CO absorption frequency. CO bending is defined as a bending of the Fe–C–O unit so that the angle between the Fe–C and C–O bonds is less than 180°, heme tilting is defined as movement of the Fe–C–O unit off the normal to the heme plane while keeping the angle between the Fe–C and C–O bonds 180°, while heme buckling is a rotation of the heme pyrroles around the Fe–N bonds.

The anisotropy of the bound ^{13}CO depletions for Hb M ^{13}CO Boston following 30% photolysis was measured to be -0.140 ± 0.003 for the β subunits and -0.142 ± 0.002 for the α subunits at a delay time of 10 ps. These values are almost identical within our experimental uncertainty and correspond to values for the angle between the normal to the plane containing the heme absorption dipoles and the CO bond of 21° ± 1° for the β subunits and 20° ± 1° for the α subunits after correction for the extent of photolysis (Hansen et al., 1989).

These angles are obtained by equating the measured anisotropy, given in eq 1, to the correlation function of the transition dipole orientation:

$$r(t) = -0.2\langle P_2(\cos\alpha)\rangle\langle P_2[\hat{\mu}(0)\,\hat{\mu}(t)]\rangle \qquad (4)$$

where $\hat{\mu}(t)$ is a vector fixed normal to the heme plane at time t and α is the angle between the C–O axis and the normal to the heme plane. The various factors relating to the validity of this equation applied to heme proteins have been discussed previously (Locke et al., 1991). At earliest times, prior to any reorientation, $\hat{\mu}(0)\,\hat{\mu}(t) = 1$, which from the definition $2P(x) = 3x^2 - 1$, yields the result

$$\langle\cos^2\alpha\rangle = (^1/_3)(1 - 10r(0)) \qquad (5)$$

where $\langle ... \rangle$ implies an average over the equilibrium distribution of angles. Thus, the angles quoted are the quantities $\cos^{-1}\{\langle\cos^2\alpha\rangle^{1/2}\}$, which are referred to in the text as "average" angles. It should be realized that the measure of anisotropies depends on the equilibrium distribution of angles, since it is concievable that the same $r(t)$ could be observed for each subunit but that the equilibrium distribution of angles in the two cases could be different. In the ensuing discussion, we are assuming that both subunits have a relatively narrow distribution of angles. There is evidence to support the assumption that the distribution of angles between CO and the heme plane normal is sharply peaked in carbonmonoxy hemoglobins. The bandwidth of bound CO absorptions indicates the range of interactions between CO and its environment. For free hemes in solution and some heme proteins, these may be considerable giving rise to IR bandwidths up to 30 cm^{-1} (Maxwell & Caughey, 1976, 1978). The bandwidths of the absorptions for Hb M Boston CO are 8 cm^{-1}, indicating that there is a relatively uniform environment for the CO and hence a narrow distribution in the CO location in both subunits. Ormos et al. (1988) reported the spread of orientations for the 1945-cm^{-1} band in low-temperature studies of Sperm whale MbCO to be 26°–30°, though it is uncertain whether the distribution at low temperatures is comparable to the distribution at high temperatures.

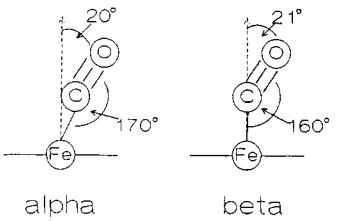

FIGURE 3: Schematic representation of the geometry of CO in the α and β subunits of Hb M Boston CO in accordance with the results of Nagai et al. (1991), which indicate that the Fe–C–O unit is less bent in the α subunits, and the present results which indicate that CO is bound at an angle of 20° and 21° from the heme plane normal in the α and β subunits, respectively. The heme plane is represented by the solid line through the Fe, the heme plane normal is represented by the dashed arrow, and Fe, C, and O atoms are represented by open circles as indicated.

The observed anisotropy was nearly identical at delay times of 1 ps and 10 ps for the α subunits, indicating that the heme in these subunits does not undergo substantial motion on a picosecond time scale as found previously for normal human Hb A CO (Locke et al., 1991). Despite a separation of 20 cm^{-1} in the bound CO IR absorption peaks, the CO is bound at a similar angle to the heme plane. Geometries of the bound CO in the α and β subunits of Hb M Boston CO consistent with our experimental results and those of Nagai et al. (1991) are shown schematically in Figure 3. In this representation the angle of the CO to the heme plane normal is nearly the same for both subunits but the extents of tilting and bending of the CO differ. As will be argued below, another factor responsible for the large difference in the positions of the bound CO IR absorptions is the change of the polar interactions between the bound CO and amino acids in the heme pocket upon substitution of a tyrosine for the distal histidine in the α subunits.

The variations in the bound CO stretching frequencies and the negative correlation between the Fe–C and C–O stretching frequencies observed for CO bound to iron porphyrins has been attributed to Fe d → CO π^* back donation (Li & Spiro, 1988). Upon binding CO, there is an overlap between the iron d_{xz} and d_{yz} orbitals and the CO π^* antibonding orbitals. As the mixing of these orbitals increases, the bond order of the Fe–C bond is increased and the bond order of CO decreases due to enhanced electron density in the CO antibonding orbitals. The extent of mixing will depend upon the electron donating ability of the ligand trans to the CO and the geometry of the bound CO. Ligands which readily donate electron density will increase the electron density on the iron, encouraging mixing of the iron d orbitals with the CO antibonding orbitals. The geometry of the bound CO will also affect the extent of backbonding. If the carbon atom of CO remains on the normal to the heme plane, then bending of the CO bond will decrease the extent of backbonding by making the mixing of the iron d orbitals and the CO antibonding orbitals less energetically favorable (Hoffmann

et al., 1977). Other theoretical treatments have also addressed the effects of CO geometry on the Fe–C and C–O stretching frequencies and reached conflicting conclusions. Calculations by Nagai et al. (1991) have shown that if the force constants for CO and Fe–C stretching are not changed then bending the CO leads to a decrease in the CO and an increase in the Fe–C stretching frequencies. They used normal-coordinate calculations for an isolated three body oscillator (Fe–C–O) using Wilson's GF matrix method (Wilson, 1939) and considered the C–O and Fe–C stretching, Fe–C–O bending, and Fe–C and C–O stretching interaction constants in their calculations. Li and Spiro (1988) considered the L–Fe–C–O unit in their calculations, in which L represents the proximal histidine, and used the Fe–L force constant in addition to the constants used by Nagai et al. (1991). In contrast to Nagai et al. (1991), they concluded that Fe–C and C–O stretching frequencies both decreased upon CO bending. These results disagree with experimental findings which indicate an inverse relationship between Fe–C and C–O stretching frequency changes for heme–CO adducts, and Li and Spiro concluded that bending was not a primary distortion in heme proteins. The primary origin for the conflicting conclusion is the assignment of the weak Raman band around 580 cm^{-1}; Li and Spiro (1988) regarded it as a fundamental while Nagai et al. (1991) treated it as an overtone. In addition to this, possible changes in the force constants upon the geometrical changes were not incorporated in both calculations. Recently, Stavrov et al. (1993) used the vibronic theory to calculate the effects of CO geometry on the frequency of the CO stretch and concluded that both CO bending and tilting will reduce the bound CO stretch frequency. Li and Spiro (1988) also considered the effects of heme buckling and Fe–C–O tilting, which increase the extent of backbonding and move the CO off the heme plane normal. Both of these distortions are believed to misalign the orbitals of the heme pyrolles with the Fe, d_{xz} and d_{yz} orbitals, decreasing the extent of porphyrin–iron mixing and hence increasing the Fe–CO orbital mixing. Recent X-ray data on human HbCO indicate that all three distortions, CO bending, Fe–C–O tilting, and heme plane buckling, occur in human Hb A CO (Derewenda et al., 1990). Finally, Augspurger et al. (1991) have shown that electric fields can either increase or decrease the CO stretch frequency depending on the orientation of the CO with the field, while Sakan et al. (Sakan et al., 1993) have noted that a linear relationship exists between the hydropathy index (Kyte & Doolittle, 1982) of amino acid substitutions for the distal histidine and the bound CO absorption frequency for recombinant human MbCO. Thus, it is evident that any discussion of the effects of the heme pocket of the Fe–C and CO IR absorption frequencies must also include polar interactions with the environment.

We will now consider what effects the distal histidine can have on the bound CO in a normal Hb subunit. In addition to its steric effects, the distal histidine appears to polarize CO and increase backbonding. Various workers have suggested that the Nε atom of the distal histidine is unprotonated and is in van der Waals contact with the CO. Overlap of the sp_2 orbitals from Nε and the antibonding orbitals of CO increases the electron density in the CO antibonding orbitals and thus decreases the bound CO absorption frequency (Tucker et al., 1978; Fuchsman & Appleby, 1979; Makinen et al., 1979). However, it has been noted that such a mechanism should also decrease the extent of mixing of the Fe d and CO π^* orbitals and thus decrease the Fe–C bond strength (Tucker et al., 1978; Ramsden & Spiro, 1989). This is not observed experimentally, as the observed inverse correlation between the Fe–C and CO stretch frequencies for heme–CO complexes indicates that as the CO bond is strengthened the Fe–C bond is weakened. Thus, it is currently uncertain how extensive mixing of the distal histidine Nε sp_2 and CO π^* orbitals is. The effects of electric fields on the CO IR absorption frequency have also been studied (Augspurger et al., 1991; Oldfield et al., 1991; Park et al., 1991). The electric field effect can be schematically pictured by considering the resonant structures of CO.

$$\underset{\text{(a)}}{C^-\equiv O^+} \longleftrightarrow \underset{\text{(b)}}{C{=}O} \longleftrightarrow \underset{\text{(c)}}{C^+{-}O^-}$$

A negative potential near the CO oxygen atom will favor resonance a and increase the CO bond strength while a negative potential near the CO carbon atom will favor resonance c and decrease the bond strength. Oldfield et al. (1991) suggested that the four bound CO absorption bands found in heme proteins arise from the different electric fields the four conformations of the distal histidine (arising from rotation of the histidine about the Cβ–Cγ bond and protonation of Nε or Nδ) impose on the bound CO. In their calculations, the Nε protonated form had a lower bound CO frequency than the Nδ protonated form when the histidine ring was in the conformation observed in X-ray structures. This result suggests that the Nε of histidine is closer to the CO carbon atom than oxygen atom, which is not what is observed in the X-ray structure of HbCO (Derewenda et al., 1990). Evidence from steady-state IR spectra indicates that the heme pocket environments of HbCO and MbCO differ in solution and crystal (Brown et al., 1983; Potter et al., 1985), and the position of the distal histidine observed in crystal structures is not necessarily its position in solution. It should also be noted that there is some uncertainty as to whether the distal histidine Nε or Nδ is protonated in solution. Neutron diffraction studies of sperm whale MbO_2 and MbCO indicate that for MbO_2 the distal histidine Nε is protonated and forms a hydrogen bond with the bound oxygen, while in MbCO crystals the distal histidine Nδ is protonated (Hanson & Schoenborn, 1981; Phillips & Schoenborn, 1981). Ab initio calculations (Ramini & Boyd, 1981) and experimental studies (Richards & Wallis, 1979) indicate that the Nε–H tautomer of histidine is favored over the Nδ–H tautomer. The neutron diffraction studies (Hanson & Schoenborn, 1981) and the work of Brown et al. (1983) could suggest that the extent of protein hydration affects which of the distal histidine nitrogens is protonated. Brown et al. (1983) observed the intensities of the 1966 cm^{-1} and 1944 cm^{-1} bound CO IR absorption bands for MbCO as a function of protein hydration. They observed that as the protein becomes less hydrated the intensity of the 1966-cm^{-1} band increased and the intensity of the 1944-cm^{-1} band decreased. Similar results were also observed for HbCO. Together with the neutron diffraction studies, these results could imply that at low hydration Nε is unprotonated and leads to a 1966-cm^{-1} absorption for MbCO while in solution Nε is protonated and the 1944-cm^{-1} band predominates. In this model, Nε would be closer to the CO oxygen atom as observed in X-ray structures. It is evident from the above considerations that experimental and theoretical results support the claim that polar interactions play a major role in affecting the bound CO absorption frequency in heme proteins. Currently, however, due to uncertainty in the structure of the heme pocket in solution it is not possible to describe precisely how the polar interactions of the distal histidine influence the bound CO absorption.

Hb M Iwate and Hb M Hyde Park are proteins in which the proximal histidine is replaced by a tyrosine in the α and β subunits, respectively. In these proteins, CO is believed to bind on the proximal side of the heme in the abnormal subunits (Nagai et al., 1991). For the CO adducts of both of these proteins the resonance Raman peaks for the bound CO stretches are indistinguishable from the normal subunit peaks and fall at 1952 cm^{-1} for Hb M Hyde Park CO and 1948 cm^{-1} for Hb M Iwate CO, close to the resonance Raman peaks observed for human Hb A CO (Nagai et al., 1991). In these proteins there is no histidine near the bound CO in the abnormal subunits yet the resonance Raman bands fall near those found for Hb A CO, in contrast to the peaks for the abnormal subunits in Hb M Boston CO and Hb M Saskatoon CO which fall at 1972 cm^{-1}. Currently, the reasons for this difference are unclear. As noted previously, both geometry and the polarity of the environment will affect the bound CO absorption frequency. The proximal side of the heme pocket in the abnormal subunits of Hb M Iwate CO and Hb M Hyde Park CO contains a serine and a tyrosine. Both of these amino acids are less polar than histidine but they might be in a position to affect the bound CO frequency.

CONCLUSIONS

Using transient IR spectroscopy, we have shown that the bound CO in the subunits of Hb M Boston CO lies 20° ± 1° from the normal to the heme plane defined by the $\pi \rightarrow \pi^*$ electronic transitions in the α subunits and 21° ± 1° in the β subunits. The IR absorptions of bound ^{13}CO fall at 1925 cm^{-1} in the α subunits and 1907 cm^{-1} in the β subunits. This separation is believed to arise from a change in the polar interactions between the bound CO and the heme pocket in the α subunits which have a tyrosine substituted for the distal histidine and to a less bent structure for the Fe–C–O unit in the α subunits. Previous workers (Nagai et al., 1991) had suggested that either CO does not photodissociate in the α subunits or if, it does, it rebinds at a much faster rate than in the β subunits. Recent time-resolved resonance Raman studies (Y. Sakan, M. Nagai, T. Ogura, and T. Kitagawa, 1992, unpublished experiment) indicate that CO photodissociates from the α subunits and ~60% undergoes geminate recombination, in contrast to the β subunits in which ~40% of the photodissociated CO recombines geminately, and CO which escapes from the protein appears to rebind faster to the α subunits than to the normal β subunits. Our measurements indicate that CO photodissociates from both subunits with the same quantum yield and that neither type of subunit exhibits significant recombination within 1 ns (see Figures 1 and 2).

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