Biochemistry

 Volume 28, Number 8 April 18, 1989

Accelerated Publications

Resonance Raman Evidence That Distal Histidine Protonation Removes the Steric Hindrance to Upright Binding of Carbon Monoxide by Myoglobin†

Jeremy Ramsden‡ and Thomas G. Spiro*

Department of Chemistry, Princeton University, Princeton, New Jersey 08544-1009

Received January 6, 1989; Revised Manuscript Received February 16, 1989

ABSTRACT: The resonance Raman band assigned to Fe–CO stretching in the sperm whale myoglobin CO adduct shifts from 507 cm^{-1} at neutral pH to 488 cm^{-1} at low pH, in concert with a shift of the C–O stretching infrared band from 1947 to 1967 cm^{-1} (Fuchsman & Appleby, 1979), while the 575-cm^{-1} Fe–C–O bending RR band loses intensity. The pK_a that characterizes these changes is ~4.4. The vibrational frequencies at low pH are well modeled by the protein-free CO, imidazole adduct of protoheme in a nonpolar solvent while those at high pH are modeled by the adduct of a heme with a covalent strap (Yu et al., 1983) which inhibits upright CO binding. It is inferred that the Fe–C–O unit changes from a tilted to an upright geometry when the distal histidine is protonated, because its side chain swings out of the heme pocket due to electrostatic repulsion with a nearby arginine residue. A different protonation step (pK_a = 5.7), which has been shown to modulate the CO rebinding kinetics (Doster et al., 1982) as well as the optical spectrum (Fuchsman & Appleby, 1979), is suggested to involve a global structure change associated with protonation of histidine residues distant from the heme.

We report resonance Raman (RR) spectroscopic evidence for a change in the geometry of CO bound to sperm whale myoglobin (Mb) from a tilted to an upright configuration, the transition being associated with a protonation equilibrium having a pK_a of ~4.4. This conformation change, which is suggested to be associated with the swinging away of the distal histidine from the vicinity of the bound CO, is of interest in connection with current thinking about the role of distal residues in the dynamics of heme protein ligation (Olson et al., 1988; Braunstein et al., 1989; Moore et al., 1989).

Figure 1 shows the 500-cm^{-1} region of the RR spectrum of MbCO, obtained with 413.1-nm excitation. At pH 6.95, one sees a prominent band at 507 cm^{-1} and a somewhat weaker one at 575 cm^{-1}, which have been assigned by Tsubaki et al. (1982) to the ν(Fe–C) stretching and δ(Fe–C–O) bending modes of the FeCO unit on the basis of ^{13}C and ^{18}O isotope shifts. [The ν(Fe–C) frequency reported by Tsubaki et al. was slightly higher, 512 cm^{-1}.] We confirmed these band assignments by comparing the spectrum with ^{13}CO bound to Mb; the 507 and 575 cm^{-1} shifted down 3 and 16 cm^{-1} (cf. 3- and 14-cm^{-1} shifts reported by Tsubaki et al.). As the pH is lowered, a new band grows in at 488 cm^{-1}, at the expense of the 507-cm^{-1} band, while the δ(Fe–C–O) band loses intensity relative to the nearby weak bands (551 and 581 cm^{-1}), which have been assigned to modes of the porphyrin ring (Choi et al., 1983). [Actually the 581-cm^{-1} band is in Fermi resonance with δ(Fe–C–O) (Tsubaki et al., 1982), losing intensity when the latter is shifted out of resonance upon ^{13}CO substitution (Figure 1). The band that remains at low pH is weaker and slightly lower in frequency than at neutral pH.]

Fuchsman and Appleby (1979) have previously reported the pH dependence of the ν(C–O) stretching infrared (IR) band. Two bands, 1947 cm^{-1} (neutral pH) and 1967 cm^{-1} (low pH), were found to be connected by a protonation step with a pK_a of 4.5. We confirmed these observations. In Figure 2 the intensities of the 507-cm^{-1} RR band and the 1947-cm^{-1} IR band are plotted against pH. It is clear that the RR and IR spectra are responding to the same protonation process. Our data are fit better with a titration curve having an inflection at pK_a = 4.3 rather than 4.5, but the difference is within the experimental error. We choose 4.4 as the best value since it

† This work was supported by NIH Grant GM 33576.

* Author to whom correspondence should be addressed.

‡ Present address: Abteilung Biophysikalische Chemie, Biozentrum, CH-4056 Basel, Switzerland.

0006-2960/89/0428-3125$01.50/0

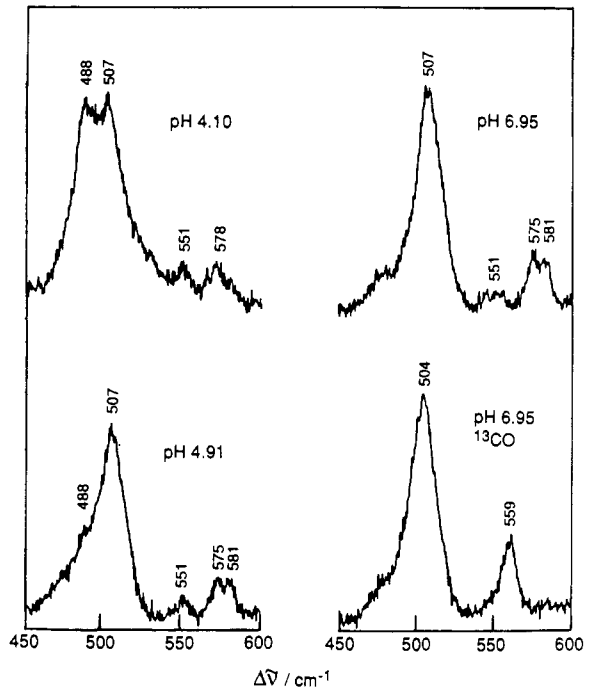

FIGURE 1: RR spectra with 413.1-nm Kr^+ laser excitation of MbCO (1 mM) at the indicated pH values. The last panel is of a sample made with ^{13}CO. Sperm whale Mb (Sigma) was dissolved in deaerated 0.1 M acetate buffer at the desired pH, centrifuged, deoxygenated for 30 min by stirring beneath an Ar stream, reduced with sodium dithionite (4-fold excess), and equilibrated by stirring under a stream of CO for 15 min. [^{13}CO was admitted to a vessel containing the reduced solution (free volume ~100 mL), which was stirred for 15 min.] Spectra were obtained in backscattering from spinning NMR tubes with a Spex 1401 double monochromator equipped with a cooled photomultiplier (RCA) and photon-counting electronics.

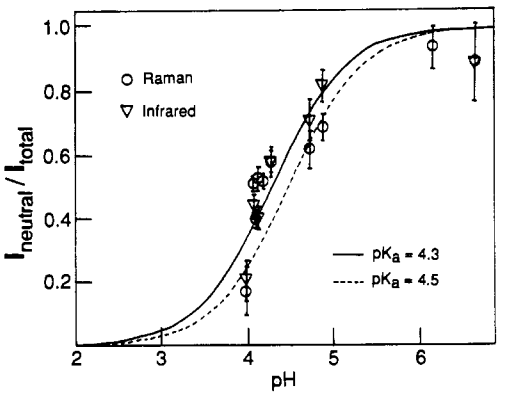

FIGURE 2: pH titration curve obtained by plotting the peak height of the neutral form of MbCO divided by the sum of the peak heights of the neutral and low pH forms using the ν(Fe–C) RR peaks (○) and ν(C–O) IR peaks (∇). Error bars were calculated from the uncertainty in the peak height readings due to the noise. The IR data were obtained on the same solutions contained in a 0.1-mm cell with CaF_2 windows by using a Digilab FTIR spectrometer. Lines are drawn for theoretical one-proton titration curves with pK_a's of 4.3 and 4.5.

corresponds to the pK_a of what is likely to be the distal histidine (see below) as reported by Wilbur and Allerhand (1977).

Table I lists the ν(Fe–C) and ν(C–O) frequencies for the neutral and low pH forms of MbCO along with those of suitable model complexes. All of these species obey the same inverse correlation between ν(Fe–C) and ν(C–O) that has been found experimentally to describe most of the available data on heme protein and model porphyrin CO adducts with imidazole as the sixth ligand (Li & Spiro, 1988; Kerr & Yu, 1988; Uno et al., 1987; Tsubaki et al., 1986; Paul et al., 1985). This inverse relationship is explained by the back-donation

Table I: Protein and Analogue CO Adduct Vibrational Frequencies (cm^{-1})

species	ν(C–O)	ν(Fe–C)	ν(Fe–C)$_{calcd}$[a]	ref
Mb neutral pH	1947	507	505	*b*
Mb low pH	1967	488	490	*b*
FeSP-15 (N-MeIm)[c]	1945	506	506	*d*
FePPDME (ImH)[e]	1960	495	495	*f*

[a] Calculated from the observed ν(C–O) by using eq 1. [b] This work and Fuchsman and Appleby (1979). [c] Heme with intermediate length "strap", *N*-methylimidazole complex in benzene/methylene chloride (Yu et al., 1983). [d] Yu et al. (1983). [e] Iron(II) protoporphyrin dimethyl ester, imidazole complex in methylene chloride (Evangelista-Kirkup et al., 1986). [f] Evangelista-Kirkup et al. (1986).

model of FeCO bonding, in which electronic influences that increase the donation of Fe dπ electrons to the CO π^* antibonding orbitals increase the FeC bond order but decrease the CO bond order, by proportional amounts. The equation describing the linear relation given by Li and Spiro (1988) is

$$\nu(\text{Fe–C}) = 1965 - 0.750\nu(\text{C–O}) \quad (\text{cm}^{-1}) \qquad (1)$$

Table I lists the ν(Fe–C) values calculated from the experimental ν(C–O) values using this relationship. The agreement with the observed ν(Fe–C) values is within experimental error. Significant deviations from this relationship result when the sixth ligand is a significantly stronger or weaker donor than imidazole (Li & Spiro, 1988). It can therefore be concluded that the protonation step associated with the RR and IR spectral changes in MbCO does not alter the Fe–proximal imidazole bond.

The frequencies observed for the low pH form of MbCO are similar to those seen for protein-free heme CO adducts with imidazole ligands when dissolved in solvents of low polarity. Table I lists data for the CO, imidazole adduct of Fe(II) protoporphyrin dimethyl ester in methylene chloride (Evangelista-Kirkup et al., 1986). ν(Fe–C) and ν(C–O) are slightly higher and lower, respectively, than the low-pH MbCO frequencies. The ν(C–O) frequency is quite sensitive to the solvent. The lower the solvent polarity, the less the back-bonding since the resulting negative charge on the CO is not stabilized as it is by a polar solvent. ν(C–O) rises to 1969 cm^{-1} (Caughey, 1980) when the protoheme adduct is dissolved in benzene [ν(Fe–C) has not been determined in this solvent]. Thus the low-pH form of MbCO resembles a heme-CO, imidazole complex in an environment with the polarity of benzene.

The neutral form of MbCO has been modeled by Yu et al. (1983) using porphyrins with a covalent transannular strap that inhibits upright binding by CO. They found an increase in ν(Fe–C) and a decrease in ν(C–O) with decreasing strap length (increasing steric hindrance). These changes reflect increased back-donation to CO, which may result from decreased back-bonding to the porphyrin ring when the FeCO unit is tilted (Li & Spiro, 1988). A porphyrin with an intermediate length strap gave frequencies that are within experimental error of those seen in the neutral form of MbCO (Table I). In addition, this species showed significant activation of the δ(Fe–C–O) RR band, as is also seen in the neutral form of MbCO. This band is not activated in unhindered heme-CO adducts, and its intensity has been stressed by Yu et al. (1983) to be an indication of off-axis CO binding in response to steric hindrance.

The crystal structure of MbCO (Kuriyan et al., 1986) shows the oxygen atom of the CO ligand to be significantly displaced from the heme normal due to nonbonded interaction with the distal histidine (His-64) side chain. Although the structure

was refined in terms of a bent FeCO unit, the vibrational data are incompatible with a significant degree of Fe–C–O bending (Yu et al., 1983; Li & Spiro, 1988). It has been argued (Li & Spiro, 1988) that the steric interaction is accommodated by relatively small angular distortions involving tilting of the FeCO unit and buckling of the heme, as well as Fe–C–O bending, with tilting being the dominant contributor. Thus the neutral MbCO frequencies are in accord with expectation for a significant degree of CO tilting due to the nonbonded interaction with the distal histidine (Yu et al., 1983). It may be that the polar character of the imidazole side chain also contributes to the ν(Fe–C) elevation and ν(C–O) depression, even though there is no H-bond to the bound CO (Hanson & Schoenborn, 1981). We note that Fuchsman and Appleby (1979) attributed the ν(C–O) lowering to a donor interaction from the nearby imidazole N atom to the C atom of the bound CO (Maxwell & Caughey, 1976). Such an interaction is unlikely, however, since it would weaken the FeC as well as the CO bond, whereas ν(Fe–C) is accurately predicted by eq 1.

In the low-pH form of MbCO the distal histidine should be protonated. One might have expected the result to be an H-bond to the bound CO. In that case back-bonding would have increased, resulting in a further elevation of ν(Fe–C) and depression in ν(C–O), as is seen in the low-pH form of the CO adduct of horseradish peroxidase (HRP) (Evangelista-Kirkup et al., 1986). Clearly this is not observed for MbCO at low pH. Not only is ν(Fe–C) low and ν(C–O) high but δ(Fe–C–O) is not activated, as it is for HRP at low pH. We infer that the distal histidine no longer restricts the CO in the low-pH form. A useful model for this form may be the Mb "doorstop" structure (Ringe et al., 1984), in which a bulky phenyl ligand forces the distal histidine out of the heme pocket and into the solvent. Protonating the distal histidine might induce a similar displacement because of electrostatic repulsion with a nearby arginine residue (Arg-45). We note that Arg-45 displays conformational flexibility; in the MbCO crystal structure two alternative conformations were found for the Arg-45 side chain (Kuriyan et al., 1986). Moreover, the His-64 side chain, although refined in a single conformation, had unusually high thermal parameters. Protonation might reasonably be expected to induce movement of this side chain out of the heme pocket. With the His-64 side chain out of the way, the remaining residues in contact with the bound CO would all be hydrophobic, consistent with the nonpolar environment indicated by the ν(Fe–C), ν(C–O) frequencies.

Doster et al. (1982) observed that the rate of CO binding to Mb increases when the pH is lowered from neutrality, with an inflection at pH 5.7. They suggested this transition to result from His-64 protonation. Wilbur and Allerhand (1977), however, assigned ^{13}C NMR resonances titrating with pK_a's of 4.4 and 4.6 to His-64 and -93. If His-64 were protonated at pH values between 5.7 and 4.5, the ν(C–O) and ν(Fe–C) bands should have been perturbed, contrary to observation, since the imidazole N atom is next to the bound CO. Thus the spectroscopic evidence points strongly to a pK_a of 4.4 for His-64. Consequently, the pK_a = 5.7 protonation that modulates the CO rebinding kinetics must involve a more distant site. Interestingly, Fuchsman and Appleby (1979) found the same pK_a for a change in the MbCO optical spectrum, implying a change in the heme environment. Gurd and coworkers (Botelho et al., 1978; Botelho & Gurd, 1978) assigned proton NMR resonances to several Mb histidine residues titrating between pH 5 and pH 9. His-113 and -119 had pK_a values of 5.5 and may be responsible for the process that influences the rebinding and the optical spectrum. These residues are on the H helix and the GH corner (Takano, 1977a,b) and are remote from the heme. We suggest that their protonation may induce a global change in the protein structure which alters the heme environment.

Additional complexity regarding the factors controlling the FeCO geometry is indicated by other results from vibrational spectroscopy. Thus Brown et al. (1983) found that dehydration of MbCO shifts ν(C–O) to 1967 cm^{-1}, suggesting upright FeCO. Champion and co-workers have observed partial conversion of the ν(Fe–C) band from 507 to 490 cm^{-1} when MbCO solutions are frozen (Reinisch et al., 1983) or in MbCO single crystals (Morikis et al., 1988). This last observation is particularly striking since it implies that there are alternative dispositions of the His-64 side chain in the crystal even though the X-ray diffraction data refines to a single conformation (Kuriyan et al., 1986). The high temperature factors suggest, however, that a disposition away from the bound CO may be accessible under some conditions of crystallization. Perhaps the water activity is a critical variable in view of the dehydration results of Brown et al. (1983). It is also possible that factors other than the His-64 disposition influence the FeCO geometry.

Added in Proof

Prof. Paul Champion has kindly sent us a manuscript (Morikis et al., 1989), which describes extensive studies of the ν(Fe–C) RR band and the ν(C–O) IR band of MbCO as a function of pH and of distal histidine replacement by site-directed mutagenesis. The results are consistent with those we report here but are more extensive. Evidence is reported for minority populations of "closed" and "open" conformations (tilted and upright in our nomenclature) at low and high pH, and separate pK_a's, 3.8 and 6.0, are estimated for them (our pK_a of 4.4 would represent a weighted average in this four-state model).

Acknowledgments

We are grateful to Dr. R. Czernuszewicz for help with the figures.

References

Botelho, L. H., & Gurd, F. R. N. (1978) *Biochemistry 17*, 5188.

Botelho, L. H., Friend, S. H., Matthew, J. B., Lehmann, L. D., Hanania, G. I. H., & Gurd, F. R. N. (1978) *Biochemistry 17*, 5197–5205.

Braunstein, D., Ansari, A., Berendzen, J., Cowen, B. R., Egeberg, K. D., Frauenfelder, H., Hong, M. K., Ormos, P., Sauke, T. B., Scholl, R., Schulte, A., Sligar, S. G., Springer, B. A., Steinbach, P. J., & Young, R. D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Brown, W. E., Sutcliffe, J. W., & Pusinelli, P. D. (1983) *Biochemistry 22*, 2914–2923.

Caughey, W. S. (1980) in *Methods for Determining Metal Ion Environment in Proteins: Structure and Function of Metalloproteins* (Darnall, D. W., & Wilkins, R. G., Eds.) pp 95–115, Elsevier North-Holland, New York.

Choi, S., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 3683–3692.

Doster, W., Beece, D., Bowne, S. F., Dilorio, E. E., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., Winterhalter, K. H., & Yue, K. T. (1982) *Biochemistry 21*, 4831–4839.

Evangelista-Kirkup, R., Smulevich, G., & Spiro, T. G. (1986) *Biochemistry 25*, 4420.

Fuchsman, W. H., & Appleby, C. A. (1979) *Biochemistry 18*, 1309-1321.
Hanson, J. C., & Schoenborn, B. P. (1981) *J. Mol. Biol. 153*, 117-146.
Kerr, E. A., & Yu, N.-T. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. III, Chapter 2, Wiley Interscience, New York.
Kuriyan, J., Wilz, S., Karplus, M., & Petsko, G. A. (1986) *J. Mol. Biol. 192*, 133-154.
Li, X.-Y., & Spiro, T. G. (1988) *J. Am. Chem. Soc. 110*, 6024.
Maxwell, J. C., & Caughey, W. S. (1976) *Biochemistry 15*, 388.
Moore, J. N., Hansen, P. A., & Hochstrasser, R. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Morikis, D., Sage, T. J., Rizos, A. K., & Champion, P. M. (1988) *J. Am. Chem. Soc. 110*, 6341-6342.
Morikis, D., Champion, P. M., Springer, B. A., & Sligar, S. G. (1989) *Biochemistry* (in press).
Olson, J. S. Mathews, A. J., Rohlfs, R. J., Springer, B. A., Egeberg, K. D., Sligar, S. G., Tame, J., Renaud, J.-P., & Nagai, K. (1988) *Nature 336*, 265-266.
Paul, J., Smith, M. L., & Paul, K.-G. (1985) *Biochim. Biophys. Acta 832*, 257.
Reinisch, L., Srajer, V., & Champion, P. M. (1987) *Bull. Phys. Soc. 33*, 1412.
Ringe, D., Petsko, G. A., Kerr, D., & Ortiz de Montellano, P. R. (1984) *Biochemistry 23*, 2-4.
Takano, T. (1977a) *J. Mol. Biol. 110*, 537-568.
Takano, T. (1977b) *J. Mol. Biol. 110*, 569-584.
Tsubaki, M., Srivatsa, R. B., & Yu, N.-T. (1982) *Biochemistry 21*, 1132-1144.
Tsubaki, M., Hiwatashi, A., & Ichikawa, Y. (1986) *Biochemistry 25*, 3563.
Uno, T., Nishimura, Y., Tsuboi, M., Makino, R., Iizuka, T., & Ishimura, Y. (1987) *J. Biol. Chem. 262*, 4549.
Wilbur, D. J., & Allerhand, A. (1977) *J. Biol. Chem. 252*, 4968.
Yu, N.-T., Kerr, E. A., Ward, B., & Chang, C. K. (1983) *Biochemistry 22*, 4534-4540.

Cytochrome *c* Oxidase: Evidence for Interaction of Water Molecules with Cytochrome *a*

Massimo Sassaroli,[‡] Yuan-chin Ching, Siddharth Dasgupta,[§] and Denis L. Rousseau*
AT&T Bell Laboratories, Murray Hill, New Jersey 07974
Received December 29, 1988; Revised Manuscript Received February 17, 1989

ABSTRACT: The resonance Raman spectra of cytochrome *c* oxidase in protonated buffer compared to that in deuterated buffer indicate that water molecules are near the heme of cytochrome *a*. Differences in widths of the heme line at 1610 cm^{-1}, after short exposure to D_2O, and, additionally, of the heme line at 1625 cm^{-1}, after long exposure, can be accounted for by changes in resonance vibrational energy transfer between modes of cytochrome a^{2+} and the bending mode of water molecules in the heme pocket. On the basis of the assignment of these modes, we place one water molecule near the vinyl group and one water molecule near the formyl group of the cytochrome *a* heme. These water molecules may play several possible functional roles.

Cytochrome *c* oxidase is the terminal enzyme in the electron transport chain and serves as the catalytic site for the reduction of O_2 to H_2O (Wikström et al., 1981). Each monomeric unit contains two heme groups (cytochrome *a* and cytochrome a_3) and at least two copper atoms. Cytochrome a_3 and an associated copper atom (Cu_B) form the dioxygen binding site, whereas cytochrome *a* and Cu_A are the primary electron-acceptor sites from cytochrome *c*. An additional function of the multicenter enzyme is to translocate protons across the inner mitochondrial membrane. Despite the fact that this is now well established (Wikström et al., 1981; Wikström & Krab, 1979; Wikström, 1984; Thelen et al., 1985; Puettner et al., 1985; Sarti et al., 1985), the mechanism of the translocation activity is not yet understood and it is even uncertain as to which centers are involved, although some specific hypotheses have been proposed (Babcock & Callahan, 1983; Gelles et al., 1986).

To illucidate the key features of proton translocation, we have studied the effect of deuterated buffers on the enzyme by monitoring the resonance Raman spectra of the heme groups (Argade et al., 1986b). This technique identifies the vibrational modes of the heme that are sensitive to deuteration. This sensitivity may result from exchange of atoms on the heme or from vibrational coupling between heme atoms and exchangeable atoms on nearby groups. The effect of deuteration on the heme modes has been reported earlier, although neither the data nor their interpretation was in agreement (Argade et al., 1986b; Copeland & Spiro, 1986). We have now made a series of high signal-to-noise resonance Raman scattering measurements and carried out a detailed analysis to determine accurately the frequencies and widths of the deuteration sensitive lines. The results indicate that spectral lines corresponding to heme vibrational modes of cytochrome a^{2+} are broader in buffered H_2O than in buffered D_2O. On the basis of a model of vibrational coupling, we conclude from these new observations that water molecules are present near

* To whom correspondence should be addressed.
‡ Present address: Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY 10029.
§ Present address: Department of Chemistry, California Institute of Technology, Pasadena, CA.

0006-2960/89/0428-3128$01.50/0