brief communication

Low pH myoglobin photoproducts

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ABSTRACT Recently, there has been interest in determining the conditions under which the iron-histidine bond ruptures in myoglobin at low pH, so that the effect of proximal heme ligation can be studied. A 220-cm^{-1} Raman mode, assigned to iron-histidine stretching, is clearly visible after photolysis of aqueous MbCO samples below pH4 at room temperature (Sage et al. *Biochemistry.* 30:1237–1247). In contrast, Iben et al. (*Biophys. J.* 59:908–919) do not observe this mode upon photolysis of a pH3 MbCO sample in a glycerol/water glass at low temperature. In order to account for both the low temperature and the room temperature experiments, Iben et al. suggest a scheme involving an unusual protonation state of the proximal histidine. Here, we discuss some inconsistencies in their explanation of the room temperature results and offer instead a simple modification of an earlier model. In addition, circular dichroism data are presented that indicate partial unfolding of MbCO in aqueous solution below pH4, and raise questions about the claim of Iben et al. that MbCO remains folded in 75% glycerol at pH3.

INTRODUCTION

The process by which small ligands migrate through the protein matrix and bind to the active site of heme proteins has been the subject of intense scientific investigation. Much remains unknown, but progress has been made in understanding the structural features on the proximal and distal sides of the heme that control the final binding step at the heme iron. It is well known that ligand binding induces a conformational change of the heme from a "domed" structure with the iron displaced from the mean plane of the porphyrin to a more planar conformation with the iron nearer the heme plane, and it is likely that this motion makes a significant contribution to the energetic barrier to ligand binding (1). In addition, ligand binding requires a spin transition of the iron that may play an important role in controlling reaction rates.

One way to address the roles of heme doming and iron spin experimentally is to study ligand binding to four-coordinate hemes, which contain an $S = 1$ iron in a planar heme. Evidence for enhanced binding kinetics to four-coordinate heme comes from studies of model compounds in which the proximal ligand is reversibly removed by protonation at low pH (2, 3). Analogous experiments in heme proteins must take into account the fact that substantial conformational changes at reduced pH, due to electrostatic destabilization of the native structure, may also affect ligand binding kinetics. Thus, previous kinetic investigations of myoglobin (4, 5) at low pH have employed rapid mixing techniques in an attempt to kinetically stabilize a species in which (*a*) the proximal bond is broken due to protonation of the histidine, but (*b*) the protein retains its native conformation. The attribution of enhanced CO binding kinetics observed under these conditions to heme tetra-coordination remains uncertain, however, because resonance Raman data recorded using a similar rapid mixing protocol (6) contain a 1357-cm^{-1} peak characteristic of five-coordinate reduced hemes. We have also argued (7) that protein conformational changes probably occur within the mixing time and may affect the results of pH jump kinetics measurements.

In principle, flash photolysis is a preferable method for studying ligand rebinding to four-coordinate hemes, because it should allow observation of the geminate phase. However, although MbCO is partially unfolded below pH4 (8) (also see below), the iron-histidine bond remains intact (6, 7, 8, 9), and rebinding to a four-coordinate heme can only be observed if the proximal bond ruptures before CO rebinding. Two recent studies (7, 9) have addressed this issue but arrived at apparently contradictory conclusions: at room temperature, the 220-cm^{-1} mode associated with iron-histidine stretching is clearly present in the 10-ns photoproduct spectrum (7), while the absence of this mode in the spectrum of photolyzed MbCO in a glycerol/water glass at 100-K has been interpreted as evidence for rupture (or weakening) of the iron-histidine bond within 30 ps of photolysis (9). The purpose of this paper is to try to reconcile these apparently contradictory observations. We point out some inconsistencies in a previous attempt (9) to explain both high and low temperature observations and suggest a simple modification of the scheme originally proposed

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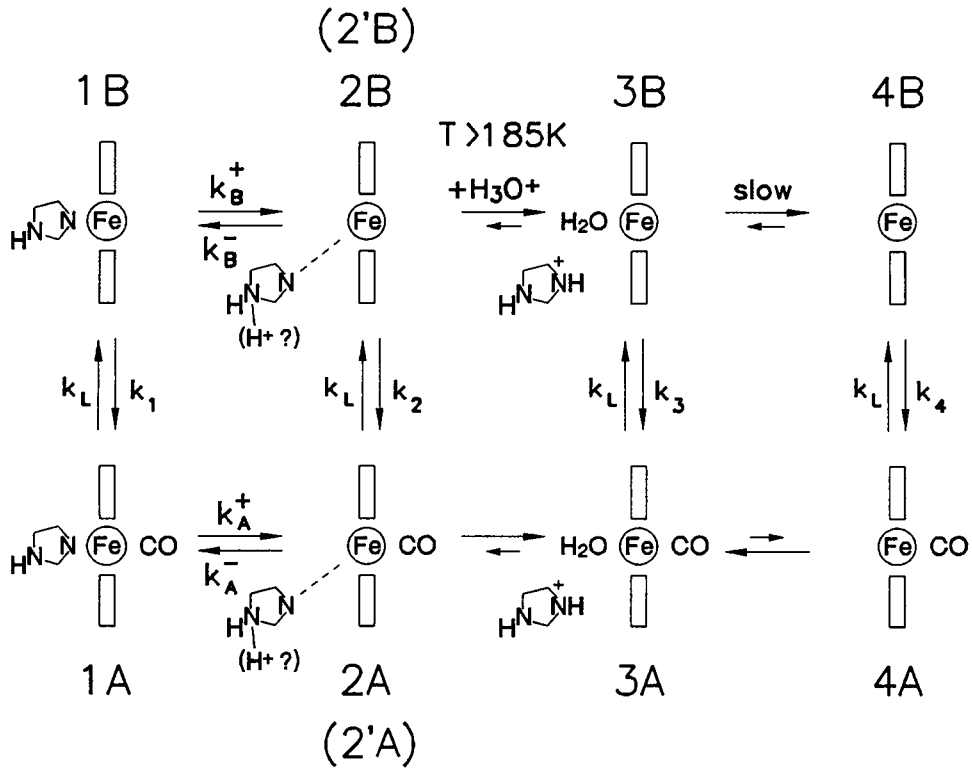
to describe the room temperature measurements (7) that is also consistent with the low temperature observations (see Scheme 1).



The assignment of proximal ligation in the room temperature studies (7, 8) is based on the observation of characteristic Raman frequencies in both photolyzed and CO-bound states. Thus, the spectrum of a low pH MbCO sample, partially photolyzed and probed with 10-ns pulses in a spinning cell, shows a 220-cm^{-1} iron-histidine mode associated with a histidine-ligated photoproduct (species 1B). The iron-histidine mode is not directly observed (10) in the CO-bound state (species 1A), but the Fe-CO stretching frequency at 491 cm^{-1} is characteristic of a sterically unhindered CO bound *trans* to a nitrogenous ligand (11). Under extended illumination with a continuous wave laser, the iron-histidine mode disappears on a millisecond time scale, producing a species (3B) that retains a characteristic high spin ferrous ν_4 band at 1357 cm^{-1} and is clearly distinct from the four-coordinate deoxyMb species (4B) observed in equilibrium below pH4 (7, 8). A species similar to 3B, observed after a rapid pH drop in deoxyMb, was attributed to a five-coordinate ferrous heme in which the histidine is replaced by a weak ligand, possibly water (6). The growth of an Fe-CO peak at 526 cm^{-1} in the bound fraction of the low pH sample under extended illumination at room temperature is also consistent with increased population of a species (3A) with a weak ligand bound *trans* to the CO. In order to explain the ultrafast ($\gtrsim 10^{10}$ s^{-1}) CO rebinding rate deduced from the difficulty in photolyzing MbCO below pH4, we postulated (7) the existence of a rapidly-rebinding minority four-coordinate deoxyMb species (2B) in fast exchange with 1B, which allows fast rebinding through the channel 1B → 2B → 2A → 1A.

Photolysis of Mb at low temperatures produces a species with no iron-histidine mode but with a typical five-coordinate high spin frequency ν_4 = 1357 cm^{-1} (9), similar to the species 3B produced under extended illumination at room temperature. Since it seems unlikely that significant rearrangements of the proximal ligand and protein conformation occur at low temperature (i.e., 2B → 3B probably does not proceed below ~185 K), Iben et al. (9) proposed a different scheme to describe their results. This scheme invokes an unusual situation in which histidine remains bound to the iron in MbCO, but the δ-nitrogen of the imidazole becomes doubly protonated at low pH (species 2'A). Upon photolysis, Iben et al. (9) suggest that either (*a*) the iron-histidine bond breaks, but the proximity of the charged histidine pushed the iron out of the heme plane toward the distal side and the iron remains high spin or (*b*) the bond remains intact, but the presence of the extra proton on N_δ leads to a drastic change in either the

frequency or the coupling strength of the iron-histidine mode, such that it does not appear with appreciable intensity in the observed frequency range. To our knowledge, the existence of either a doubly-protonated histidine nitrogen or a high spin four-coordinate heme is unprecedented, and this scheme must be regarded as speculative. Nevertheless, it does provide a consistent description of the low temperature measurements (9).

Iben et al. (9) also make an ingenious attempt to describe the room temperature results within the same scheme. According to their scenario, the original sample is predominantly in the protonated form 2'A, with a small admixture of 1A (i.e., $k_A^+ \gg k_A^-$ at pH3). Upon photolysis, a substantial fraction of CO will escape to the solvent from the slowly rebinding minority photoproduct 1B, while the protonated photoproduct 2'B will rebind CO before it has a chance to escape from the vicinity of the heme. After reestablishment of the equilibrium between 1A and 2'A, continued excitation of the heme will supposedly cause population to build up in the unprotonated photoproduct state 1B, thus giving rise to the iron-histidine mode observed in the room temperature experiments (8).

There appear to be two problems with this interpretation of the room temperature measurements. First, since 1B is observed during photolysis with 10-ns pulses at room temperature (7), CO escape to the solvent, which takes ~200 ns at pH7 (12), is probably not a relevant factor. (Since the sample is in a spinning cell, repetititve pulsed excitation of the same sample volume is also not an issue.) A more fundamental point is that reequilibration of 1A and 2'A within the 10-ns pulse width requires $k_A^+ + k_A^- \gg (10\ \text{ns})^{-1}$, while the existence of an observable population of 1B requires $k_B^+ < (10\ \text{ns})^{-1}$. Since the assumed initial conditions require $k_A^+ \gg k_A^-$, it follows that $k_A^+ \gg k_B^+$. It is not clear to us why the rate of protonation of N_δ would be so strongly affected by the presence of CO bound *trans* to the histidine. If anything, the π-acceptor nature of the CO ligand should reduce the electron density on N_δ and make protonation less likely in the CO-bound state.

However, it may be possible to reconcile the low temperature and room temperature measurements by using a combination of the two schemes (7, 9) that retains the notion that species 2B is a high spin deoxy heme with an unobservable iron-histidine mode. In this scheme, experimental variables such as pH, temperature, and the presence of glycerol are likely to alter the equilibrium between 1A and 2A and thus influence the intensity of the iron-histidine mode observed in the photoproduct. For example, the measurements of Sage et al. (7) were carried out in aqueous solution at $T = 300$ K and pH3.6, while those of Iben et al. (9) were performed using a glycerol cosolvent at $T = 100$ K and pH3. Since the relative intensity of the photoproduct iron-histidine mode at room temperature is noticeably reduced at pH2.4 relative to pH3.6 (7), the pH difference (along with thermal and solvent effects) may contribute to the differences between the room temperature and the cryogenic observations. It should be noted that at least one of the reported low temperature spectra (Fig. 3 *c* of reference 9) does show a weak peak near the frequency expected for the iron-histidine mode at 100 K. In addition, we note that the relative populations of photoproducts with (1B) and without (2B) the iron-histidine mode may be altered by the temperature dependence of the geminate recombination rate k_2 (Scheme 1). For example, the photoproduct 2B will contribute to the 100 K spectrum if $k_2 < k_L$, but an increase of this rate with temperature can lead to the conditions $k_2 \gg k_L$ at 300 K, with a significantly reduced population of 2B.[1]

Although Scheme 1 is reasonably consistent with the available experimental data (7, 9), several kinetic and spectroscopic observations suggest that caution is necessary in the assignment of the proximal ligation state in 2A and 2B. Since the enhanced geminate rates observed experimentally (9) suggest a weak or absent proximal ligand, the Fe-CO stretching frequency of 2A is expected to be in the range 525–530 cm^{-1} (11). However, the low temperature Raman spectrum shows only the 491 cm^{-1} Fe-CO peak usually associated with strong histidine ligation. Similar ambiguities persist in the interpretation of rapid-mixing measurements, where the deoxyMb species present after a rapid pH drop was originally assigned as four-coordinate on the basis of absorption measurements (5) and more recently assigned as five-coordinate on the basis of Raman measurements (6). Further progress in this area will require additional model compound studies in order to clarify the relationship between spectroscopic observables and the iron coordination. In particular, the uncertainties surrounding proximal ligation in states 2A and 2B must be resolved before kinetic investigations of CO binding to myoglobin below pH4 can be interpreted with confidence.

Finally, we are compelled to point out that a meaningful analysis of any low pH study must be predicated on knowledge of the protein conformation, because it is well known (13, 14) that there is a substantial loss of α-helical structure in metmyoglobin below pH4. In aqueous solutions, the MbCO Soret band undergoes only a 1–2-nm blueshift below pH4, in contrast to the

[1]For example an Arrhenius barrier height of ~6 kcal/mol will lead to a factor of 100 change in k_2 between 100 and 300 K. Since myoglobin at pH7 is at least 90% photolyzed at $T = 100$ K (9) as well as at 300 K (7), we expect that $k_1 \ll k_L$ at both temperatures.

dramatic changes seen for metMb and deoxyMb (8). Nevertheless, the global protein structure is drastically altered for MbCO as well as for metMb in aqueous solution at low pH. This conclusion is based on the intensity of the circular dichroism (CD) feature at 220 nm, which provides a semi-quantitative measure of the α-helical content (see Fig. 1). In contrast, Iben et al. (9) suggest that "the presence of glycerol serves to stabilize MbCO in its folded structure" at pH3, apparently based on the lack of drastic changes in the MbCO absorption spectrum upon lowering the pH. However, one of the principal points of our earlier work (8) is that the heme absorption of MbCO is not a sensitive indicator of the global protein structure. Thus, the claim (9) that the globin remains folded in 75% glycerol at pH3 is unfounded in the absence of direct evidence of the secondary structure. This issue is particularly acute in cryogenic glycerol samples, because low temperatures may lead to further changes in global conformation. For instance, "cold denaturation" of myoglobin can be observed even near 270 K when the native state is destabilized at low pH (14). The conformation of myoglobin at pH3 at the glass transition temperature of a glycerol/water solvent (< 200 K) is difficult to predict with any certainty.

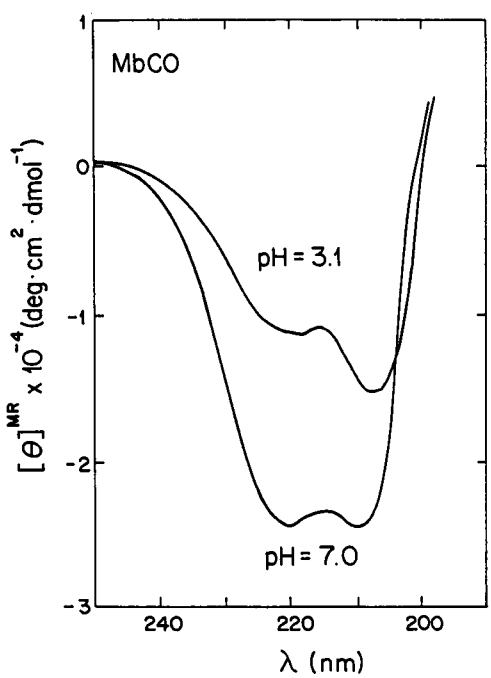


FIGURE 1 Circular dichroism spectra of MbCO at pH7 and 3.1, recorded on a Jasco Inc. J-500 spectropolarimeter (Easton, MD). Visible absorption spectra of both samples have a Soret peak at 420 nm. Samples contain 1 μM concentrations of protein in 0.1 M potassium phosphate buffer.

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REFERENCES

1. Šrajer , V., L. Reinisch, and P. M. Champion. 1988. Distributed coupling and the binding of ligands to heme proteins. *J. Am. Chem. Soc.* 110:6656–6670.
2. Cannon, J., J. Geibel, M. Whipple, and T. G. Traylor. 1976. Dual pathways of heme protein model compound reactions with carbon monoxide. *J. Am. Chem. Soc.* 98:3395–3396.
3. Geibel, J., C. K. Chang, and T. G. Traylor. 1975. Coordination of myoglobin active site models in aqueous solution as studied by kinetic methods. *J. Am. Chem. Soc.* 98:5924–5926.
4. Giacometti, G. M., T. G. Traylor, P. Ascenzi, M. Brunori, and E. Antonini. 1977. Reactivity of ferrous myoglobin at low pH. *J. Biol. Chem.* 252:7447–7448.
5. Coletta, M., P. Ascenzi, T. G. Traylor, and M. Brunori. 1985. Kinetics of carbon monoxide binding to monomeric hemoproteins: role of the proximal histidine. *J. Biol. Chem.* 260:4151–4155.
6. Han, S. H., D. L. Rousseau, G. Giacometti, and M. Brunori. 1990. Metastable intermediates in myoglobin at low pH. *Proc. Natl. Acad. Sci. USA.* 87:205–209.
7. Sage, J. T., P. Li, and P. M. Champion. 1991. Spectroscopic studies of myoglobin at low pH: heme ligation kinetics. *Biochemistry.* 30:1237–1247.
8. Sage, J. T., D. Morikis, and P. M. Champion. 1991. Spectroscopic studies of myoglobin at low pH: heme structure and ligation. *Biochemistry.* 30:1227–1237.
9. Iben, I. E. T., B. R. Cowen, R. Sanches, and J. M. Friedman. 1991. CarboxyMb at pH3. Time-resolved resonance Raman study at cryogenic temperatures. *Biophys. J.* 59:908–919.
10. Wells, A. V., J. T. Sage, D. Morikis, P. M. Champion, B. Springer, and S. G. Sligar. 1991. The iron-histidine mode of myoglobin revisited: resonance Raman studies of isotopically labelled E. coli expressed myoglobin. *J. Am. Chem. Soc.* 113:9655–9660.
11. Yu, N.-T., and E. A. Kerr. 1988. Applications of Raman Spectroscopy to Biological Systems, Vol. 3. T. G. Spiro, editor. John Wiley and Sons, New York. 39–95.
12. Henry, E. R., J. H. Sommer, J. Hofrichter, and W. A. Eaton. 1983. Geminate recombination of carbon monoxide to myoglobin. *J. Mol. Biol.* 166:443–351.
13. Hermans, J., Jr., D. Puett, and G. Acampora. 1969. On the conformation of denatured proteins. *Biochemistry.* 8:22–30.
14. Privalov, P. L., Yu. V. Griko, S. Yu. Venyaminov, and V. P. Kutyshenko. 1986. Cold denaturation of myoglobin. *J. Mol. Biol.* 190:487–498.