Acid-Induced Transformations of Myoglobin. Characterization of a New Equilibrium Heme-Pocket Intermediate†

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ABSTRACT: The pH dependence of the absorption and resonance Raman (RR) spectra of the deoxy and met forms of myoglobin (Mb) has been examined in detail. The spectral data were acquired at a number of different pHs (12) in the 2.6−7.6 range. RR spectra were obtained for both the low- and high-frequency regions by using a variety of excitation wavelengths ranging from the UV to the green. The data obtained for deoxyMb indicate that a spectroscopically distinct intermediate (I′) exists at equilibrium in the pH 3.5−4.5 range. The I′-form of metMb could not be identified. The Soret absorption maximum of the I′-form of deoxyMb is at ~426 nm compared with the value of 435 observed for the native (N) form and 383 nm observed for the so-called unfolded (U′) form which occurs in the pH 2.6−3.5 range. The absorption and vibrational spectra of the I′-form of deoxyMb observed at equilibrium are very similar to those of the intermediate that appears within a few milliseconds in pH-jump experiments. The RR data indicate that the structure of the heme group in the I′-form is distinctly different from that of either N- or U′-forms. The iron−histidine bond, characteristic of the N-form, is ruptured in both the I′- and U′-forms as is evidenced by the absence of the RR band due to the stretching vibration of this unit. In the I′-form, the histidine ligand is replaced by a relatively strongly bound, exchangeable water molecule. This ligand is absent in the U′-form. The aquo ligand of the five-coordinate heme in the I′-form is identified by a RR band at 411 cm^{-1} which undergoes a 15−17 cm^{-1} downshift in deuteriated buffer solutions. In contrast, none of the RR bands of the N- and U′-forms exhibit any significant isotope sensitivity. The properties of the I′-form and the conditions under which it is generated strongly suggest that this form corresponds to the molten globule intermediate of apoMb.

The acid-induced unfolding/refolding of myoglobin (Mb)[1] and its derivatives has been extensively investigated. Fluorescence, circular dichroism (CD), nuclear magnetic resonance (NMR), viscosimetric, calorimetric, and electrometric titrations on holo- and/or apoMb indicate that the acid-induced transformation from the native (N) form to the unfolded (U) form proceeds through an intermediate (Kirby & Steiner, 1970; Bismuto et al., 1983; Irace et al., 1986; Privalov et al., 1986; Griko et al., 1988; Hughson & Baldwin, 1989; Hughson et al., 1990, 1991; Goto & Fink, 1990; Barrick & Baldwin, 1993; Jennings & Wright, 1993; Shin et al., 1993a,b; Waltho et al., 1993; Griko & Privalov, 1994). The N-forms of holo- and apoMb, observed under neutral and mildly acid conditions (pH 4.5−7.0), are characterized by ~80% and ~55% α-helix content, respectively. These helicities are indicative of a highly compact structure with an extended hydrophobic interior. In contrast, the U-forms, observed under highly acidic conditions (pH <2.0), exhibit only a small residual α-helix content and high intrinsic viscosity (Griko et al., 1988). Together, these observations indicate that the very low pH U-forms have a predominantly random-coil conformation. The heme group of holoMb is also dissociated from the protein at very low pH (Antonini & Brunori, 1971).

In the transition region between the N- and U-forms (pH 2.5−4.5), Mb exhibits more complicated behavior. The detailed features of the pH titration curves of the holo- and apoproteins also differ in the transition region. In the pH 2.5−3.5 range, apoMb exhibits properties characteristic of the U-form. In contrast, a well-defined intermediate (I) form is observed in the pH 3.5−4.5 range (Hughson et al., 1990). This form exhibits a relatively high helicity, approximately 60−65% of the α-helix content of the N-form of the apoprotein. However, the amide groups of the I-form are relatively poorly protected compared with those of the N-form (Hughson et al., 1990; Jennings & Wright, 1993; Shin et al., 1993a,b; Waltho et al., 1993). Together, these observations indicate that the I-form assumes a somewhat open structure with reduced hydrophobicity in the interior. This open structure arises from substantial unfolding of the B and E helices. In contrast, the A, G, and H helices appear to remain folded in an N-like conformation. No information is available on the F helix which contains the proximal histidine (F8) residue that serves as the only covalent link between the heme group and the protein. It has been proposed that the I-form of apoMb observed at equilibrium corresponds to the molten globule intermediate (Hughson

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[1] Abbreviations: CCD, charge-coupled device; CD, circular dichroism; I, intermediate form of apomyoglobin characterized in the pH 3.5−4.5 range via circular dichroism and nuclear magnetic resonance studies; I′, intermediate form of deoxymyoglobin characterized in the pH 3.5−4.5 range via absorption and resonance Raman studies; Mb, myoglobin; N, native form of myoglobin; U, unfolded form of myoglobin that occurs below pH 2.0; U′, unfolded form of myoglobin characterized in the pH 2.6−3.5 range via absorption, circular dichroism, and resonance Raman studies.

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et al., 1991). Recent kinetic studies have shown that a transient species with properties similar to those of the I-form exists on the folding pathway of the protein (Jennings & Wright, 1993).

The structural properties of holoMb in the transition pH region are not as well characterized as those of the apoprotein. CD studies have shown that the holoprotein loses ~50% of its α-helix content as the pH is decreased from 5.0 to 4.0 (Bismuto et al., 1983; Irace et al., 1986). The residual helicity, which is indicative of a partially folded structure, is retained at pH 3.5, where the apoprotein exhibits characteristics of the U-form. The absorption spectrum of the heme group of holoMb also tracks the transformation between the N- and U-forms (Acampora & Hermans, 1967; Puett, 1973; Bismuto et al., 1983; Irace et al., 1986; Privalov et al., 1986; Sage et al., 1991). As the pH is lowered, the Soret maximum blue shifts. At very low pH (<2.0), the spectrum is characteristic of an aggregated heme in aqueous solution. Opinions are mixed concerning the appropriate description of the absorption spectrum in the transition-pH region. Most workers have described the absorption spectrum observed below pH 3.5 as being characteristic of an aggregated heme, identical to that of the U-form. On the other hand, Champion and co-workers (Sage et al., 1991) noted that the spectrum observed in the pH 2.6−3.5 range is not actually identical to that of the U-form although it has similar features. These workers also reported variable pH, resonance Raman (RR) studies of holoMb derivatives. The RR features observed in the pH 2.6−3.5 range support the argument that the protein is not in a true U-form. The RR data indicate that the heme is closely associated with the binding pocket in the pH 2.6−3.5 range and exists as a four-coordinate, intermediate spin species. (This in turn implies that the iron−histidine bond is broken in this pH range; however, no direct evidence was obtained in support of this proposal.) These results, together with the fact that holoMb exhibits a large residual helicity in the pH 2.6−3.5 range, led Champion and co-workers to propose that the holoprotein species present in this pH range corresponds to the I-form of the apoprotein (Sage et al., 1991). From here on, the holoprotein species observed in the pH 2.6−3.5 range will be designated the U′-form in order to distinguish it from the U-form.

Time-resolved absorption techniques have also been used to monitor the effect of pH on the properties of the heme group of holoMb (and hemoglobin) (Giacometti et al., 1977; Ascenzi et al., 1981; Coletta et al., 1985, 1988; Han et al., 1990). pH-jump experiments on deoxyMb show that altering the pH from neutral to acid conditions (pH 2.3−3.9) results in absorption changes in the Soret region on a time scale of less than 10 ms. The Soret maximum of the transient form is blue-shifted to 426 nm. This absorption spectrum is distinctly different from that of either the N-form (λ_{max} = 435 nm) or the U′-form (λ_{max} = 383 nm) observed under static conditions. From here on, the low-pH transient intermediate will be designated the I′-form. The ligand-binding affinity of the I′-form is different from that of the N-form. The I′-form exhibits significantly enhanced affinities for carbon monoxide and nitric oxide (Ascenzi et al., 1981; Coletta et al., 1988). These enhanced affinities were suggested to be due to the formation of a four-coordinate heme resulting from cleavage of the iron−histidine bond. The iron ion of four-coordinate hemes is more in-plane than that of five-coordinate species (Scheidt, 1978), and this leads to smaller activation barriers for ligand binding (Geibel et al., 1975; Carman et al., 1976).

Rousseau and co-workers have used time-resolved, pH-jump RR techniques to probe the I′-form of deoxyMb (Han et al., 1990). The RR features of this form are different from those of either the N-form or the U′-form. The time-resolved RR data demonstrate that the iron−histidine bond is cleaved in the I′-form. This is evidenced by the absence of an RR band due to the iron−histidine stretching vibration, ν_{Fe-His}. The breakage of the iron−histidine bond could lead to the formation of a four-coordinate heme as is proposed for the structure of the cofactor in the U′-form (Sage et al., 1991). However, the skeletal-mode frequencies of the I′-form are not consistent with those of a four-coordinate species. Instead, these features are more typical of a five-coordinate heme. As a consequence, Rousseau and co-workers proposed that the histidine ligand of the N-form is replaced by a new axial ligand in the I′-form of deoxyMb (Han et al., 1990). This new ligand was suggested to be water; however, no direct evidence was reported in support of this proposal.

The static and time-resolved absorption and RR data previously reported for Mb at low pH provide insight into the structural characteristics of the heme pocket. These data indicate that at least two distinct heme-pocket intermediates are formed at low pH: the transient I′-form and the equilibrium U′-species. The question remains whether the U′-form occurs on the kinetic unfolding pathway and whether the I′-form exists at equilibrium. In order to explore the latter issue, we undertook a detailed reinvestigation of the effects of pH on the static absorption and RR spectra of deoxy- and metMb. During the course of this investigation, spectral data were acquired at a number of different pHs (12) in the 2.6−7.6 range. RR spectra were obtained in both the low- and high-frequency regions by using a variety of excitation wavelengths ranging from the UV to the green. These studies provide new information concerning the distribution of heme-pocket intermediates present under low-pH equilibrium conditions. They also provide insight into the structures of these intermediates and suggest correlations between these structures and those of the protein as a whole during the acid-induced transformations.

MATERIALS AND METHODS

Sperm whale Mb (Sigma, type II) was purified by standard procedures (Antonini & Brunori, 1971). The Mb samples were prepared by solubilizing metMb in either phosphate (pH 5.5−7.6) or acetate (pH 2.6−5.5) buffer. The protein concentration was ~0.1 mM for the UV−Vis absorption and RR experiments. A concentration of ~0.5 mM was used to obtain spectra of the weak, near-infrared charge-transfer absorption, band III. In the pH 5.5−7.6 range, metMb was added to the phosphate buffer solutions which were previously prepared at the desired pH. In all cases, the pH measured after addition of the protein was found to be identical to that of the original buffer solution. In order to explore the effects of ionic strength, spectral data were acquired at a number of pH values in the 5.5−7.6 range for samples in both 1 mM and 10 mM buffers; data were also acquired at selected pH values in 100 mM buffer. In the pH 2.6−5.5 range, the samples were prepared by first solublizing metMb in either 1 or 10 mM pH 4.4 acetate

buffer and then adjusting the pH to the desired value via slow addition of acid or base. This method was used because addition of the protein directly to very low pH buffer solution resulted in aggregation. Addition of acid or base to the buffered solution alters the final buffer concentration particularly in the very low pH regime. However, the total buffer concentration in the pH region of particular interest (pH 3.5−4.5) falls within the range used to acquire data in phosphate buffer (1−100 mM). In all cases, the metMb samples prepared at different pHs and buffer concentrations were equilibrated for ~2 h. DeoxyMb was prepared in deoxygenated buffers under strictly anaerobic conditions. The heme was reduced by adding a slight excess of buffered sodium dithionite solution using the syringe−septum technique. The intermediate- and low-pH forms of deoxyMb were generally stable for many hours under these conditions. The integrity of the deoxyMb samples was checked at frequent intervals during the course of the RR experiments (generally 2−3 h) by monitoring the position and intensity of the Soret band and the positions of the marker bands in the high-frequency region of the Soret-excitation RR spectrum. No changes in the spectral signatures were observed within the time scale of the experiments. However, prolonged exposure to high acid results in significant denaturation of the protein as is evidenced by the appearance of protein precipitate.

The absorption spectra were recorded with a HP8452 diode spectrophotometer. The RR spectra were acquired with a triple spectrograph (Spex 1877) equipped with a holographically etched 2400 groove/mm grating in the third stage. The samples were contained in either a spinning cell or a capillary tube. The scattered light was collected in a 90° configuration by using a 50 mm f/1.2 Canon camera lens. The excitation wavelengths were provided by the discrete outputs of a Krypton ion (Coherent Innova 200-K3) or argon ion (Coherent Innova 400-15UV) laser. A 1152 × 298 pixel, front-illuminated, UV-enhanced charge-coupled device (CCD) was used as the detector (Princeton Instruments, LN/CCD equipped with a EEV1152-UV chip). The laser powers were typically 5 mW or less. The frequencies were calibrated by using the known frequencies of indene and CCl_4. The frequencies are accurate to ±1 cm^{-1} for strong and/or isolated bands. The slit widths were set for ~2 cm^{-1} resolution at a Raman shift of 1375 cm^{-1}. No smoothing was performed on any of the spectra.

RESULTS

DeoxyMb. The results of the static absorption and RR studies on deoxyMb are summarized below. We first discuss the pH dependence of the absorption spectra. We then describe the characteristics of the high-frequency RR spectra. Finally, we discuss the low-frequency RR spectra and the dependence of these spectra on the isotopic composition of the solvent/buffer system.

(1) Absorption Spectra. The pH dependence of the absorption spectra of deoxyMb is shown in Figure 1. The spectra of the N- and U′-forms are consistent with those previously reported (Antonini & Brunori, 1971; Eaton et al., 1978; Makinen & Churg, 1983; Sage et al., 1991). At pH 6.9, the Soret maximum occurs at 435 nm. The Q-state and band III absorptions occur at 555 and 764 nm, respectively. At pH 3.1, the Soret band is very broad and blue-shifted to

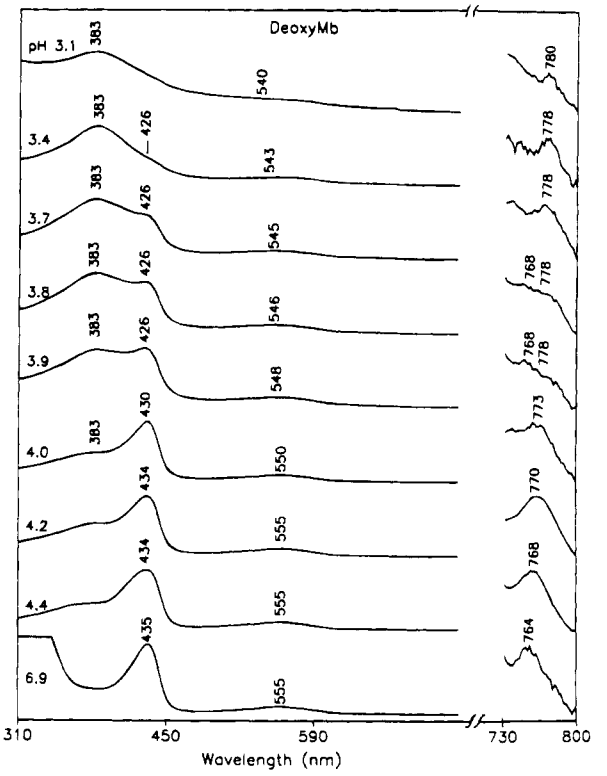

FIGURE 1: Absorption spectra of deoxyMb as a function of pH. The relative intensities of the different spectra do not represent the actual values but are scaled for pictorial clarity. The spectra in the 700−800-nm region are scaled by a factor of 10^3 in order to show the extremely weak band III absorption.

383 nm. The ϵ_{max} of the Soret absorption of the U′-form is also significantly less than that of the N-form. Under highly acidic conditions, the Q-band maximum is blue-shifted to ~540 nm. In contrast, band III is red-shifted to ~780 nm.

At intermediate pH values, the absorption spectra are qualitatively characteristic of a superposition of the spectra of the N- and U′-forms. However, closer inspection of the data shown in Figure 1 reveals that this is not the case. The spectral features are consistent with the presence of another species whose absorption characteristics are different from those of either the N-form or the U′-form. The presence of the new species is most apparent in the pH-titration characteristics of the Soret band and is not easily identified in the behavior of the Q-state and band III absorptions. As the pH is lowered, the Soret band gradually blue shifts. At pH 4.0, the Soret maximum is observed at ~430 nm; at pH 3.8, the maximum is at ~426 nm. The blue shift in the Soret maximum observed in the pH 4.2−3.8 range cannot be attributed to the contribution of the U′-form to the total band contour because the shift occurs at pH values where this form does not make a substantial contribution to the spectrum (Figure 1). Instead, the blue shift appears to be due to simultaneous contributions from the N-form and the new species which exhibits a Soret maximum at ~426 nm. Below pH 4.0, absorption features characteristic of the N-form are not clearly observed. In this region, the absorption spectrum appears to be a superposition of spectra of the new species and the U′-form. The intensity of the 426-nm band maximizes in the pH 3.8−4.2 range and persists as the pH is decreased below 3.8. Below pH 3.7, this feature loses intensity as the 383-nm band of the U′-form becomes

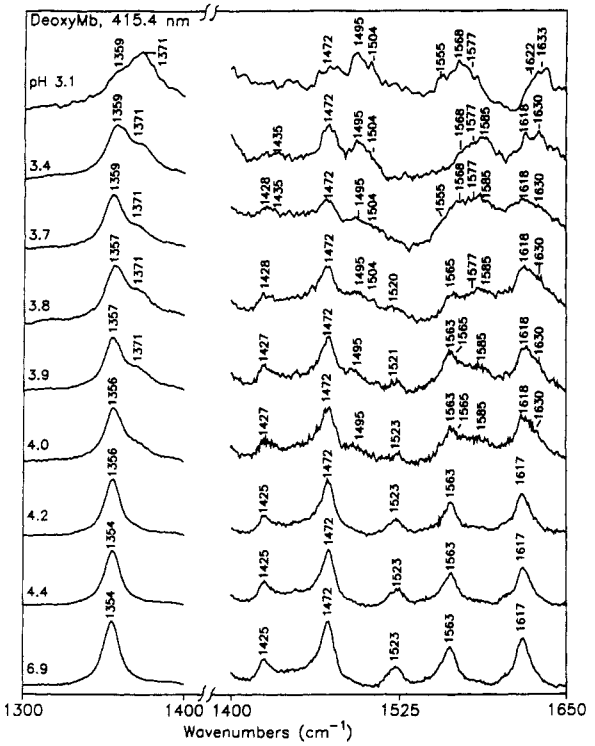

FIGURE 2: High-frequency regions of the Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of deoxyMb as a function of pH. The intensities of the different spectra do not represent the actual values but are scaled for clarity. The spectra in the 1300−1400-cm^{-1} region are scaled by a factor of 10^{-1} for pictorial clarity.

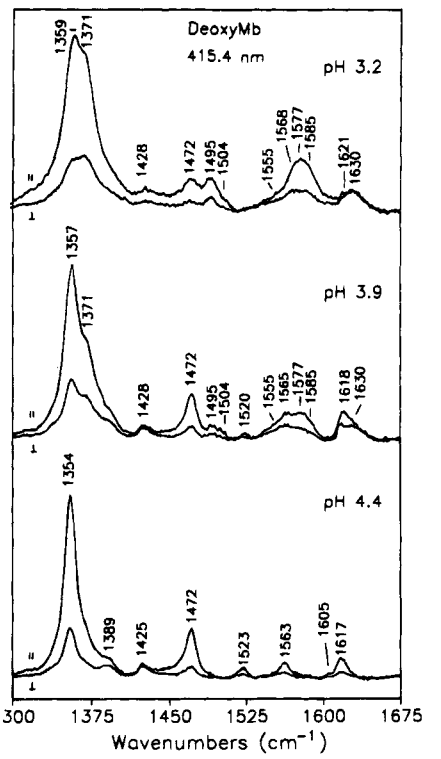

FIGURE 3: Parallel and perpendicular components of the high-frequency Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of deoxyMb at pH 3.2, 3.9, and 4.4. The intensities of the different spectra do not represent the actual values but are scaled for clarity.

more pronounced. Additional studies indicate that all of the above noted spectral changes are reversible. These changes are also independent of the buffer concentration used in the experiments (1−100 mM).

The wavelength of the new Soret band observed in our static experiments (426 nm) is the same as that of the I′-form observed in pH-jump experiments (Giacometti et al., 1977; Ascenzi et al., 1981; Coletta et al., 1985, 1988; Han et al., 1990). The results of the RR studies reported herein (vide infra) confirm that the 426-nm-absorbing species identified in this study is in fact the same species as that observed in the transient RR experiments. Accordingly, the I′-form appears to be present even under equilibrium conditions. The known extinction coefficient of the I′-form (Giacometti et al., 1977) can be used to estimate the relative populations of the three forms in equilibrium. At pH 4.0, where the population is approximately the greatest, the ratio of the populations of the N-, I′-, and U′-forms is approximately 5:3:2. Outside the pH 3.8−4.2 range, the contribution of the I′-form is detectable; however, the amount of this form present is too small to make a reliable population estimate.

(2) High-Frequency RR Spectra. The high-frequency regions of the Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of deoxyMb obtained in the pH 3.1−6.9 range are shown in Figure 2. Spectra were also obtained at several other pH values in this range; however, these spectra did not reveal any features not shown in Figure 2. For clarity, these spectra are not included in the figure. The polarized RR spectra obtained with λ_{ex} = 415.4 nm at pH 3.2, 3.9, and 4.4 are shown in Figure 3. The RR spectra obtained at several different excitation wavelengths ranging from the UV to

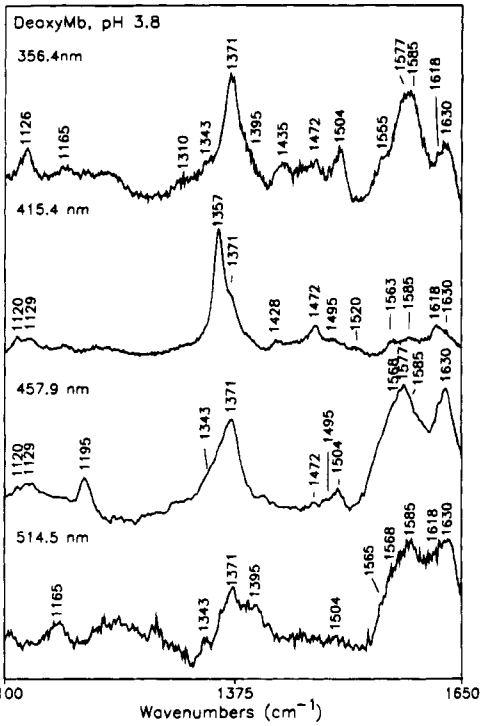

FIGURE 4: High-frequency regions of the RR spectra of deoxyMb at pH 3.8 acquired with several excitation wavelengths in the UV to green regions. The intensities of the different spectra do not represent the actual values but are scaled for clarity.

green are shown in Figure 4. RR spectra were also obtained at several other excitation wavelengths in this region; however, these spectra did not reveal any features not shown in Figure 4. These spectra are not included in the figure for clarity.

Table 1: Frequencies (cm^{-1}), Polarization, and Assignments[a] for Selected RR Bands of N-, I′-, and U′-Forms of DeoxyMb

assignment	obsd polarization	N-form	I′-form	U′-form
ν_{vinyl}	p	1617	1619	1622
ν_{10}	dp	1605	*b*	1630
ν_{37}	p	1583[c]	1585	1577
ν_2	p	1563	1565	1568
ν_{11}	dp	n.o.[d]	1551	1555
ν_{38}	p	1523	n.o.	n.o.
ν_3	p	1472	1494	1504
$\delta_{(=CH_2)sym}$	dp	1423	1430	1435
ν_4	p	1355	1359	1371
ν_{15}	dp	754	*b*	750
ν_7	p	671	674	678
pyr. fold	p	500	490	*b*
pyr. fold	dp	475	*b*	475
$\nu_{Fe-OH_2(OD_2)}$	p		411 (394)	
$\delta_{C_\beta C_a C_b(vinyl)}$	p	405	*b*	410
$\delta_{C_\beta C_1 C_2(propionate)}$	p	371	374	378
ν_8	p	341	*b*	345
ν_9	p	n.o.	270	n.o.
pyr. tilt	p	238	240	244
ν_{Fe-His}	p	218		

[a] Assignments for the N-form are derived from Abe et al. (1978), Choi et al. (1982a,b), Choi and Spiro (1983), Sitter et al. (1985), and Hu et al. (1993). [b] Band not identified due to spectral congestion. [c] Observed in Q-state excitation RR experiment. [d] Band not observed.

The Soret-excitation RR spectra of deoxyMb observed in the range pH 4.4−6.9 agree with those previously reported for the N-form (Rousseau & Friedman, 1988). These spectra are dominated by strong, polarized RR bands due to ν_{vinyl} (1617 cm^{-1}), ν_2 (1563 cm^{-1}), ν_{38} (1523 cm^{-1}), ν_3 (1472 cm^{-1}), and ν_4 (1354 cm^{-1}). The frequencies, polarization characteristics, and assignments for these and other selected RR bands of the N-form are summarized in Table 1. Below pH 4.4, the RR signature of deoxyMb changes in a complicated fashion. Bands broaden, disappear, and are replaced by new features (Figures 2 and 3). The detailed appearance of the RR spectra observed at low pH is also highly dependent on the excitation wavelength (Figure 4). The complicated appearance of the low-pH RR spectra is attributed to the simultaneous contribution of bands from the N-, I′-, and U′-forms (vide infra). The frequencies, polarization characteristics, and assignments for the RR bands of the latter two forms are compared with those of the N-form in Table 1. The assignments for the I′- and U′-forms were made by analogy to those of the N-form.

The UV excitation wavelengths used in our RR studies fall on the blue side of the composite Soret band contour observed for deoxyMb at low pH and to the blue side of the Soret absorption maximum of the U′-form (~383 nm). Consequently, the U′-form should be the predominant contributor to the UV excitation RR spectra. This in turn allows for unambiguous assignment of the RR bands of the U′-form. The UV excitation RR spectra we observe for deoxyMb at low pH are similar to those previously reported by Champion and co-workers (Sage et al., 1991). These spectra exhibit prominent polarized bands due to ν_2 (1568 cm^{-1}), ν_{37} (1577 cm^{-1}), ν_4 (1371 cm^{-1}), and ν_3 (1504 cm^{-1}). Depolarized bands assignable to ν_{10} (1630 cm^{-1}), ν_{11} (1555 cm^{-1}), and $\delta_{(=CH_2)}$ (1435 cm^{-1}) are also observed. The frequencies of the ν_3 and ν_4 modes of the U′-form are significantly higher than those of their counterparts of the N-form (Table 1). The frequencies of these skeletal vibrations led Champion and co-workers to propose that the acid-induced transformation converted the five-coordinate, high-spin heme of the N-form into a four-coordinate, intermediate spin species (Sage et al., 1991).

The violet excitation wavelengths used in our RR studies fall in the vicinity of the Soret absorption maximum of the I′-form (~426 nm) and between the absorption maxima of the N-form (~435 nm) and U′-form (~383 nm). Consequently, scattering from all three forms can potentially contribute to the RR spectra. Inspection of the violet excitation RR spectra observed at intermediate pH values (3.8−4.2) reveals that these spectra are more complicated than those observed with UV excitation (Figure 2; Figure 4, cf. top and second traces). As the pH is lowered below 4.4, features due to the N-form persist in the spectra; however, new polarized RR bands appear at 1585 and 1495 cm^{-1}. The ν_{38} (1523 cm^{-1}) band of the N-form weakens and eventually disappears. As the pH is lowered, the position of the ν_4 band also undergoes a systematic shift to higher wavenumbers. At pH 4.4, the ν_4 band is at 1354 cm^{-1}, characteristic of the N-form (Table 1). At pH 4.2, ν_4 shifts to 1356 cm^{-1}. The ν_4 band continues to shift as the pH is lowered to pH 3.8, where it is observed at 1359 cm^{-1}. The ν_4 band persists at 1359 cm^{-1} as the pH is lowered further. Similar behavior is observed for the ν_{vinyl} band which falls in the 1617−1622-cm^{-1} range. We attribute the polarized RR bands observed at 1618, 1585, 1495, and 1359 cm^{-1} to the ν_{vinyl}, ν_{37}, ν_3, and ν_4 modes of the I′-form (Table 1). The depolarized bands observed at 1555 and 1430 cm^{-1} are assigned to the ν_{11} and $\delta_{(=CH_2)}$ modes of this species.

The fact that the new RR bands observed for deoxyMb at low pH (4.2 and below) are due to a new species different from either the N- or the U′-forms is further exemplified in the excitation wavelength dependence of the spectral features (Figure 4). For example, the N-form typically yields strong scattering with λ_{ex} = 457.9 nm which falls on the red side of the Soret maximum (Figure 1). However, RR bands characteristic of the N-form are absent or extremely weak at this excitation wavelength at pH 3.8. Indeed, the spectral features observed with λ_{ex} = 457.9 nm seem to be more characteristic of the U′-form. The relative intensities of RR bands attributed to the I′-form also appear to track each other as the excitation wavelength is varied and exhibit a different excitation wavelength dependence than those attributed to the U′-form. This is illustrated in the behavior of the ν_3 and ν_4 modes (I′-form, 1495 and 1359 cm^{-1}; U′-form, 1504 and 1371 cm^{-1}).

Comparison of the pH-induced spectral changes observed in our static RR experiments with those observed in the time-resolved, pH-jump studies (Han et al., 1990) reveals a number of common features. These features include upshifts in both the ν_{vinyl} and ν_4 modes, the appearance of a new band at ~1585 cm^{-1}, and the disappearance of the 1523-cm^{-1} (ν_{38}) band. In addition, the low-frequency RR band due to the ν_{Fe-His} mode is absent from our low-pH spectra (vide infra) which is also a characteristic of the I′-form observed in the pH-jump experiments (Han et al., 1990). Collectively, these observations strongly suggest that the new species observed in the two different studies are the same. The fact that the RR bands attributed to the I′-form exhibit maximum intensities with excitation near the 426-nm absorption band further strengthens this argument. It should be noted, however, that there is not a one-to-one match between the static and time-resolved spectra observed at low pH. This is not surprising

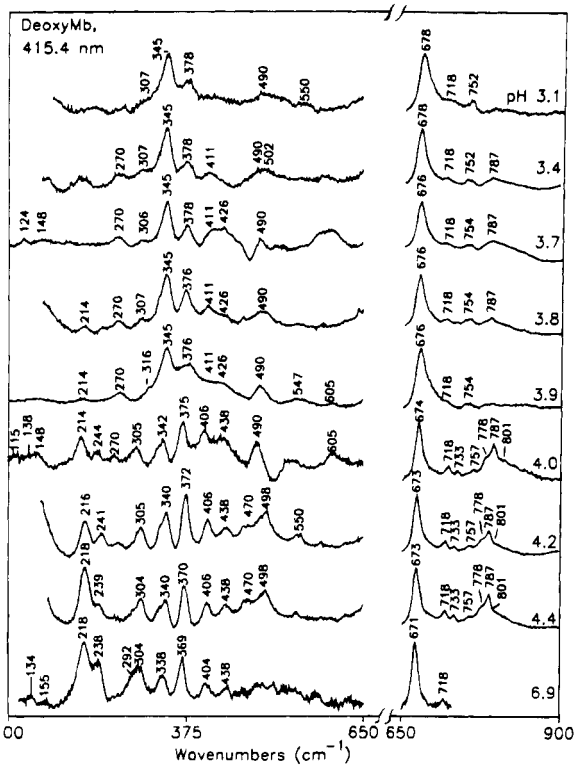

FIGURE 5: Low-frequency regions of the Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of deoxyMb as a function of pH. The intensities of the different spectra do not represent the actual values but are scaled for clarity. The spectra in 650−900-cm^{-1} region are scaled by a factor of 10^{-1} for pictorial clarity.

given that the former experiments reflect equilibrium conditions whereas the latter do not.

In addition to the RR studies described above, a number of other experiments were performed on deoxyMb. These studies explored the reversibility of the acid-induced changes observed in the spectra and the effects of ionic strength. In one experiment, the pH of the sample was cycled from 4.4 to 3.2 and then back to 4.4 by adding small amounts of sodium acetate or acetic acid. These studies showed that all of the pH-induced changes observed in the RR spectra are reversible. This observation is consistent with the behavior of the absorption features. The pH cycling experiments also eliminate the possibility that any of the spectral features attributed to deoxyMb are actually due to metMb and/or oxyMb impurities. The RR signatures of these latter species are different from those of deoxyMb (Sitter et al., 1985; Kerr & Yu, 1988). The RR data acquired in different concentration buffers (1−100 mM) showed that the features of the spectra do not depend on this factor.

(3) Low-Frequency RR Spectra. The low-frequency regions of the Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of deoxyMb obtained in the pH 3.1−6.9 range are shown in Figure 5. Spectra were also obtained at several other pH values in this range; however, these spectra did not reveal any features not shown in Figure 5. For clarity, these spectra are not included in the figure. The frequencies, polarization characteristics, and assignments of selected low-frequency modes of the N-, I′-, and U′-forms are listed in Table 1. As is the case for the high-frequency modes, the assignments for the low-frequency vibrations of the I′- and U′-forms were made by analogy to those of the N-form.

The low-frequency RR spectrum observed for the N-form of deoxyMb is similar to that previously reported (Choi & Spiro, 1983; Rousseau & Friedman, 1988). These spectra are characterized by bands due to both in-plane and out-of-plane deformations of the heme group. A particularly important feature of the RR spectrum of the N-form is the ν_{Fe-His} mode observed at 218 cm^{-1} (Argade et al., 1984; Kitagawa, 1988; Wells et al., 1991). Low-frequency RR spectra have not previously been reported for deoxyMb at low pH. Champion and co-workers attempted to obtain such spectra by using UV excitation; however, they reported that the spectral quality was compromised by significant interference from the Rayleigh wing (Sage et al., 1991). We also found this to be the case. However, we were able to obtain reasonable quality low-frequency spectra of deoxyMb at low pH by using violet excitation. The high-frequency RR data indicate that the U′-form is the predominant contributor to the violet excitation spectrum at pH 3.1 (vide supra). Inspection of Figure 5 (top trace) reveals that the low-frequency RR spectrum of the U′-form is characterized by relatively few bands. The most prominent features are polarized bands observed at 678 and 345 cm^{-1} which are attributed to ν_7 and ν_8 (Table 1). The frequencies of these bands are slightly higher than those observed for the N-form. The most striking aspect of the low-frequency RR spectrum of the U′-form is the absence of any band assignable to the ν_{Fe-His} mode. The absence of this band is consistent with the breakage of the iron−histidine bond at low pH.

Inspection of the RR data shown in Figure 5 reveals that the low-frequency spectra exhibit complicated pH-dependent signatures. This behavior parallels that observed for the high-frequency RR features. As the pH is lowered below 4.4, the RR bands characteristic of the N-form lose intensity and/or shift and new features appear. Some of the new RR features are due to scattering from the U′-form. However, the RR spectra observed in the intermediate pH range (3.7−4.0) range cannot be accounted for by a superposition of the spectra of the N- and U′-forms. For example, new RR bands are observed at 490, 411, and 270 cm^{-1} which are not observed in the spectra of either the N-form or the U′-form. These bands are attributed to the I′-form of the protein whose population maximizes in the pH 3.8−4.2 range (vide supra). Inspection of the RR spectra observed in this pH range also reveals that the band due to the ν_{Fe-His} mode is absent. The result is consistent with breakage of the iron−histidine bond in the I′-form and is consistent with the proposal that the intermediate species observed in the equilibrium and time-resolved RR experiments are the same.

In order to test whether water might be associated with the heme group in low-pH forms of deoxyMb, we compared the RR spectra of the protein in H_2O versus D_2O buffer solutions. The low-frequency regions of the Soret-excitation (λ_{ex} = 415.4 nm) spectra obtained for deoxyMb in these buffers at pH 6.9, 3.8, and 3.2 are compared in Figure 6. The high- and low-pH spectra are dominated by scattering from the N- and U′-forms. The I′-form is the dominant contributor at intermediate pH although the N- and U′-forms also contribute (vide infra). The RR band observed at 411 cm^{-1} is a particularly characteristic feature of the I′-form. Comparison of the spectral data shown in Figure 6 reveals that isotopic substitution has no significant effect on the RR spectra of either the N-form or the U′-form (right and left panels, respectively). [The only noticeable spectral change is the slight downshift (~2 cm^{-1}) of the ν_{Fe-His} mode of the N-form that occurs in D_2O buffer. This downshift has been

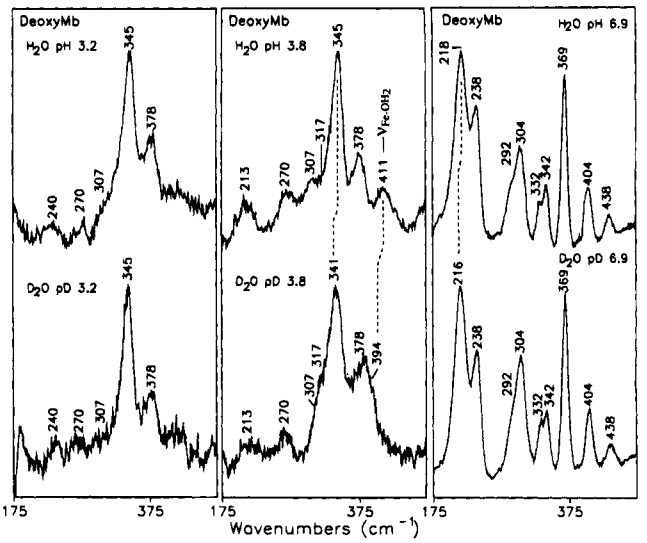

FIGURE 6: Low-frequency regions of the Soret-excitation (λ_{ex} = 415.4 nm) RR spectra of deoxyMb in H_2O and D_2O buffers at pH 3.2, 3.8, and 6.9.

previously observed and has been attributed to deuteriation of the exchangeable protons on the proximal histidine ligand and/or deuteriation of the labile protons of other amino acids in the near vicinity (Argade et al., 1984).] In contrast, the low-frequency RR spectra observed at intermediate pH (Figure 6, middle panel) exhibit significant isotope sensitivity. The most striking effect of isotopic substitution is the absence of the 411-cm^{-1} band of the I′-form. This band downshifts by 15−17 cm^{-1} and appears as a shoulder on the porphyrin band at 378 cm^{-1}. The exact magnitude of the downshift is difficult to determine because of the overlap of the two bands. In addition to the shift of the 411-cm^{-1} band, the ν_8 mode of the heme group (345 cm^{-1}) downshifts noticeably (~4 cm^{-1}) in D_2O buffer. None of the other low- (or high-) frequency RR bands exhibit any significant isotope sensitivity.

The isotope sensitivity of the RR bands of the I′-form strongly suggests that water serves as an axial ligand to heme iron as was originally proposed by Rousseau and co-workers (Han et al., 1990). Both the frequency of the 411-cm^{-1} band and the magnitude of its deuteriation-induced downshift are consistent with those expected for an iron−water stretching vibration, ν_{Fe-OH_2}. The ν_{Fe-OH_2} vibrations of iron−aquo complexes typically occur near 400 cm^{-1} (Nakamoto, 1986). A deuteriation shift of ~16 cm^{-1} is predicted for the ν_{Fe-OH_2} mode if the iron and water species are approximated as a diatomic oscillator. (It is also conceivable, although less likely, that H_3O^+ is the axial ligand under acidic conditions. If so, a 21-cm^{-1} deuteriation shift is predicted.) The fact that the ν_8 (345 cm^{-1}) mode of the heme group also exhibits a deuteriation shift indicates that this mode is coupled to the ν_{Fe-OH_2} vibration. Normal coordinate calculations indicate that the ν_8 vibration contains metal−pyrrole nitrogen stretching character (Abe et al., 1978; Li et al., 1990). In a nonplanar, five-coordinate heme, this mode can mix with the metal−axial ligand stretching vibration (Proniewicz et al., 1991).

MetMb. The fact that an intermediate form is observed in the static absorption and RR spectra of deoxyMb prompted us to carefully examine the pH-dependent behavior of the spectral signatures of the met form of the protein. The

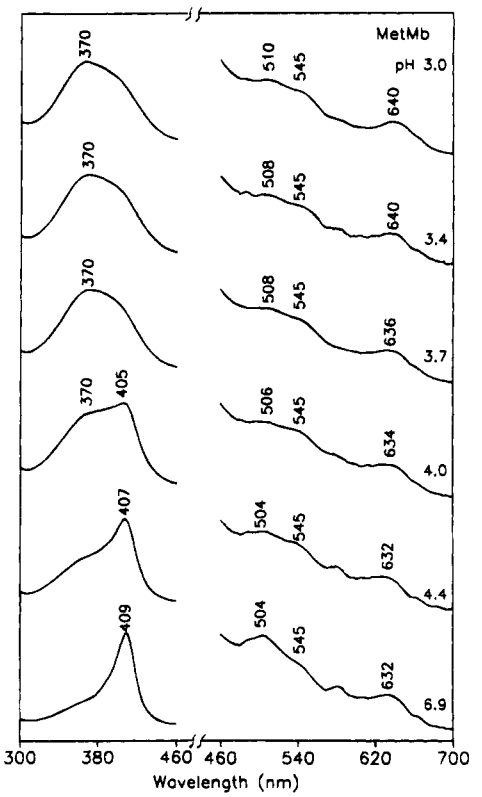

FIGURE 7: Absorption spectra of metMb as a function of pH. The relative intensities of the different spectra do not represent the actual values but are scaled for pictorial clarity. The spectra in the 460−700-nm region are scaled by a factor of 10^1 in order to enhance the weak Q-band features.

protocol for these studies was similar to that described above for deoxyMb. We first discuss the pH dependence of the absorption spectra and the describe the characteristics of the RR spectra.

(1) Absorption Spectra. The pH dependence of the absorption spectrum of metMb is shown in Figure 7. The spectra of the N- and U′-forms are consistent with those previously reported (Antonini & Brunori, 1971; Acampora & Hermans, 1967; Puett, 1973; Bismuto et al., 1983; Irace et al., 1986; Privalov et al., 1986; Sage et al., 1991). The Soret maximum of the N-form occurs at 409 nm; the maximum for the U′-form is very broad and centered at ~370 nm. At intemediate pH values, the absorption spectra of metMb appear to be a superposition of the spectra of the N- and U′-forms. Unlike the case of deoxyMb, there is no definitive evidence for an I′-form. Nevertheless, the presence of an intermediate cannot be completely ruled out. The acid-induced shift in the Soret maximum of metMb (39 nm) is less than that for deoxyMb (52 nm), and the broad absorption contour of the U′-form strongly perturbs the blue side of the Soret band even at relatively low populations of the U′-form. The spectral features of the I′-form could easily be obscured in the complicated band profile observed at intermediate pH values.

(2) RR Spectra. The high-frequency regions of the Soret-excitation (λ_{ex} = 406.7 nm) RR spectra of metMb obtained in the pH 3.1−6.9 range are shown in Figure 8. The general features of these spectra are similar to those previously reported for metMb at neutral and low pH (Sage et al., 1991). The frequencies and assignments for selected high-frequency RR bands of these forms are summarized in Table 2. As

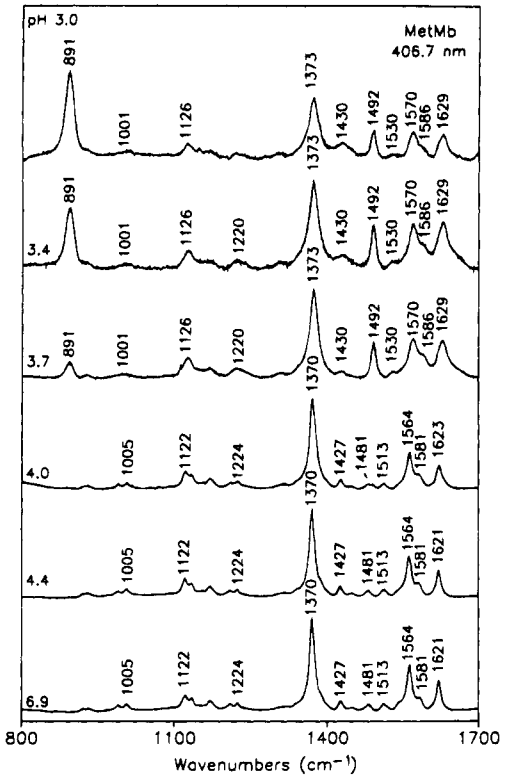

FIGURE 8: High-frequency regions of the Soret-excitation (λ_{ex} = 406.7 nm) RR spectra of deoxyMb as a function of pH. The intensities of the different spectra do not represent the actual values but are scaled for clarity.

Table 2: Frequencies (cm^{-1}), Polarization, and Assignments[a] for Selected RR Bands of N- and U′-Forms of MetMb

assignment	obsd polarization	N-form	U′-form
ν_{10}	dp	1611[b]	1629
ν_{vinyl}	p	1621	1623 (sh)
ν_{37}	p	1581	1586
ν_2	p	1564	1570
ν_{11}	dp	1544	*c*
ν_{38}	p	1513	1530
ν_3	p	1481	1492
$\delta_{(=CH_2)sym}$	dp	1427	1430
ν_4	p	1370	1373
ν_{13}	dp	1224	1220
ν_{44}	p	1122	1126
?	*d*		891

[a] Assignments for the N-form are derived from Abe et al. (1978), Choi et al. (1982a,b), Choi and Spiro (1983), Sitter et al. (1985), and Hu et al. (1993). [b] See Spiro et al. (1979). [c] Band not observed. [d] Depolarization ratio not determined.

the pH is lowered, the spectral features characteristic of the N-form are replaced by the signatures of the U′-form. As is the case for the absorption spectra of metMb, the RR spectra observed in the pH 3.8−4.2 range appear to be a superposition of the spectra of the N- and U′-forms. No RR bands are observed that can be definitively ascribed to the I′-form. It should be noted, however, that the frequencies of the strong RR bands of the N- and U′-forms of metMb are not substantially different. These differences are in general much smaller than those that distinguish the N-, I′-, and U′-forms of deoxyMb. For example, the frequencies of the ν_4 modes of the N- and U′-forms of metMb differ by only 3 cm^{-1} (Table 2). In the case of deoxyMb, this difference is 17 cm^{-1} (Table 1). If the frequencies of the I′-form of metMb were to fall between those of the N- and U′-forms, it is unlikely that these features would be resolved in the RR spectra. It is also possible that the excitation wavelengths chosen for the RR studies on metMb do not result in significant scattering from the I′-form. The best choice of exciting lines to probe for the I′-state of metMb (presuming one exists) is not obvious in the absence of any absorption features to guide in this selection.

DISCUSSION

The characterization of the structural and functional properties of low-pH heme-pocket intermediates is important because these properties are dictated by the local, and perhaps global, structure of the protein matrix. Ultimately, all of these factors must be linked together in order to produce a complete picture of the protein at the molecular level. The absorption and RR spectra of the low-pH forms of deoxyMb reported herein, in conjunction with previous work, indicate that at least two distinct heme-pocket intermediates (I′- and U′-forms) occur along the pathway between the N- and U-forms. The properties of the I′-form appear to be similar to those of the intermediate which forms on the millisecond time scale in pH-jump experiments.

The existence of the I′-form of holoMb under equilibrium conditions has not been previously reported. The failure to observe this form is in large part due to the fact that virtually all previous studies of the low-pH forms of Mb have focused on the metprotein and not the deoxy species (Acampora & Hermans, 1967; Puett, 1973; Bismuto et al., 1983; Irace et al., 1986; Privalov et al., 1986). Thus far, the I′-form has only been identified in deoxyMb. We, like others, cannot find any spectroscopic signatures indicative of an I′-form of metMb. As far as we know, only a single static spectroscopic study has been reported for deoxyMb at low pH (Sage et al., 1991). The I′-form was not detected in this work. There are several reasons why this could be the case. First, the detailed characteristics of the composite absorption band contour are sensitive to the exact values of both the pH and the ionic strength. It is possible that the specific conditions used in the previous study precluded a clear identification of the 426-nm signature of the I′-form. Second, the presence of the I′-form is more readily detected in the RR than in the absorption spectra. Nevertheless, the I′-form is only clearly visible if the sample is excited with violet excitation wavelengths. The previous RR study of the low-pH forms of deoxyMb used primarily UV excitation wavelengths where the I′-form is not readily detected.

Given that deoxyMb is prepared from pH-equilibrated metMb, it seems reasonable that an I′-form of the metprotein should also exist. As was previously noted, there are several possible explanations for why the I′-form of metMb might not be detected. These explanations hinge on the choice of experimental conditions and/or the ability to distinguish the spectral characteristics of the I′-form. The latter explanation seems most reasonable given the large number of studies that have been conducted on the metprotein. It is also conceivable, although seemingly unlikely, that conversion of the heme group from the ferric to ferrous states induces the transformation to the I′-form. Additional studies are needed to address this issue.

Nature of the Heme Pocket in the Low-pH Forms. The heme group of the N-form of deoxyMb is a five-coordinate, high-spin complex with a histidine axial ligand (Takano,

1977a; Phillips, 1981; Kitagawa, 1988; Rousseau & Friedman, 1988). In both the I′- and U′-forms, the iron−histidine bond is broken. Water replaces the axial histidine ligand in the I′-form of the protein. The heme group of the U′-form exhibits spectral features characteristic of a four-coordinate, intermediate spin complex (Sage et al., 1991). The relatively high frequency observed for the ν_{Fe-OH_2} vibration of the I′-form (411 cm^{-1}) suggests that the water ligand is relatively tightly bound to the metal ion. This could influence the spin state of the metal ion. The frequencies of the ring skeletal modes of the heme group are reasonably reliable indicators for the spin state of the iron atom (Spiro, 1983). The ν_2, ν_3, and ν_4 modes of the N-form are observed at 1563, 1472, and 1354 cm^{-1}, respectively. In the U′-form, these modes upshift to 1568, 1504, and 1371 cm^{-1}. The frequencies of the ν_2, ν_3, and ν_4 modes of the I′-form occur at 1565, 1495, and 1359 cm^{-1}. The frequency of the ν_2 mode of the I′-form is midway between its N- and U′-form counterparts. The ν_3 frequency of the I′-form is closer to that of the U′-form than the N-form. On the other hand, the ν_4 frequency of the I′-form is closer to that of the N-form. The fact that the skeletal-mode frequencies of the I′-form are not clearly characteristic of either the high-spin N-form or the intermediate spin U′-form does not permit a definitive identification of the spin state of the metal ion. Indeed, the skeletal-mode frequencies observed for the I′-form may signify that the heme exists in an $S = 1, 2$ spin admixture. This would be consistent with the relatively strong association of the water ligand indicated by the relatively high frequency of the ν_{Fe-OH_2} vibration. In this regard, Cheng et al. (1994) have recently reported X-crystallographic and magnetic data for a mono-aquo porphyrin model complex, [Fe^{III}(octaethylporphyrin)(OH_2)]ClO_4. The Fe−OH_2 bond length in this species is extremely short (2.045 Å) and the shortest known for any neutral O-donor ligands of iron(III) porphyrinates (range 2.069−2.134 Å). The short Fe−OH_2 bond length results in a quantum mechanically admixed $S = {}^3/_2$, ${}^5/_2$ ground state for the mono-aquo complex.

The fact that a water molecule appears to be relatively strongly associated with the ferrous heme group in the I′-form of deoxyMb is unusual. In the N-form of deoxyMb, water resides in the distal pocket but is not bonded to the heme iron (Takano, 1977b; Phillips, 1981). Apparently, the loss of the axial histidine ligand, in conjunction with the characteristics of the heme pocket, drives the formation of a five-coordinate species. It is not clear whether the water ligand resides on the proximal or distal side of the heme group. This is illustrated in Figure 9. Regardless, the isotope sensitivity of the ν_{Fe-OH_2} vibration indicates that the water ligand is exchangeable. This result is consistent with a less compact structure for the protein under acidic conditions. The strong water−ligand affinity exhibited by the ferrous heme of the I′-form of deoxyMb parallels the behavior observed for the mono-aquo model complex (Cheng et al., 1994). Water is also associated with the ferric heme in proteins under neutral as well as acidic conditions (although less strongly than for the mono-aquo model complex). For example, the Fe−OH_2 bond length in metMb is ~2.1 Å (Takano, 1977b). Water is also found at coordinating distances in a number of distal and proximal histidine mutants of metMb (Quillan et al., 1993; Tang et al., 1994; Barrick, 1994).

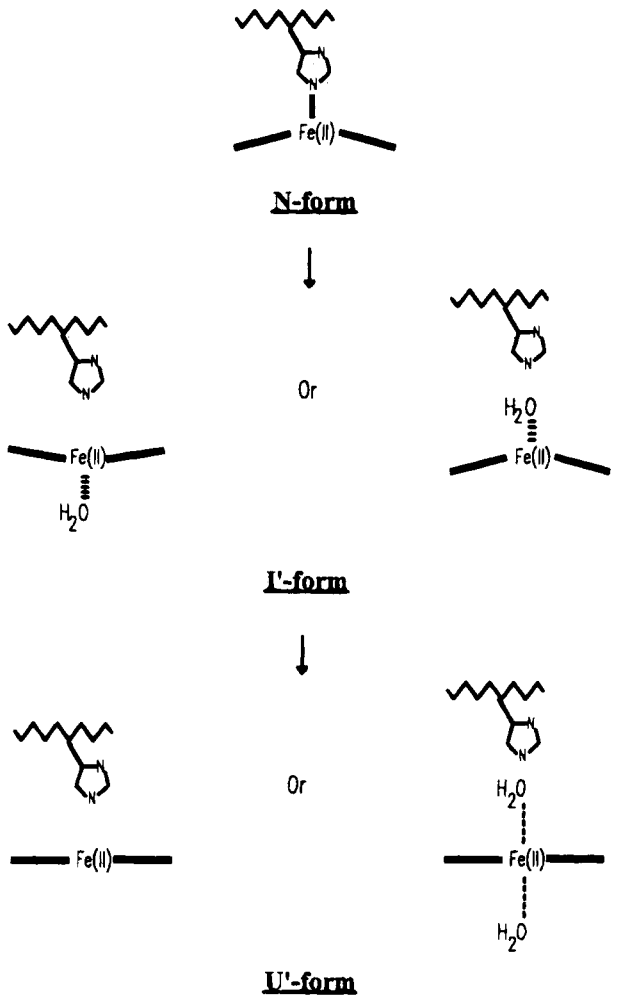

FIGURE 9: Possible heme−axial ligand structures in the N-, I′-, and U′-forms of deoxyMb.

The fact that the I′-form of deoxyMb contains a five-coordinate heme suggests that the four-coordinate form is unstable under acidic conditions. This result would appear to be inconsistent with the proposed structure of the U′-form of the protein (Sage et al., 1991). It is possible that the protein environment at lower pH which stabilizes the U′-form relative to the I′-form also promotes the dissociation of the water ligand. On the other hand, if the protein structure becomes more open at lower pH (Hughson et al., 1990; Jennings & Wright, 1993; Shin et al., 1993a,b; Waltho et al., 1993), it is not obvious why the ligand would dissociate. In fact, it seems more likely that a second water molecule would be associated with the heme group under these conditions. This is illustrated in Figure 9. A *very weakly* coordinated bis-aquo complex might be expected to exhibit properties similar to those of a four-coordinate species. The ν_{Fe-OH_2} modes of such a complex would be expected at very low frequencies and might not be detected in deuterium isotope experiments. It should be emphasized, however, that the difference between a four-coordinate complex and a six-coordinate species with *very weak* axial ligands is primarily semantic.

Relationship between the Heme-Pocket Intermediates and the Global Protein Structure. On the basis of the information available at the time, Champion and co-workers suggested that the U′-form of holoMb corresponds to the I-form of the apoprotein (Sage et al., 1991). However, comparison of the full body of data now available indicates that the I′-form of

holoMb actually exhibits properties more in common with those of the I-form of the apoprotein. In this regard, kinetic experiments on apoMb indicate that a species with properties similar to those of the equilibrium state I-form is generated within milliseconds during protein folding (Jennings & Wright, 1993). The I′-form observed in pH-jump experiments is generated on the same time scale (Giacometti et al., 1977; Ascenzi et al., 1981; Coletta et al., 1985, 1988; Han et al., 1991). In contrast, the U′-form appears to occur only after longer times (Sage et al., 1991). The maximum populations of the I- and I′-forms also occur in the same pH range (3.8−4.2) (Hughson et al., 1990). The population of the U′-form maximizes in a lower pH range (2.6−3.5).

The I′-form of holoMb exhibits other properties that are generally consistent with those of the I-form of the apoprotein. For example, the heme pocket of the I′-form contains an exchangeable water molecule that serves as a ligand to the iron. A more open structure, characteristic of the I-form, might favor the formation of this type of heme intermediate. Disruption of either the E helix or the F helix could allow solvent access to the heme group (distal and proximal sides, respectively; Figure 9). The E helix of the I-form of apoMb is known to be unfolded (Hughson et al., 1990). No data are available on the F helix. The absence of the iron−histidine bond in the I′-form of holoMb is certainly indicative of a local structural perturbation in the F helix and might signal a more global perturbation.

The similarities between the properties of the I′-form of holoMb and the I-form of the apoprotein strongly suggest that these species are related. The question remains as to how the U′-form fits into the general picture. It is clear that the structure of the heme group of the U′-form is different from that of the I′-form. These structural differences could arise either from local changes in the heme pocket or from more global changes in protein structure. At this time, insufficient structural information is available for the holoprotein to resolve this issue. The most quantitative information concerning the structure of Mb at low pH has come from studies of the apoprotein. These studies do not provide any evidence that two structurally distinct intermediates occur at low pH. The data obtained for the apoprotein could be construed to suggest that the protein structure is similar in the I′- and U′-forms. However, it is also possible that the absence of the heme cofactor in apoMb blurs the distinction between the two intermediates. Further studies are needed to address these issues.

SUMMARY AND CONCLUSIONS

The absorption and RR spectra obtained for deoxyMb indicate that a previously unidentified intermediate (I′-form) exists at low pH under equilibrium conditions. The spectroscopic signatures of this intermediate are similar to those of the intermediate that appears within a few milliseconds in pH-jump experiments. The population of the I′-form maximizes in the pH 3.8−4.2 range where it exists in equilibrium with the N- and U′-forms of the protein. The structure of the heme group in the I′-form is distinctly different from that of N- or U′-forms. The proximal histidine serves as the axial ligand in the N-form. The iron−histidine bond is ruptured in both the I′- and U′-forms. In the I′-form, the histidine ligand is replaced by a relatively strongly bound, exchangeable water molecule. This ligand is absent in the U′-form. The equilibrium properties of the I′-form, in conjunction with the kinetics of its formation, suggest that this form could correspond to the molten globule intermediate of apoMb. Collectively, the results establish a possible link between the local structure of the heme pocket and the protein as a whole during the unfolding/refolding process.

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