\textbf{1H NMR Study of the Dynamics of the pH Modulation of Axial Coordination in Aplysia limacina (Val(E7)) and Sperm Whale Double Mutant His(E7) \rightarrow Val, Thr(E10) \rightarrow Arg Metmyoglobin}

\textbf{ROLE OF DISTAL HYDROGEN BONDING IN LIGATION*}

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The molecular and electronic structure, thermodynamics, dynamics, and mechanism of interconversion of the pH-modulated reversible equilibria of Aplysia limacina metmyoglobin, (metMb), have been investigated by \textit{1H} NMR spectroscopy. The four identified species which interconvert slowly on the NMR time scale (lifetime > 1 ms) are metMbOH (B) at alkaline pH, five coordinate metMb (N) at acidic to neutral pH, an acidic form, A, near pH \~{}4 and an extremely low pH form, D, attributed to an equilibrium unfolded species. The presence of strong distal hydrogen bonding by Arg (E10) to bound hydroxide is detected via a significant solvent isotope effect on the metMbOH (B) hyperfine shifts. Integration of the peak intensities provides pK values of 7.7 and 4 for the B \rightleftharpoons N and N \rightleftharpoons A equilibria, respectively. Saturation transfer via chemical exchange is observed for B \rightleftharpoons N and N \rightarrow A, where the rates for forming metMbOH (B) and the acidic form A from N are base- and acid-catalyzed, respectively, while the reverse rates are first-order. The much slower interconversion rate for N \rightleftharpoons B in A. limacina metMb than His(E7) containing mammalian metMb is attributed to the fact that a ligand bond is broken rather than just proton transferred and that the equilibrium involves a major rearrangement of the orientation of Arg(E10). This conclusion is supported by \textit{1H} NMR data for the sperm whale double mutant His(E7) \rightarrow Val/Thr(E10) \rightarrow Arg metmb, which exhibits a pK \approx{} 8.7 for the equilibrium between five-coordinate metMb (N) and metMbOH (B) with an even slower interconversion rate than in A. limacina metMb. This double mutant metMbOH (B) exhibits hydrogen bonding by Arg (E10) with coordinated hydroxide similar to that in A. limacina metMbOH. The slow but acid-catalyzed rates of conversion of A. limacina metMb (N) to the acid species A with significantly weakened bonding of the heme iron to the axial His(F8) residue is consistent with protonation of an inaccessible residue and/or a structural change accompanying the protonation equilibrium. It is concluded that metMb will coordinate water strongly only when there is a distal hydrogen bond acceptor residue, while the hydroxide ion is coordinated strongly only if there is a distal hydrogen bond donor residue.

The ferric or met derivatives of myoglobin, Mb, and hemoglobin, Hb, are non-physiological forms, but their structural and ligation properties reflect on important functional properties of these proteins (1–7). Whether the sixth coordination position is occupied by a water molecule, hydroxide ion, or is vacant depends on the solution conditions and the hydrogen bonding properties and/or polarity of distal residues. The dynamics and thermodynamics of the ligation of common anions that bind to metMb, such as azide, can reveal details on pH-modulated structural changes in the active site (2–5). For the majority of Mbs and Hbs which possess a distal His(E7), the neutral pH, high-spin form binds a water molecule (I in Fig. 1) (8) that is converted to a bound hydroxide in the alkaline form (II in Fig. 1) which exhibits a low-spin high-spin equilibrium (1, 3, 6, 9–11). The pK for the reversible transition has been attributed (7) to the hydrogen bonding by the ubiquitous His(E7) in both forms (i.e. I and II in Fig. 1).

The invertebrate Mb from the sea hare Aplysia limacina possesses Val69 at helical position E7 (12), and previous optical and ligation rate studies (3–5) indicated it lacked a bound water below pH 7 in metMb (125 in Fig. 1). The absence of a bound water was quantitatively established by the crystal structure of A. limacina metMb (13) and confirmed in solution by \textit{1H} NMR and resonance Raman spectroscopy (11, 14). While the Val(E7) residue cannot participate in the usual hydrogen bond interaction with the ligand, Arg95 at helical position E10 in A. limacina Mb has been shown to orient into the pocket and hydrogen bond to the ligands in metMbF, metMbN3, and metMbO2.

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\textit{**} The abbreviations used are: Mb, myoglobin; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; ppm, parts/million; Hb, hemoglobin; metMb, ferric myoglobin; metHb, ferric hemoglobin; WT, wild type; VR-Mb, the sperm whale and double point mutant His84(E7) \rightarrow Val, Thr95(E10) \rightarrow Arg.}

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FIG. 1. Modes of axial coordination in metMb in water at different pH and in the presence of distal hydrogen bonding acceptors and donors. I, water coordinator at acidic to neutral pH with the distal residue providing a hydrogen bond acceptor; II, vacant coordination site, such as in A. limacina metMb at acidic to neutral pH; III, hydroxide coordination at weakly alkaline pH with the distal residue providing a hydrogen bond donor; IV, the weak interaction between the imidazolium ring of a protonated His(F8) and the iron; this is a possible structure of species A for A. limacina metMb identified by $^1$H NMR.

Affinity at low pH distinct from that due to the conventional neutral ↔ alkaline transition, and this increased ligand affinity has been proposed to reflect the protonation of His(F8), and hence a perturbation of the iron-axial His(F8) bond (4). A resonance Raman spectroscopic investigation of A. limacina metMb, however, has failed to find evidence for an additional low pH (4) species in a ligation state different from that characterized by x-ray crystallography at pH 7 (11).

**MATERIALS AND METHODS**

MetMb was prepared by the bucal muscle of the mollusc *A. limacina* as previously reported. (13, 31) The purified protein was stored at $-20^\circ$C until used. The artificial sperm whale double mutant VR-metMb was expressed, isolated, and purified as described by Cutruzzoli et al. (2). The proteins were concentrated in an Amicon ultrafiltration cell to $\sim$3 mM for *A. limacina* metMb and $\sim$0.6 mM for sperm whale VR-metMb sample, and the solutions exchanged with $^2$H$_2$O in 0.1 M NaCl as needed. The sample pH was adjusted with 0.2 M HCl and NaO'H, and measured with a Beckman 3550 pH meter using an Ingold 620 micro-combination electrode. The pH readings of $^2$H$_2$O are not corrected for the isotope effect.

$^1$H NMR spectra were recorded on Nicolet NT-360 and General Electric 0-500 NMR spectrometers operating at 360 and 500 MHz, respectively. Data were collected using 16,384 data points over a 40 kHz bandwidth (at 360 MHz) and 8,096 data points over a 62 kHz bandwidth (500 MHz). Typical spectra consisted of $2 \times 10^5$ transients for *A. limacina* metMb and $5 \times 10^5$ transients for sperm whale VR-metMb. The saturation transfer spectra were recorded by applying a decoupler presaturation pulse of 50 ms to the desired resonance; saturation as a function of time showed that the steady-state was achieved for 230 ms saturation. Corresponding reference spectra were collected under identical conditions but with the decoupler pulse off-resonance; on- and off-resonance frequencies were alternated every 512 scans. These spectra consisted of $3 \times 10^5$ transients for *A. limacina* metMb and $3 \times 10^5$ transients for sperm whale VR-metMb; the pulse repetition rate was 11.8 s$^{-1}$. The signal-to-noise ratio was improved by exponential apodizing that introduced 30-50 Hz line broadening. Non-selective $T_1$ values were determined by the inversion-recovery method ($180^\circ - \tau - 90^\circ$). The $T_1$ values were determined from the initial slope of semilogarithmic plots of intensity versus $\tau$; the $T_1$ values are reproducible to ±15%. Although the intrinsic relaxation time demands a selective $T_1$ experiment, comparison in iso-electronic sperm whale metMb$_2$H$_2$O has shown that the selective and non-selective $T_1$ values differ inconsequentially for a methyl in the presence of the strong paramagnetic relaxation contribution (32). Chemical shifts are referenced to DSS through a methyl in the presence of the strong paramagnetic relaxation contribution (32). Chemical shifts are referenced to DSS through a methyl in the presence of the strong paramagnetic relaxation contribution (32). Chemical shifts are referenced to DSS through a methyl in the presence of the strong paramagnetic relaxation contribution (32).
of resonance Y, the intensity of peak X before, \( I(X) \), and after, \( I(Y) \), saturating is related to the exchange rate:

\[
h_x = \frac{1}{T_1(X)} \left[ \frac{I_d(X)}{I(X)} - 1 \right]
\]  

(Eq. 1)

The ratio \( I_d(X)/I(X) \) was measured in the range 12 to 1.3, and is judged accurate to \( ±15\% \). A similar expression for \( h_y \) is obtained upon saturating peak X. In each case \( T_1(X), T_1(Y) \) are the longitudinal relaxation times in the absence of exchange. Conversely, the exchange rate \( h_x \), if not directly measurable, can be obtained from \( h_x \) from the relation:

\[
f_x f_h = f_y h_y
\]  

(Eq. 2)

where \( f_x, f_y \) are the mole fractions of species \( X, Y \) obtained directly from relative peak areas.

RESULTS

A. limacina metMb

**pH Effects on \(^1\text{H} \) NMR Spectrum**—The 360 \(^1\text{H} \) NMR spectra of \( A. \) limacina metMb in \( \text{H}_2\text{O} \) at 30 °C as a function of pH are illustrated in Fig. 2. At pH 6 (Fig. 2A) the spectrum is dominated by the species for which the x-ray crystal structure has been reported, \((13)^2\) which is designated as \( N \) (for neutral); this species is five-coordinated in the crystal \((13)^2\) (II in Fig. 1). The previously assigned signals for the major isomer are labeled \( N_i \), where \( i \) indicates the heme position or amino acid sequence origin; the signal \( N_{is} \) arises from the axial His(F8) \( C=\text{H} \) (14, 24). Upon raising the pH into the alkaline region, the peaks \( N_i \) lose intensity while a new species gives rise to a set of peaks \( B_i \) (species designated \( B \) for the basic species) as shown in Fig. 2B, which dominates at pH 8.7 (Fig. 2C). The species \( B \) has been shown (14) to exhibit all of the NMR properties associated with the hydroxide complex (i.e. \( III \) in Fig. 1). Integration of methyl peaks \( N_{a} \) and \( B_i \) as a function of pH yields the mole fractions \( B \) and \( N \), \( f_{N}/f_{B} \), as shown on the right side of Fig. 3, which reflect a \( pK \) -7.7 ± 0.1 for the equilibrium \( N \rightleftharpoons B \). This value agrees well with that previously reported based on optical data in \( \text{H}_2\text{O} \) (3). In the pH range 6.5–9, where the peak positions of both acidic (A) and basic (B) species are \( pH \)-independent (Fig. 4), the \( \text{H}_2\text{O} \) NMR spectra in \( \text{H}_2\text{O} \) and \( \text{H}_2\text{O} \) at the same nominal \( pH \) values exhibit essentially the same relative amounts of \( N \) and \( B \). However, while the methyl resonances of \( A. \) limacina metMb (N) exhibit the identical chemical shifts in \( \text{H}_2\text{O} \) and \( \text{H}_2\text{O} \), as reported in detail previously (28), those of metMbOH (B) exhibit significantly smaller, albeit \( pH \)-independent, shifts in \( \text{H}_2\text{O} \) (spectra not shown) than \( \text{H}_2\text{O} \). The shift difference is approximately 0.5 ppm, and the values for individual methyl peaks is indicated in Table I.

Lowering the pH into the strongly acidic region leads to similarly dramatic changes in the \( \text{H}_2\text{O} \) NMR spectrum (Fig. 2, B‘-E’), where the peaks for the neutral \( pH \) form, \( N \), exhibit both small shift changes (Fig. 4) as well as intensity loss, while a new set of peaks grows in which we label \( A \) for the species \( A \), (for acidic); only three such methyl peaks are resolved, \( A_{a}, A_{b}, A_{c} \) (Fig. 2D‘) (see below). Upon lowering the \( pH \) below 4, an additional resonance is detected (labeled \( D \)) for an apparently denatured species we label \( D \). This peak is a broad (~2 kHz) composite and must represent all of the methyls and possibly some single protons (Fig. 2E‘). Integration of peaks \( A_{a}, A_{b}, A_{c} \) as a function of \( pH \) yields the mole fraction \( f_{A}, f_{D} \) which indicates a \( pK \) -4 (left side of Fig. 3) for the \( N \rightleftharpoons A \) transition. The presence of species \( D \) is accounted for approximately by attributing to peak D the area for four methyls, \(^3\) which allows an estimate of the mole fraction of species \( D, f_{D} \) (left side of Fig. 3). However, in contrast to metMbOH (B), and like metMb (N), the chemical shifts of species \( A \) are indistinguishable in \( \text{H}_2\text{O} \) and \( \text{H}_2\text{O} \) (Table I).

Thus the \( \text{H} \) NMR spectra in Fig. 2 reveal \( pH \)-modulated equilibria involving four species as given by:

\[
D \rightleftharpoons A \rightleftharpoons N \rightleftharpoons B
\]

(Eq. 3)

where the rates of interconversion are also indicated. Since each of the four species exhibit resolved sets rather than averaged resonances, the exchange rates are all slow on the \( \text{H} \) NMR time scale (~10\(^{-7}\) s\(^{-1}\) based on chemical shift differences as small as 4 kHz). The slower rate of the neutral \( \rightleftharpoons \) alkaline transition in \( A. \) limacina relative to sperm whale metMb has been noted earlier (3, 14). The data in Fig. 4 show that the basic metMbOH form, \( B \), exhibits \( pH \)-independent shifts. However, the species \( N \) or metMb exhibits \( pK \) values near 6 and 4 which weakly modulate the shifts particularly for the propionate resonances; these \( pK \) values likely arise from the rapid protonation of these heme side chains also observed in the metMbCN complex (33). The acidic species \( A \) exhibits inflections indicative of \( pK \) values well below \( pH \) ~4, which could arise from titration of numerous carboxylates close to or remote from the active site. The chemical shifts for each of the molecular species \( A, N, \) and \( B \) in the flat \( pH \) region are listed in Table I.

Assignment of Resonances in \( A \) and \( B \)—The location and identity of peaks for these complexes are readily obtained by the saturation-transfer observed when the exchange rate, \( k_x \), is comparable to the intrinsic relaxation rate (29). This is illustrated in Fig. 5 for the equilibrium \( N \rightleftharpoons B \). Saturation, in turn, of the four assigned methyls for \( N \) leads to saturation-transfer via chemical exchange to the respective methyls of metMbOH, as shown in Fig. 5, B–E. The four signals with less than one proton intensity in Fig. 5A arise from a minor species which has the heme rotated by 180° about the \( \alpha,\gamma \)-meso axis, as described previously (14, 33); the peaks are labeled \( n \), for the minor component. Saturation of the major component single proton peaks \( N_i \) yield the location\(^4\) of the major component metMbOH peak \( B \), (not shown). The shifts for the complex \( B \) are listed in Table I.

The assignment of the resonance for \( A \) at \( pH \) 4.5 is illustrated in Fig. 6. Saturation of the metMb methyl peaks \( N_{a}, N_{b}, \) and \( N_{c} \) yields magnetization transfer to the respective methyl peaks in the \( A \) form (Fig. 7, B–D). The analog to \( N_{a} \), (peak \( A_{a} \)), as well as the minor component peaks, \(^5\) \( A_{a}, A_{b}, \) are similarly located, although those assignments require multiple controls to correct for off-resonance effects (not shown).

Saturation of the well-resolved His(F8) \( C=\text{H} \) peak \( N_{a} \) for

\(^3\) Note that the possibly significant uncertainty in the area of peak \( D \) does not influence the accuracy of the \( pK \) for the \( N \rightleftharpoons A \) transition obtained separately from the better defined areas of individual methyl peaks \( N_{a} \) and \( A_{a} \).

\(^4\) Unpublished optical data on the \( pH \) reversible equilibria of \( A. \) limacina metMb are in agreement with the present NMR data. Whereas the \( pK \) value (~7.7) of the \( N \rightleftharpoons B \) transition is essentially insensitive to solvent composition (for the optical data; see Ref. 3), the \( N \rightleftharpoons A \) equilibrium is affected by anions. Indeed the \( pK \) value of the \( A \rightleftharpoons N \) process increases from ~3.4 in 2 mM Tris (F. Ascoli, P. Ascoli, and M. Bruno, unpublished optical data), to ~4.0 in 0.1 M NaCl (present NMR data), to 4.4–4.7 in acetate 2.0/0.1 M (Ref. 4).

\(^5\) Saturation of the minor component methyl peaks (14) \( n \), results in the location and assignment of the methyls for the minor component at basic \( pH \) (in analogy to major component \( B \)), with shifts; \( b_{a} \), 40.5, \( b_{b} \), 38.0, \( b_{c} \), 30.0, \( b_{d} \), 28.3 ppm at \( pH \) 7.72, 30 °C; at acidic \( pH \) (in analogy to major component \( A \)) with shifts \( a_{a} \), 70.4, \( a_{b} \), 72.0, \( a_{c} \), 51.8, \( a_{d} \), 56.3 ppm at \( pH \) 4.1 at 30 °C.
**Acid ↔ Alkaline Transitions**

**FIG. 2.** 360 MHz $^1$H NMR spectra 3 mM of *A. limacina* metMb in $^2$H$_2$O in 0.1 M NaCl at 30 °C at: A, pH 6.0, with the dominant species metMb, N, whose peaks N, have been assigned by isotope labeling and nuclear Overhauser effects (14); B, pH 7.2, showing the appearance of a set of peaks B, for species B (metMbOH); C, pH 8.7, with a predominance of species B (metMbOH) with peak B, $B'$, pH 5.1, which shows the emergence of a new set of peaks A, for a species designated A; C', pH 4.1, and D', pH 3.6, which shows further in intensity of peaks A, at the expense of peak N, but also the appearance of a broad composite peak labeled D which is centered at 55 ppm; E', pH 3.2, where peak D increased intensity at the expense of both peak N, and A. The subscript $i$ refers to heme position (1–8), and amino acid sequence ($i$ = F8 is for C$_8$H of His(F8)).

metMb (N) results in saturation transfer to a peak at 12.5 ppm, as illustrated in Fig. 6F, locating the same proton, $A_{F8}$, in the acidic or A form. The chemical shifts for the heme methyl and axial His C$_8$H resonances, $A_i$, are listed in Table I.

**Dynamics of Interconversion**—The quantitative consideration of the saturation transfer yields the rates of exchange for the various species (29). The necessary intensity data are obtained directly from the spectra as in Figs. 5 and 6, where the intensity of the detected resonance is $I(X) - I_0(X)$ in the difference trace, with the reference trace (in Figs. 5A and 6A) yielding the $I_0(X), I(X)$ needed from Equation 1. At pH 9.5 and 6.0 the protein is >95% metMbOH (B) and metMb (N), respectively. A $T_1$ determination for the optimally resolved methyl peaks $N_3$ (at pH 6.0) and $B_3$ (at pH 9.5) yield 3.5 ± 0.6 and 6.0 ± 1.0 ms, respectively, and represent the necessary intrinsic $T_1$ values needed in Equation 1. For the equilibrium $N \rightleftharpoons B$, $k_N$ and $k_B$ in Equation 3 were obtained directly from the reciprocal saturation of $B_3$ and $N_3$, respectively, in the pH range 6.6–7.5. The ratio of $k_B$ and $k_N$ is consistent with the observed mole fraction in Fig. 3. The rate of $k_B$ is found essentially pH-independent (to ±15%), while the rate $k_N$ increases strongly with pH, indicating base catalysis, as shown on the right side of Fig. 7. These pH rate profiles allow interpretation on the basis of the simple scheme:

$$k_B = k_1, \quad k_N = k_2[OH^-]$$

(Eq. 4)

which is the same mechanism as that reported for the sperm...
whale metMb (9, 10). The straight lines in Fig. 7 represent \( k_1 \) and \( k_2 \), and yield a pK \( \approx 7.8 \). The rate constants at 30 °C are given in Table II where the data can be compared to similar data for WT sperm whale metMb at 25 °C (10).

Both species A and N are significantly populated at any pH where A is clearly detected, so that the necessary intrinsic \( T_1 \) for a resonance for A could not be directly determined. However, the available \( T_1 \) for N3 allows the measurement of the saturation-transfer to N3 upon saturating A3 (Fig. 6E). This rate, \( k_{A3} \), was found to be essentially independent of pH in the pH range 3.7-4.5, as shown in Fig. 7. The reverse rate, \( k_{N3} \), is obtained via Equation 2 and the relative mole fraction in Fig. 3, which yield rates that increase rapidly at lower pH indicative of acid catalysis. This pH rate profile allows interpretation on the simple kinetic scheme:

\[
k_{A} = k_{1}, \quad k_{N} = k_{5}[H^{+}] \quad \text{(Eq. 5)}
\]

for which the straight lines on the left side of Fig. 7 yield the \( k_1 \), \( k_{5} \) values listed in Table II, and a pK \( \approx 4.0 \). Saturation of the resonance of A failed to result in any saturation transfer to D, indicating that the interconversion is too slow to measure by \(^1\text{H} \) NMR.

Sperm Whale VR-metMb

The pH influences on the \(^1\text{H} \) NMR spectra of 0.5 mM protein in \(^2\text{H}_2\text{O} \) in 0.1 M NaCl at 25 °C is illustrated in Fig. 8. At acidic to neutral pH, the spectrum is dominated by a species with chemical shifts diagnostic of a high-spin ferric form (21-24) which we designated N, with methyl peaks N1 with mean shift of 78 ppm and a strongly upfield broad resonance at ~25 ppm (Fig. 8, A and B). The non-selective \( T_1 \) values for the peak N1 is 3 ± 1 ms. There is no evidence for additional species below pH 6, although the heme methyl peaks exhibit some line broadening at pH 5 (not shown). The pattern of the four heme methyl shifts is very similar to that of two assigned metMb complexes (14, 25), so that the assignments 8-CH3, 5-CH3, 3-CH3, 1-CH3 in order of increasing upfield bias can be assumed. Increasing the pH into the alkaline region leads to intensity loss of peaks N and the appearance of a new set of peaks B, for the metMbOH species similarly labeled B (Fig. 8, C and D). Integration of peaks N and B, reflect a pK \( \approx 8.5 \) which is consistent with optical data (2). The pH independent shifts for methyl peak B, are listed in Table I; a non-selective \( T_1 \) ~6 ± 1 ms was obtained for methyl peak N2 and N1. However, unlike N, the species B exhibits a significant isotope effect on the heme methyl shifts, as listed in Table I.

The resolution of the spectra for the species N and B without significant line broadening dictates that the exchange rate is slow on the NMR time scale (<10⁴ s⁻¹), and hence much more similar to that in \( A. limacina \) than WT sperm whale metMb (10). Saturation of peak N2 in the pH range 8-9 leads to detectable (1-3% saturation) of the peak labeled B2 (not shown). The minimal but detectable saturation transfer was not possible to quantitate sufficiently to obtain information on the pH influence on the rate. However, the 1-3% saturation of peak B3 indicates that the rate \( k_{N_B} \) ~4 s⁻¹ at the pK \( \approx 8.7 \). This rate is a factor ~10 slower than for \( A. limacina \) metMb.

**DISCUSSION**

**Structure of the Complexes**—Four molecular species of \( A. limacina \) metMb which interconvert slowly on the \(^1\text{H} \) NMR time scale are identified. The dominant species at neutral (N) and alkaline pH (B) are clearly the previously characterized five coordinated metMb and six coordinate metMbOH forms, respectively. While the pure metMbOH (B) exhibits a \(^1\text{H} \) NMR spectrum too congested to even resolve most of the single proton peaks, the location and assignment of these resonances is readily achieved by saturation-transfer from the well-resolved resonances of metMb (N), whose resonances are well downfield from their counterparts in B. A similar strategy has been used to assign the Dolabella auricolaria metMbOH spectrum (30). The detection of a substantial isotope effect on the heme methyl contact shifts of metMbOH, (Table I), moreover, can be taken as direct evidence for a distal hydrogen bond interaction (26, 28).

Hence we conclude that, in contrast to metMb (A), metMbOH (B) has the Arg(E10) oriented into the heme pocket like in \( A. limacina \) metMbF, metMbN4, and metMbCN (15-18), with the guani-
FIG. 4. Plot of chemical shifts of *A. limacina* metMb in $^2$H$_2$O and in 0.1 M NaCl at 30 °C as a function of pH: the low-field resonances are labeled B, N, A, for molecular species B (metMbOH), N (metMb), and A, where *i* refers to the position of the heme (1–8), or the amino acid sequence (*i* = F8 is His (F8) C$_2$H). The solid lines simply connect the data points and do not represent fits to pK values.

**TABLE I**

$^1$H NMR chemical shifts for the various pH forms of *A. limacina* metMb and sperm whale VR-metMb

<table>
<thead>
<tr>
<th>Peaks Q $^<em>^</em>$ at pH 4.2</th>
<th>metMb(N) at pH 6.0</th>
<th>metMbOH(B) at pH 9.0 $^*$</th>
<th>Species A</th>
<th><em>A. limacina</em></th>
<th>VR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-CH$_3$</td>
<td>55.1 (&lt;0.1)$'$</td>
<td>57.2 (&lt;0.1)$'$</td>
<td>27.5 (&lt;0.5)$'$</td>
<td>25.2 (&lt;0.4)$'$</td>
<td>51.9 (&lt;0.1)$'$</td>
</tr>
<tr>
<td>3-CH$_3$</td>
<td>77.8 (&lt;0.1)</td>
<td>74.3 (&lt;0.1)</td>
<td>35.9 (&lt;0.7)</td>
<td>29.3 (&lt;0.5)</td>
<td>66.1 (&lt;0.1)</td>
</tr>
<tr>
<td>5-CH$_3$</td>
<td>88.7 (&lt;0.1)</td>
<td>84.3 (&lt;0.1)</td>
<td>39.5 (&lt;0.4)</td>
<td>34.7 (&lt;0.4)</td>
<td>69.3 (&lt;0.1)</td>
</tr>
<tr>
<td>8-CH$_3$</td>
<td>95.4 (&lt;0.1)</td>
<td>96.9 (&lt;0.1)</td>
<td>38.0 (&lt;0.5)</td>
<td>35.6 (&lt;0.3)</td>
<td>72.5 (&lt;0.1)</td>
</tr>
<tr>
<td>Ave CH$_3$</td>
<td>79.3 (&lt;0.1)</td>
<td>78.2 (&lt;0.1)</td>
<td>35.2 (&lt;0.5)</td>
<td>31.2 (&lt;0.4)</td>
<td>64.9 (&lt;0.1)</td>
</tr>
<tr>
<td>His(F8)CH$_3$</td>
<td>22.7</td>
<td>31.2</td>
<td>a</td>
<td>a</td>
<td>12.5</td>
</tr>
</tbody>
</table>

$^{*}$Q = N, B, A for the molecular species N, B and A, respectively.

$^*$Saturation of the single proton resonance N, of metMb (N) leads to the location and identity of the following peaks: B$_5$, 34.3, B$_6$, 25.0, B$_{6}'$, 33.4, B$_7$, 27.2, B$_{7}'$, 25.2.

$^*$Shift difference, in ppm, between $^2$H$_2$O and $^1$H$_2$O is given in parentheses; negative shifts indicate upfield bias in $^2$H$_2$O relative to $^1$H$_2$O.

$^*$Not assigned.
The $^1$H NMR spectral parameters support (21, 23–25) a five-coordinate structure for sperm whale VR-metMb species $N$ (i.e. $II$ in Fig. 1) as observed in the crystal structure (34). The absence of a coordinated water is further confirmed by the lack of isotope effect on the hyperfine shifts of the species $N$ for the double mutant (Table I). The chemical shifts for the alkaline species, $B$, on the other hand, are diagnostic for metMbOH, and the observation of a substantial isotope effect on the heme methyl peak $B_n$ in $A$. limacina metMb (Fig. 6, Table I), when compared to the heme methyl contact shifts, indicates that the axial bond is not broken, but is much weaker in $A$ than $N$ (metMb), which could conceivably result from an interaction between the iron and the protonated His(F8) imidazole ring, as shown in $IV$ of Fig. 1. On the other hand, the absence of a species similar to $A$ for sperm whale VR-metMb at pH 5 indicates that this titratable group is much less accessible in the double mutant.

The species, $D$, for $A$. limacina metMb detected at partial population at the lowest pH exhibits $^1$H NMR properties that suggest a denatured species, but one that is in equilibrium with both $A$ and $N$ (Fig. 3), and which reversibly refolds upon elevating the pH. $A$. limacina Mb has been shown to be remarkably stable and exhibits reversible unfolding at elevated temperature and in the presence of standard denaturing agents (38–40). The present data suggest that the protein can reversibly unfold also at acidic pH.

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Dynamics of Interconversion—The mechanism of the conventional acid ↔ alkaline transition in $A$. limacina is the same as that for sperm whale metMb (9, 10). However, the rates of interconversion are over two orders of magnitude slower in $A$. limacina metMb than sperm whale metMb. The pK in $A$. limacina metMb ($\sim7.7$) is different from that observed for sperm whale metMbH$_2$O ($\sim9.0$) in $H_2$O (10) and the change in pK of 1.3 results from a decrease in $k_5$ of $8 \times 10^4$-fold and a decrease in $k_6$ of $4 \times 10^5$-fold relative to sperm whale WT metMb (Table II). While the saturation transfer is too weak
Acid ↔ Alkaline Transitions

FIG. 6. Saturation transfer $^1$H NMR spectra of $A$. limacina metMb in $^2$H$_2$O and in 0.1 M NaCl at 30 °C and pH 4.5; under these conditions the protein exists as $-12\% A$, $-83\% N$ (metMb), and $-5\% D$. A, low-field reference spectrum with labeled resonances. B-D, saturate metMb, N, peaks $N_8$, $N_7$, $N_5$; note saturation transfer to species A peaks $A_8$, $A_7$, and $A_5$, respectively. E, saturate A species peaks $A_6$; note saturation transfer to metM, N, peak $N_6$. F, saturate axial His(F8) CBH peak $N_F^A$; note saturation transfer to the same proton peak in species A, $A_F^A$. The experiment in F was carried out at pH 3.9 where the population of A is increased and the ability to detect saturation transfer is improved. The peak $A_F^A$ is not readily detected in the reference spectrum because of the proximity of the intense diamagnetic region. Off-resonance saturation is labeled by a closed circle (○).

FIG. 7. Plot of the exchange rates for $A$. limacina metMb, $k_A$, $k_B$, for the equilibrium $N \rightleftharpoons B$ (triangles) and $k_N^A',k_B^A$ for the equilibrium $N \rightleftharpoons A$ in Equation 3 (squares) as a function of pH obtained by saturation transfer via Equation 1 and the $T_1$ values in Table I (open marker) and Equation 2 (closed markers). The straight lines are the best fits to pH independent $k_0 = k_A$, $k_B \longrightarrow k_1$, base catalyzed (with unit slope) $k_0 = k_2 [\text{OH}^-]$ and acid catalyzed (with unit slope) $k_0 = k_3 [\text{H}^+]$. The resulting rate constants are listed in Table II.
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TABLE II

| Rate constants for the interconversion of pH modulated species for A. limacina metMb, sperm whale WT metMb, and the double mutant VR-metMb |
|---|---|---|
| In 
| A. limacina | Sperm whale WT | Sperm whale VR |
| $k_1$, s$^{-1}$ (B $\rightarrow$ N) | $2.0 \pm 0.8 \times 10^4$ | $1.6 \pm 0.6 \times 10^5$ | $4 \pm 2$ |
| $k_2$, M$^{-1}$ s$^{-1}$ (N $\rightarrow$ B) | $3.2 \pm 1.1 \times 10^3$ | $1.3 \pm 0.5 \times 10^5$ | $2 \pm 1 \times 10^5$ |
| $k_1'$, s$^{-1}$ (A $\rightarrow$ N) | $2.0 \pm 0.8 \times 10^4$ | $1.3 \pm 0.5 \times 10^5$ | $2 \pm 1 \times 10^5$ |
| $k_2'$, M$^{-1}$ s$^{-1}$ (N $\rightarrow$ A) | $2.0 \pm 0.9 \times 10^6$ | $1.3 \pm 0.5 \times 10^5$ | $2 \pm 1 \times 10^5$ |

*Data at 30 °C obtained from the rate data in Fig. 6 and the mechanism in Equation 5.

*Data at 25 °C taken from Ref. 10.

*Estimated for $k_3$ at the pK (~8.5) assuming the mechanism is given by Equation 4.

Fig. 8. 500 MHz $^1$H NMR spectra of 0.5 mM sperm whale VR-metMb in $^2$H$_2$O and in 0.1 M NaCl at 25 °C, pH 5.9, with the sole species as VR-metMb and designated N with methyl peak N, and His(F8) CαH peak NF8 (A). B, pH 7.5, showing the appearance of a set of peaks B, for species B (VR-metMbOH). C, pH 8.6 where the areas of methyl peak N, and B, are similar. D, pH 9.7 where the species B (VR-metMbOH) is fully formed.

to establish the mechanism for the N ↔ B interconversion in sperm whale VR-metMb, the observation of the same mechanism, (i.e. Equation 4) for both A. limacina and WT sperm whale metMb (9, 10) suggests that the same mechanism applies to the double mutant VR-metMb. Thus the factor ~10 decrease in $k_b$ at more alkaline pH (~8.5) than in A. limacina metMb indicates a reduction in $k_2$ for the double mutant by ~160, with largely conserved $k_1$, as compared to A. limacina metMb (Table II).

The much slower interconversion N ↔ B in A. limacina metMb and the sperm whale double mutant VR-metMb than in WT sperm whale metMb can be attributed to the fact that in these former two proteins, in contrast to WT sperm whale Mb where the two species differ only in a proton (6, 7, 9, 10), the neutral (N) ↔ alkaline (B) transition involves the breaking of the iron-oxygen bond; additionally the interconversion reflects a protein conformational change in that the Arg(E10) side chain must reorient from extended into solution in metMb (N), to reinserted into the pocket and hydrogen bond in the bound hydroxide ion in metMbOH (B). This interaction is essential to provide significant stability to the hydroxide complex of ferric heme. The somewhat faster interconversion rate in A. limacina relative to that in the sperm whale VR-metMb may reflect an intrinsically more flexible nature of the former protein. It has been observed that the reorientation of the prosthetic group about its α,γ-meso axis in the folded protein is significantly more rapid in A. limacina than WT sperm whale Mb (41, 42).

The acid catalyzed mechanism of the N $\rightarrow$ A conversion supports the notion that the rate-limiting step is the protonation of some residue. The slow rate, however, suggests that the residue is not readily accessible and/or that the process involves more than just the addition of a proton. Since the protonation step clearly significantly weakens the axial bond, as reflected in the strong decrease in the His(F8) CαH contact shift in A compared to that in N, the coordinated imidazole side chain itself may reasonably be the protonation site in A. A pK ~4 for the axial His imidazole has been deduced for ferrous A. limacina MbCO and MbNO derivatives based on the rate of CO combination as well as absorbance and EPR spectroscopy (43, 44).

Role of Distal Hydrogen Bonding in metMb—Sperm whale metMb coordinates a water molecule at low to neutral pH and a hydroxide ion at alkaline pH. A. limacina metMb and sperm whale VR-metMb, with the E7 occupied by Val but the E10 occupied by Arg (12), fail to bind H$_2$O at low to neutral
pH, but readily bind hydroxide at alkaline pH. The sperm whale His(E7)Val metMb point mutant does not possess a coordinated water at low to neutral pH (24, 45). Moreover, as shown by optical spectroscopy, complexation with hydroxide ion is incomplete even at pH 11 (35), indicating that the stability of the bound hydroxide is sharply reduced in the absence of both His(E7) and Arg(E10). These results find ready interpretation in terms of the type of hydrogen bonding that is necessary to stabilize the two ligands of interest, water and the hydroxide ion (7). A coordinated water molecule serves as a hydrogen bond donor and is stabilized only by a distal hydrogen bond acceptor residue, such as the His(E7) residue imidazole with the imidazole proton on N rather than N, (i.e. I in Fig. 1). On the other hand, the Arg(E10) guanidinium group can serve only as a hydrogen bond donor, and since both the His(E7) and Arg(E10) residues imidazole with the imidazole proton on Na rather than Na, these results find stability of the bound hydroxide is sharply reduced in the absence of both His(E7) and Arg(E10). These results find ready interpretation in terms of the type of hydrogen bonding involved in the control of binding of water and hydroxyl ion

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