Nuclear Magnetic Resonance Investigation of the Electronic Structure of Deoxymyoglobin

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Received December 4, 1992

Abstract: The heme methyl resonances of sperm whale high-spin ferrous or deoxymyoglobin, Mb, have been assigned by a combination of ^1H and ^2H NMR of the protein reconstituted with specifically deuterated hemes. The complete methyl assignments were carried out on deoxyMb with the native protoheme in both the equilibrium orientation and the reversed orientation differing by 180° rotation about the α,γ-meso axis that is populated transiently upon assembly of apomyoglobin with protoheme. The ^1H NMR spectra of deoxyMb reconstituted with a variety of synthetic hemes also lead to specific assignment of pyrrole proton signals. The heme methyl assignments for the reversed protoheme orientation are also provided for the high-spin ferric or metaquo derivative. The pattern of the dominant heme methyl contact shift in the two heme orientations of low-spin and high-spin metMb are shown to be interpretable solely on the basis of the imposition of a fixed protein-based rhombic influence on the heme in-plane asymmetry, with the net equatorial bonding to the heme conserved for the altered orientations. It is shown, moreover, that the equilibrium, as well as rates of ligation, in the conversion of metaquoMb to metcyanoMb, are independent of heme orientation. The much larger magnitude as well as the distinctive pattern of the protein-imposed rhombic influence in low-spin as compared to high-spin metMb is shown to be characteristic of an orbitally degenerate ground state of the low-spin d^5 system. Analysis of the heme methyl contact shifts for the two heme orientations of deoxyMb reveals a magnitude and pattern of the protein-induced rhombic influence that strongly favor an orbital ground state derived from ^5E rather than $^5B_2$ in 4-fold symmetry. However, significant and systematic differences in the mean heme methyl contact shift and proposed E7 Val dipolar shifts in the two heme orientations provide evidence that there are likely contributions from two electronic states to the hyperfine shift pattern and that the relative contributions of the two states differ with heme orientation. Since these significant differences in the deoxyMb NMR characteristics for the two heme orientations do not manifest themselves in any ligation properties of the reduced protein, considerable caution should be exercised in interpreting heme hyperfine shifts in deoxyMb in terms of structural or functional properties.

Introduction

The hyperfine shifts exhibited by paramagnetic metalloproteins in general,[1,2] and hemoproteins in particular,[3-5] provide a wealth of unique information on the electronic, magnetic, and molecular structural properties of the active site. Crucial to extracting this information from NMR spectra are the unambiguous assignment of the resonances, an interpretive basis of the hyperfine shifts in terms of scalar contact and dipolar contributions, and information on the location of the metal unpaired spins (orbital ground state) that delocalize spin density into the heme.[1-8] The metalloproteins involved in oxygen transport and storage,[9] hemoglobin, Hb, and myoglobin, Mb, constitute the most extensively investigated systems in the low-spin ($S = {}^1/_2$) and high-spin ($^5/_2$) ferric states. The earliest definitive assignments were provided by reconstitution of Mb with isotope labeled hemes which identified the heme methyl and vinyl signals in a variety of ferric Mb, Hb derivatives.[10-13] This was followed by the application of steady-state nuclear Overhauser effect, NOE, experiments to extend the assignments to the heme propionates.[14,15] More recently modern 2D NMR methods[8] have been shown to have surprising utility in such paramagnetic systems.[16,17]

The low-spin ($S = {}^1/_2$) ferric derivatives of Mb, Hb exhibit large magnetic anisotropy and a simple π-spin delocalization mechanism which allow remarkably detailed interpretations of their hyperfine shifts.[4-7,18] Their relatively narrow lines, moreover, have allowed the most comprehensive application of modern 2D methods[16,17] and hence low-spin ferric systems constitute the most studied and best understood class of paramagnetic metalloproteins. The high-spin ($S = {}^5/_2$) ferric derivatives exhibit broad lines, but

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the large contact shifts lead to the resolution of the resonances for the coordinated heme and axial ligands,[4-8] with the result that assignments exist for a number of proteins. The dominant contact shift pattern for coordinated heme and axial ligand can be strongly correlated to molecular/electronic structural details of the active site.[12,14,15,19]

The third of the relatively stable and common oxidation/spin states of hemoproteins, high-spin iron(II), has been the least studied by ^{1}H NMR, and the pattern of the heme hyperfine shifts in proteins is obscure.[3-7] This is in spite of the fact that the high-spin ferrous or deoxy form is the only physiologically relevant paramagnetic oxidation/ligation state for Hb, Mb.[9] The very limited understanding of the heme hyperfine shifts in proteins is due to the lack of assignments. The ^{1}H NMR spectra exhibit only relatively small hyperfine shifts[4,5,20-25] which, in conjunction with the strong contribution from the field dependent Curie relaxation[25,26] and the intense diamagnetic envelope in a protein, leads to poor resolution at any field strength. In the several cases where unassigned deoxyMb, Hb spectra have been reported,[20-30] the intensity of the resonances indicate that generally only one or two of the heme methyls are resolved. However, such unassigned methyl resonances have been shown to be sensitive probes for the influence of subunit interface mutations in Hb[4,22,23] and heme shift pattern differences have been proposed[24] to reflect structural differences in deoxyMbs. The lack of information on the heme hyperfine shift pattern is in contrast to that on the axial ligand, where the large σ contact shift and unambiguous assignment[27-29] for the His F8 $N_\delta H$ have allowed the use of this resonances as a sensitive indicator for the modulation of axial bonding and a marker for Hb quaternary state.[29-31] The relevance of the electronic structure of deoxy Mb, Hb to understanding oxygen binding, together with the recent availability of synthetic point mutants for both proteins,[32,33] underscore the importance of more carefully evaluating the information content of deoxyMb, Hb heme hyperfine shifts.

In this report we pursue quantitative and complete assignment of the heme methyl signals in two deoxyMb samples selected to provide information on the interpretive basis of their hyperfine shift patterns. Studies on model compounds have indicated that the heme shifts are dominated by the contact term,[6,7] with contributions from both σ and π spin delocalization, and hence the four methyls serve as orientationally invariant reporter groups on the distribution of the unpaired spin density among the four pyrroles. As candidates for two alternate complexes for which to carry out the comparison, we select the two derivatives of

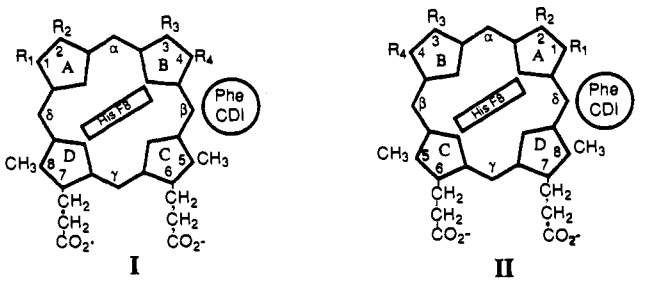

Figure 1. Orientations of a heme in the pocket of Mb that differ by a 180° rotation about the α, γ-meso axis, with (I) the crystallographically defined[34] orientation and (II) the reversed orientation for native protoheme ($R_1 = R_3 = CH_3$, $R_2 = R_4$ = vinyl). The position of the axial His 93(F8) imidazole plane and the invariant Phe 43(CD1) are shown.

sperm whale Mb with the native protoheme ($R_1 = R_3$ = methyl, $R_2 = R_4$ = vinyl in Figure 1) oriented in the pocket as defined in the crystal structure[34] shown as orientation I in Figure 1, and that with the heme rotated 180° about the α,γ-meso axis shown as orientation II in Figure I. The latter form is marginally populated (~8%) at equilibrium but occurs transiently at ~50% population in the initial stages of the assembly of heme and apoprotein, as discussed in detail previously.[11,13,35,36]

The basis for selecting these two alternate heme orientations of the same Mb is that functional studies in the reduced state[37] as well as the oxidized state (see below) show that both the thermodynamics and dynamics of ligation are unaffected by heme orientation. However, because the heme interacts predictably and differentially with the protein matrix in the two orientations (see Figure 1), it is possible to anticipate the relationship of the NMR properties between the two forms. Just as importantly, it is possible to characterize by ^{1}H NMR these same two Mb forms in each and every oxidation/spin state, most notably the low-spin metMbCN[13] and high-spin metMbH_2O,[35] and thereby compare the influence of a rotation of the protein rhombic influence by 180° about the heme α,γ-meso axis in the three paramagnetic forms. While the assignment of the heme resonances in the various native Mb complexes could likely be accomplished completely by 2D NMR methods,[8] the need to obtain the methyl assignments in the transiently populated orientation II dictates the use of specifically deuterated hemes. Moreover, in order to provide more details on the nature of the spin delocalization mechanism,[6,7] the deoxyMb reconstituted with deuteroheme ($R_1 = R_3 = CH_3$, $R_2 = R_4$ = H in Figure 1), pemptoheme ($R_1 = R_3 = CH_3$, R_2 = H, R_4 = vinyl), and isopemptoheme ($R_1 = R_3 = CH_3$, R_2 = vinyl, R_4 = H) will also be investigated.

Experimental Section

Protein Handling. Sperm whale metMb was purchased from Sigma Chemical Co. as a lyophilized powder; sodium dithionite and hydroxycobalamine were obtained from Nakarai Chemicals (Japan) and Strem Chemicals, respectively. ApoMb was prepared by the standard acid/2-butanone extraction method of Teale.[38] The heme free apoMb was reconstituted with native protoheme ($R_1 = R_3 = CH_3$, $R_2 = R_4$ = vinyl in Figure 1) and a variety of its deuterated derivatives as well as a series of completely synthetic hemes. The hemin was dissolved in minimal 0.01 M NaOH and added, with stirring, to cold apoMb in 0.1 M phosphate buffer (pH 6.7–7.0). The protein was dialyzed against distilled water at 5 °C to remove buffer and then lyophilized. The lyophilized protein was stored at −20 °C until use. For samples in which the transiently ~50% populated orientation II is to be retained for spectral investigation, the

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Table I. Chemical Shifts for Resonances in DeoxyMb Containing Various Hemes[a,b]

	protoheme[c]		deuteroheme			
assignment	I[d]	II[e]	I[d]	II[e]	pemptoheme I[d]	isopemptoheme I[d]
1-CH_3	[7.4]	16.5 [15.7]				
3-CH_3	11.2 [10.9] (50)[f]	14.1 [13.8]				
5-CH_3	16.5 [16.1] (50)	10.4 [10.1]	14.1(?)[g]		14.9(?)	16.6(?)
8-CH_3	[7.8]	18.5 [17.8]		17.4(?)		
2-H			42.0	67.0	41.0	
4-H			61.4	45.8		61.8
His F8 $C_\delta H/C_\epsilon H$	~44	40				
His F8 $N_\delta H$	78.5	78.5	78.1	77.1		
Val E11 $C_{\gamma 2}H_3$	−4.3 (20)	−4.7				
Val E11 $C_{\gamma 1}H_3$	−7.4 (6)	−10.3	−3.5	−8.3	−5.0	−6.9

[a] ^1H NMR in ppm from DSS at ^2H_2O at 25 °C (except for His F8 $N_\delta H$ in ^1H_2O), pH 8.3. [b] ^2H NMR shifts, in ppm from DSS in ^1H_2O at 25 °C, are given in square brackets. [c] Single proton frequencies for orientation I are 14.0, 13.7, ~13.5, 12.8, and 11.2 ppm and for orientation II are 14.0, 13.5, 13.3, and 12.4 ppm. [d] Heme orientation I in Figure 1. [e] Heme orientation II in Figure 1. [f] ^1H NMR T_1 values, in ms (uncertainty ±15%), are given in parentheses. [g] Assignment obtained by analogy with native deoxy Mb are indicated by (?).

dialysis of the metMbH_2O was omitted in order to prevent extensive equilibration of the protein to its unique heme orientation. For deoxyMb studies on the freshly reconstituted sample, 2 equiv of KCN were added to the hemin prior to reconstitution, leading to the orientationally disordered metMbCN with a "frozen" ratio of isomers close to 1:1 (the metMbCN complex exhibits the slowest heme reorientation rate of Mb).[36] The excess CN^- was removed by ultrafiltration. DeoxyMb was prepared at pH 8.3 in degassed solvent by the direct addition of dithionite to the metMbCN sample. ^1H NMR samples were 2 mM in 0.5 mL of 90% ^1H_2O; 10% ^2H_2O or 99.9% ^2H_2O, while ^2H NMR samples were ~1 mM in 1.5 mL of 99% ^2H depleted ^1H_2O. Sample pH was adjusted by 0.2 M ^2HCl or 0.2 M NaO^2H and measured on a Beckman 3550 pH meter with an Ingold microcombination electrode; pH values are not corrected for the isotope effect.

Deuterated and Synthetic Hemins. Since the isotope labeling studies using protoheme were carried out piecemeal over a period of about 10 years, a variety of different labeled hemins were prepared based on the nature of the particuar planned experiment. The preparations of the protoheme ($R_1 = R_3 = CH_3$, $R_2 = R_4$ = vinyl in Figure 1) deuterated at two methyl positions[39] are the same as reported previously[12,13] (with the percent deuteration in parentheses), [1-C^2H_3(~70%),3-C^2H_3-(~75%)]heme, [1-C^2H_3(70%),8-C^2H_3(90%)]heme, and [1-C^2H_3(65%),5-C^2H_3(90%)]heme. The hemes with single methyl deuteration, [5-C^2H_3-(~70%)]heme and [8-C^2H_3 (~70%)]heme, were prepared as described elsewhere.[40] Deuteroheme ($R_1 = R_3 = CH_3$, $R_2 = R_4$ = H in Figure 1), pemptoheme ($R_1 = R_3 = CH_3$, R_2 = H, R_4 = vinyl), and isopemptoheme ($R_1 = R_3 = CH_3$, R_2 = vinyl, R_4 = H) were prepared by standard methods.[41,42] The 2-fold symmetric protoheme **III** ($R_1 = R_4$ = vinyl, $R_2 = R_3 = CH_3$) and protoheme **XIII** ($R_1 = R_4 = CH_3$; $R_2 = R_3$ = vinyl) type isomers were prepared as reported previously.[43]

NMR Spectra. ^1H NMR spectra of deoxyMb samples were recorded at 200 or 300 MHz on Nicolet NTC-200 and General Electric Ω-300 spectrometers operating in quadrature mode and consisted of ~6–20 × 10^3 scans of 4096 points over a bandwidth of ±12 kHz (for ^2H_2O) or ±20 kHz (for ^1H_2O). The spectra were zero-filled to 8192 points and apodized to introduce 10 Hz line broadening. ^1H NMR spectra of metMbH_2O in ^2H_2O were recorded at 360 MHz on a Nicolet NTC-360 operating in quadrature mode and consisted of ~8–15 × 10^3 scans of 4096 points over a bandwidth of ±45 kHz. The data were zero-filled to 8192 points and apodized to introduce 20 Hz linebroadening. In all cases the solvent signal was suppressed by a presaturation pulse from the decoupler. The nonselective T_1 of resolved ^1H NMR resonances in native deoxyMb were obtained at 300 MHz using the inversion-recovery pulse sequence. ^2H NMR spectra at 76.7 MHz were recorded on a Nicolet NT-500 operating in quadrature mode with 2–10 × 10^4 scans of 4096 points over a bandwidth of ±10 kHz; the 90° pulse was 22 μs. The data were zero-filled to 8192 points and apodized to introduce 10–20 Hz line broadening. All chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, via the residual solvent signal.

Results

DeoxyMb Assignments. The resolved portions of the 200 MHz ^1H NMR trace of native deoxyMb in ^2H_2O at 25 °C and pH 8.3 is illustrated in Figure 2A; signals for methyls and single protons are labeled M_i, H_i for the domiannt A-orientation of the heme. A broad signal (line width ~1 kHz) is observed at ~45 ppm (not shown; supplementary material). Variable temperature and pH studies had shown[20] that peaks M_a, M_b exhibited intensity for methyls, with a single proton, H_e, under M_b to account for its greater intensity than M_a. Four other frequencies, H_a–H_d (listed in Table I), are observed in the 12–15 ppm window, which intensity considerations indicate contain ~5–6 single proton peaks; the signal at H_d has been shown to consist of two protons. The upfield window exhibits one strongly relaxed methyl peak M_z and one intermediate relaxed methyl peak, M_y. The shifts and T_1s for resolved lines are listed in Table I.

The ^1H NMR spectrum of deoxyMb freshly reconstituted with native protoheme is illustrated in Figure 2B, where additional peaks are observed that arise from the protein with the reversed or orientation II; resonances for this species are labeled by lower case m_i (methyls) and h_i (single protons). An additional broad peak (line width ~1 kHz) is detected at ~40 ppm (not shown; see supplementary material). Key observations are resolved obvious methyl peaks, low field m_a, and strongly relaxed upfield m_z, of which the latter must be the counterpart to M_z. The relative intensities of M_z:m_z are ~55:45 (as confirmed by ferricyanide oxidation and a CN^- trap (not shown)). Using H_d or M_z as a standard for orientation I, the peak at 16.5 in Figure 2B must be a composite of methyls for both orientations, i.e., M_a and m_b. The two new relatively intense peaks at 14.1 and 10.6 likely arise from methyls (see below) and are labeled m_c, m_d, respectively. Single proton peaks from orientation II appear at 14.9 (h_a), 13.8 (h_b), 13.6 (h_c), and 11.6 ppm (h_d), but others are likely coincident with peaks from orientation I. The ^1H NMR spectra for the samples in Figure 2 (parts A and B) each exhibit one additional peak in ^1H_2O at 78.7 ppm which can be assigned directly to the His F8 $N_\delta H$[27] (not shown; see supplementary material). The fact that a single peak with unaltered line width is observed for the orientationally disordered sample, with area indicative of one proton from each of orientations I and II, dictates that the two isomers exhibit identical $N_\delta H$ shifts.

DeoxyMb samples freshly reconstituted with [1,8-$(C^2H_3)_2$]-protoheme and [1-3-$(C^2H_3)_2$]protoheme yield the ^1H NMR traces in Figure 2 (parts C and D, respectively). Loss of intensity in Figure 2C but not Figure 2D uniquely identifies m_a as 8-CH_3, while reduction in intensity in Figure 2D but not Figure 2C identifies both m_c and M_b as 3-CH_3. The retained intensity in

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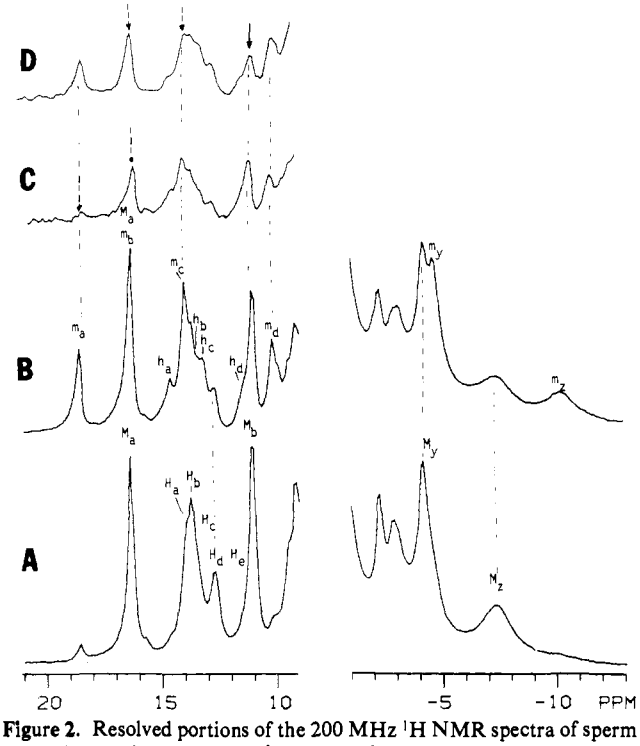

Figure 2. Resolved portions of the 200 MHz ^1H NMR spectra of sperm whale deoxyMb complexes in ^2H_2O at 25 °C, pH 8.3: (A) native protein with orientation I of protoheme, with apparent methyl peaks M_i and single proton peak positions, H_i; (B) freshly reconstituted protein that contains comparable population of protoheme in orientations I and II; the new apparent methyl (m_i) and single proton peak positions (h_i) for orientation II are labeled; (C) freshly reconstituted protein that contains comparable populations of the two heme orientations of [1,8-$(C^2H_3)_2$]-protoheme; (D) freshly reconstituted protein with comparable populations of the two orientations of [1,3-$(C^2H_3)_2$]protoheme. Methyl peaks in traces C and D that lose intensity due to deuteration for orientations I and II are indicated by solid and dotted vertical arrows, respectively.

both parts C and D in Figure 2 argue that m_d, if it is a heme methyl, must arise from 5-CH_3. Lastly, the reduction of the intensity of the composite at 16.5 ppm in both parts C and D in Figure 2, together with completely retained intensity of M_a when the sample had equilibrated (not shown; see supplementary material), is consistent with m_b as 1-CH_3 but not 3-CH_3 (see below) and demands that M_a is 5-CH_3. The ambiguities of some of the assignments in the low field region for the reversed heme orientations reflects an inherent problem with deoxyMb ^1H NMR, namely poor spectral resolution.

In order to resolve the potential ambiguity of the m_b assignment, confirm the assignment of m_d to 5-CH_3, and locate the two heme methyls not resolved in the low field region for orientation I, we turn to direct 76.7 MHz ^2H NMR detection of three freshly reconstituted deoxyMb samples containing [5-C^2H_3]protoheme, [8-C^2H_3]protoheme, and [1,3-$(C^2H_3)_2$]protoheme, whose spectra are illustrated in A, B, and C, respectively, of Figure 3. The more intense peak at 16 ppm in Figure 3A (oxidation in the presence of CN^- confirms a 3:1 A:B ratio) confirms the assignment of M_a to 5-CH_3 and establishes that the orientation II peak m_d indeed arises from the heme 5-CH_3. The trace in Figure 3B confirms the lowest field orientation II peak m_a as 8-CH_3 and locates the orientation I 8-CH_3 peak at 7.8 ppm. Lastly, the trace in Figure 3C confirms M_b as 3-CH_3, locates the orientation I 1-CH_3 under the diamagnetic envelope at 7.4 ppm, and confirms the orientation II peak m_b as 1-CH_3 and m_c as 3-CH_3, as suggested by the ^1H NMR spectra in Figure 2. Note that, while ^2H NMR spectrum in Figure 3C proves m_b, m_c arise from either 1-CH_3 or 3-CH_3, the ^1H NMR data in Figure 2 (parts B and C) are needed to differentiate 1-CH_3 from 3-CH_3. The ^2H NMR line widths of the various methyl peaks are ~60–70 Hz, as reported previously

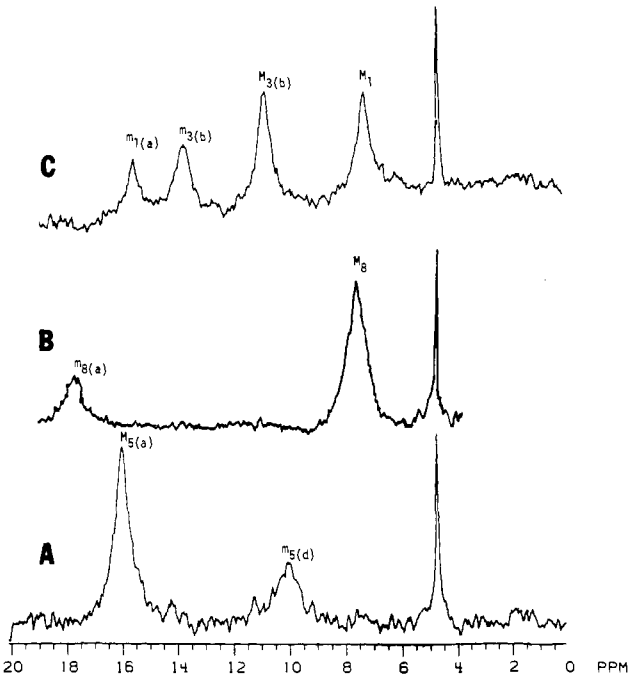

Figure 3. 76.7 MHz ^2H NMR spectra of freshly reconstituted deoxyMb in ^2H-depleted ^1H_2O at 25 °C, pH 8.4, that contains both heme orientation of (A) [5-C^2H_3]protoheme, (B) [8-C^2H_3]protoheme, and (C) [1,3-$(C^2H_3)_2$]protoheme. The methyl peaks are labeled M_i, m_i for orientations I and II, respectively. Although the degree of deuteration of the 1-CH_3 and 3-CH_3 differs slightly, the signal-to-noise is insufficient to differentiate the two orientation II signals by intensity; this distinction is achieved in the ^1H NMR spectra in Figure 2.

for both metMbH_2O and metMbCN and reflect solely quadrupolar relaxation.[44]

The ^1H NMR spectra of deoxy deuterohemeMb in ^1H_2O freshly reconstituted and after equilibration as the cyano–met complex[45] are illustrated in Figure 4, parts A and B, respectively; the lowest field peaks, H_p and h_p, are absent in ^2H_2O and can be assigned[27] to the His F8 $N_\delta H$ for orientations I and II. The chemical shifts for the lowest field obvious methyl peak, M_a and m_a, for orientations I and II, respectively, are similar to the peaks with the same label for protoheme Mb. The ^1H NMR traces of equilibrated deoxypemptohemeMb ($R_1 = R_3 = CH_3$, $R_2 = H$, R_4 = vinyl) and isopemptoheme-Mb ($R_1 = R_3 = CH_3$, R_2 = vinyl, $R_4 = H$) in ^2H_2O are shown in Figure 4, parts C and D, respectively. Comparison of traces in Figure 4 (parts A and B) identify the 2-H, 4-H peaks H_q, H_r for orientation I, and h_q,h_r for orientation II, and the essentially unaltered shifts of the only resonances in the 30–70 ppm window for pemptoheme (possesses only 2-H) and isopemptoheme (possesses only 4-H) identify the individual peaks for orientation I of deoxydeuterohemeMb as 2-H for H_r and 4-H for H_q. The chemical shifts are included in Table I.

metMb Assignments. The hyperfine shifted resonances in ^1H NMR spectra of native metMbH_2O, as shown in Figure 5A, have been completely assigned by isotope labeling[12] and NOEs,[14] and the location, but not the assignment, of the heme methyl resonances established[35] for orientation II in the freshly reconstituted sample. The individual assignments of the methyls in orientation II of metMbH_2O are illustrated in Figure 5, where traces in parts D, E, and F are those of metMbH_2O immediately reconsituted with unlabeled protohemin, [1,3-$(C^2H_3)_2$]protohemin, and [1,8-$(C^2H_3)_2$]protohemin, respectively, which leads to the unambiguous assignments of m_a, m_b, m_c, m_d to 5-CH_3, 8-CH_3, 3-CH_3, and

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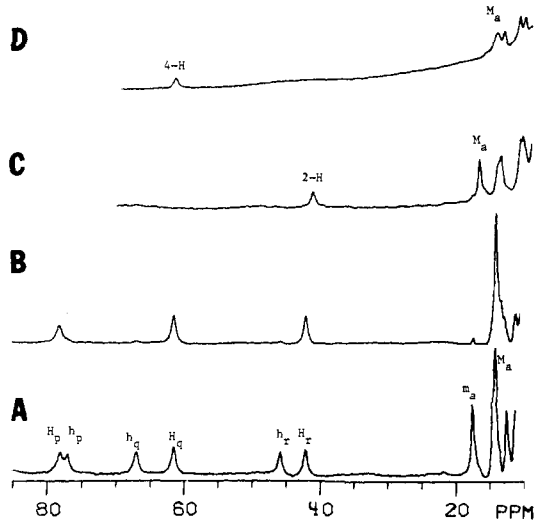

Figure 4. Resolved low-field portion of the 200 MHz ^1H NMR at 25 °C, pH 8.3 of deoxy Mb complexes reconstituted with chemically modified hemes. (**A**) Freshly reconstituted protein in ^1H_2O containing both orientations of deuteroheme ($R_1 = R_3 = CH_3$, $R_2 = R_4 = H$ in Figure 1) and (**B**) equilibrated protein in ^1H_2O with primarily orientation **I** of deuteroheme. (**C**) Equilibrated protein in ^2H_2O containing pemptoheme ($R_1 = R_3 = CH_3$, $R_2 = H$, R_4 = vinyl) in orientation **I** and (**D**) equilibrated protein in ^2H_2O containing isopemptoheme ($R_1 = R_3 = CH_3$, R_2 = vinyl, $R_4 = H$) in orientation **I**. The apparent methyls for orientations **I** and **II** are labeled M_i and m_i, respectively, and the resolved single proton peaks in orientations **I** and **II** are labeled H_i and h_i, respectively. Peak H_a in traces A and B and peak h_a in trace A are absent in ^2H_2O solution and are assigned to His F8 $N_\delta H$.

1-CH_3, respectively. The chemical shifts for the methyls in metMbH_2O for both orientations of native protoheme are listed in Table II. The ^1H NMR spectra of metMbH_2O reconstituted with the two 2-fold symmetric protohemin type isomers, protohemin III ($R_1 = R_4 = CH_3$, R_2, R_3 = vinyl) and protohemin XIII (R_1, R_4 = vinyl, R_2, $R_3 = CH_3$), are included in Figure 5, parts B and C, respectively. Comparison of the spectra of the assigned native protoheme metMbH_2O[12] in Figure 5A with that of protoheme *III* metMbH_2O in Figure 5B (which differ in 3-CH_3, 4-vinyl → 3-vinyl, 4-CH_3) reveals three peaks with essentially unaltered shifts, M_8, M_5, and M_1, which must arise from the conserved positions 8-CH_3, 5-CH_3, and 1-CH_3, respectively, and one strongly changed position, M_4, which must originate from the unique 4-CH_3 of protoheme III. Similarly, comparison of native metMbH_2O in Figure 5A and protoheme XIII metMbH_2O in Figure 5C reveals inconsequentially unchanged shifts for the conserved M_8 (8-CH_3), M_5 (5-CH_3), and M_3 (3-CH_3), and a new position for the unique M_2 (2-CH_3) of protoheme XIII metMbH_2O close to M_3. The strong similarity of the shifts for M_1 and M_4, and for M_2 and M_3, in contrast to the differences in shifts between the pairs M_1, M_4 and M_2, M_3, is noteworthy.

Lastly, while all four heme methyls for orientation **I** *and the* three resolved heme methyls for heme orientation **II** in reconstituted metMbCN have been assigned,[10,13,44] the unresolved 1-CH_3 for orientation **II** has not been reported. The ^2H NMR detection of the freshly reconstituted metMbCN with both heme orientations confirms the 3-CH_3 of orientation **I** at 4.9 ppm[44] and locates the orientation **II** 1-CH_3 at 7.9 ppm (not shown). The methyl shifts are included in Table II.

Cyanide Ligation in metMb. ^1H NMR spectroscopy is unique in providing a rapid and quantitative assessment of the relative ligand affinities and rates for the two protoheme orientations in orientationally disordered Mb in the reaction

$$\text{metMb}H_2O + CN^- \rightleftarrows \text{metMbCN} + H_2O \qquad (1)$$

Since orientations **I** and **II** yield resolved resonances in both the metMbH_2O[35] and metMbCN[13] complexes, it is possible by signal intensity to simultaneously determine the position of equilibrium 1 for the two heme orientations. Figure 6 displays the spectral region 100–80 ppm and 28–26 ppm region where the resolved 5-CH_3 (M_5,m_5) and/or 8-CH_3 (M_8,m_8) resonate for the four species. Addition of ~25% mol equiv of KCN to a freshly reconstituted metMbH_2O sample leads to the ^1H NMR spectra in Figure 6A; the ratio of M_5:m_8 in both high-spin and low-spin forms is ~6:4, although this ratio is not as clear for metMbH_2O peaks at the apodization of 10 Hz used to allow resolution of the metMbCN peak. The addition of excess of CN^- before and after recording trace A yields the ^1H NMR traces in Figure 6, parts B and C, respectively. Note the ratio of M_5:m_8 ~6:4 is observed under conditions of partial and complete ligation, establishing that cyanide ligation is described by the same (but here not numerically defined) binding constant. Comparison of traces B and C in Figure 6 confirms that negligible heme reorientation occurred while obtaining the spectrum in trace A.

The rate of the back reaction in equilibrium 1 is determined by the slow cyanide off-rate. It has been shown[46,47] that the cyanide off-rate in low-spin hemoproteins can be determined by the rate of loss of the low-spin peak intensities (and the appearance of high-spin peaks) in the presence of excess hydroxycobalamine, which has a much higher CN^- affinity than metMbH_2O.[48] Addition of a 5-fold excess of hydroxycobalamine to a sample

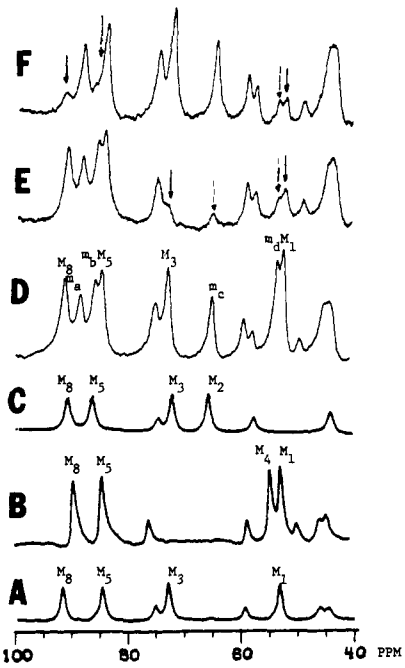

Figure 5. Low-field portions of the 360 MHz ^1H NMR spectra of metMbH_2O complexes in ^2H_2O at 25 °C, pH 6.2. (**A**) Native Mb with protoheme in orientation **I**: the previously assigned[13] methyls are labeled M_i for i = 1, 3, 5, 8. (**B**) Protein reconstituted with the 2-fold symmetric protoheme-**III** ($R_1 = R_4 = CH_3$, $R_2 = R_3$ = vinyl in Figure 1); note appearance of M_4 near M_1 and the absence of M_3 relative to trace A. (**C**) Protein reconstituted with the 2-fold symmetric protoheme-**XIII** ($R_1 = R_4$ ≈ vinyl, $R_2 = R_3 = CH_3$ in Figure 1); note new peak M_2 near M_3 and absence of peak M_1 relative to trace A. (**D**) Freshly reconstituted protein containing native protoheme in orientations **I** (~60%) and **II** (~40%); the methyl peaks for orientation **II** are labeled m_i. (**E**) freshly reconstituted protein containing [1,3-$(C^2H_3)_2$]protoheme with orientations **I** (55%) and **II** (45%). (**F**) Freshly reconstituted protein containing [1,8-$(C^2H_3)_2$]protoheme in orientations **I** (~50%) and **II** (~50%). Loss of methyl peak intensity in traces E and F for orientations **I** and **II** is indicated by solid and dotted vertical arrows, respectively.

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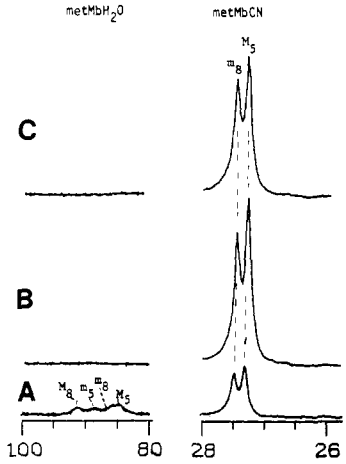

Figure 6. Portion of the 360 MHz ^1H NMR spectra in ^2H_2O at 25 °C, pH 6.5, of ~3 mM freshly reconstituted metMb containing protoheme with orientations **I** (55%) and **II** (45%); only the low-field region 100–80 ppm window, where the metMbH$_2$O M_8, M_5 and m_8,m_5 resonate, and the 28–30 ppm window, where the metMbCN M_5, m_8 resonate are shown. The methyl peaks are labeled M_i,m_i for the **I**- and **II**-orientation, with i being the heme position 5 or 8: (**A**) ~$^1/_4$ equivalent of KCN has been added to this sample to convert approximately one-quarter of the protein to metMbCN and (**B**) upon addition of excess KCN after the (**C**) prior (immediately after reconstitution) recording the trace in A. Note that the ratio of the metMbCN peaks M_8:m_8 is essentially the same on partial (trace A) and complete CN^- ligation (traces B and C), dictating that the CN^- affinity for the two orientations is the same. The only minor difference in the m_5,m_8 intensity ratio is trace B and C shows that a negligible amount of heme reorientation occurred in the metMbH$_2$O portion during the recording of trace A.

with ^1H NMR spectra as in Figure 6C (complete CN- ligation) leads over several hours to ^1H NMR spectra where the ratio of M_5:m_8 for metMbCN remains invariant with time, with both peak intensities decreasing as the analogous resonances of the disordered metMbH$_2$O emerge (not shown). Hence the CN^- off-rate is independent of heme orientation.

Discussion

Resonance Assignments. The Heme. This ^1H and ^2H NMR study of orientationally disordered sperm whale deoxyMb presents the first complete and unambiguous assignment of the heme methyls signals in any high-spin ferrous hemoprotein. The heme labeling also shows that, in contrast to monomeric *Chironomus* deoxy Hb,[21] none of the apparent upfield methyls, M_y,M_z, arise from the heme. It is noted in Table I that, where individual methyls are detected by both ^1H and ^2H NMR, the ^2H chemical shift is always smaller than the ^1H shift, with the difference correlating with the magnitude of the downfield shift. This primary isotope effect is that expected solely for the contract shift in a paramagnetic system.[49] This isotope effect provides direct evidence that the heme methyl hyperfine shifts are dominated by the contact interaction. Hence the different heme methyl shift pattern for the two orientations cannot arise from variable rhombic dipolar shift contributions. The report[24] of a steady-state NOE from M_5 to a single proton at H_a with a geminal partner at H_c confirms the 6-propionate α-CH_2 assignment. On the other hand, the present isotope labeling clearly shows that the intense composite centered at 13 ppm, in contrast to a previous proposal,[24] does not originate from a heme methyl. Previous ^1H decoupling experiments on vinyl group ^{13}C labeled deoxyMb have shown[50] that the two vinyl H_αs are under H_a, H_c and that both H_βs for one vinyl resonate to the low field of 10 ppm. This leaves one, or at most two, unassigned protons in the resolved low field region, and these peaks likely are due to the 7-propionate α-CH_2. Hence there is no evidence for any non-heme peaks in the resolved low field region.

Amino Acid Residues. The His F8 ring labile $N_\delta H$ assignments are determined solely on the basis of model compounds;[3,27] however, their shift is in a sufficiently unique spectral window so as to reasonably preclude an alternative interpretation. A broad peak (~1 kHz) due to one or two nonexchangeable protons is observed at 40 ppm in orientation I, and the peak in orientation II appears at ~45 ppm (not shown; supplementary material). Based on model compounds,[27] this peak should arise from the His F8 ring $C_\delta H$ and/or $C_\epsilon H$. The strongly relaxed and upfield hyperfine shifted three-proton peaks, M_y,M_z (and m_y,m_z), must likely originate from methyl groups on noncoordinated residue(s), as suggested previously.[24] Based on the relative relaxation times (T_1s) of heme methyls, 6 Å (R_{Fe}) from the iron, and the T_1 values for the upfield shifted methyls in Table I, the relationship[1,2]

$$T_{1i}/T_{1j} = R_{Fe-i}{}^6/R_{Fe-j}{}^6 \qquad (2)$$

leads to R_{Fe} for M_z and M_y of 4.2 and 5.2 Å. The only methyl groups that close to the iron in deoxyMb[34] are the two methyls of Val E11 (R_{Fe} ~ 4.4, 5.5 Å). Thus both upfield hyperfine shifted methyl resonances arise from a distal residue that closely approaches, but does not covalently interact, with the iron; hence the high-spin ferrous ion exhibits magnetic anisotropy.[1-7]

Comparison of the Two Heme Orientations. Functional Properties. The oxygen and CO affinities as well as the respective rates have been shown to be independent of the heme orientation in ferrous sperm whale Mb.[37] The very similar ^1H NMR shift for the few resolved resonances of orientationally disordered MbCO, in particular the nearly identical upfield ring current shift of Val E11 $C_{\gamma 1}H_3$ of –2.35, 2.29 ppm for orientations **I** and **II**,[35] respectively, directly support a conserved MbCO structure. The ^1H NMR data presented here show that both the equilibrium constant as well as the rate-limiting step in the metMbCN → metMbH$_2$O conversion, the CN^- off-rate, are inconsequentially influenced by the heme orientation. Although this is a ligand replacement reaction rather than a simple ligation,[9] the most reasonable conclusion is that the molecular/electronic properties that affect function in both metMbCN and metMbH$_2$O are conserved for the two heme orientations.

Structural Comparison in metMb. In this section we consider in detail how the dominant contact shift patterns[6,7] for the heme in ferric Mb and their sensitivity to protein environment (in particular the two heme orientations) reflect on the nature of the spin delocalization mechanism and orbital ground state of the iron. Model compounds of both low-spin and high-spin ferric heme have shown that,[6,7,51,52] in the absence of rhombic contributions from either protein or axial ligand, the pattern of the four methyl contact shifts is modulated by the inherent asymmetric distribution of the nonequivalent peripheral substituents but that the mean methyl shift is highly conserved for variable substituents at the 2,4-position. The conserved mean methyl shift, $\bar{\delta}(M_{1,3,5,8})$, $\bar{\delta}(m_{1,3,5,8})$, dictates that the amount of transferred spin (covalency) is constant but is distributed asymmetrically among the four pyrroles. This asymmetry in the model compounds represents the intrinsic asymmetry of the molecular orbital(s) of the porphyrin containing the unpaired spin(s). In both the low-spin and high-spin protoheme models,[51,52] the 5,8-CH_3 and 1,3-CH_3 are pairwise nearly equivalent, but the two pairs are clearly in distinct electronic environments (the former pair always has the larger shift).

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Upon incorporating protoheme into a protein matrix, the unpaired spin distribution is further modulated by the protein rhombic environment. Assuming a conserved protein fold for the two heme orientations, the rhombic or in-plane asymmetric perturbation exerted on the heme by the protein is rotated 180° about the meso axis or acts along orthogonal vectors with respect to the heme plane in the alternate orientations. In one orientation, the sum of the intrinsic heme and protein rhombic asymmetry, and in the other orientation, the difference, is exerted on the heme. The manner in which the protein induced in-plane asymmetry manifests itself depends on the relative magnitudes of the intrinsic heme and protein-induced rhombic perturbations.

High-spin ferric metMbH$_2$O exhibit contact shifts that arise from both σ and π spin transfer, consistent with unit unpaired spin occupation of each d orbital.[6,7,52] The NMR spectral properties of the extracted chromophore do not adequately model the protein because of significant difference in the axial ligand.[3,6,7] However, inspection of the shift data in Table II clearly shows that the protein-induced rhombic asymmetry is smaller than the intrinsic asymmetry of the heme, since incorporation into the proton matrix modulates the individual methyl shifts but does not significantly alter the pattern for the two orientations. The magnitude of the rhombic influence can be measured by the fractional spread of the 5-CH$_3$, 8-CH$_3$ contact shifts (the 5-CH$_3$, 8-CH$_3$ environments are rigorously interchanged in the two heme orientations; Figure 1), $\Delta\delta(M_{5,8})/\bar{\delta}(M_{5,8}) \sim 0.1$, which is small and not significantly different from that in a model compound.[52] The mean methyl shifts, $\bar{\delta}(M_{1,3,5,8})$, $\bar{\delta}(m_{1,3,5,8})$, and particularly for the precisely interchanged methyls, $\bar{\delta}(M_{5,8})$, $\bar{\delta}(m_{5,8})$, are clearly strongly conserved in the two heme orientations of metMbH$_2$O (Table II). For the pair 5-CH$_3$,8-CH$_3$, where the intrinsic shift difference is small, the alternate orientations actually interchange the environments and, as expected, highly conserve $\bar{\delta}(M_{5,8})$. Conversely, the 1-CH$_3$,3-CH$_3$ shifts differ in the two orientations but do not result in changes in the relative positions because the protein rhombic influence is significantly smaller than the intrinsic difference between the two methyl shift at positions 1,4 and 2,3.

This latter conclusion is unequivocally demonstrated by the methyl peak positions and resulting assignment for the protoheme III and protoheme XIII–metMbH$_2$O complexes shown in Figure 5, parts B and C. Note that for these two 2-fold symmetric heme type isomers, M_8, M_5 shifts, are essentially unchanged from that of native protoheme (type isomer IX), with the unique M_4 shift in protoheme III very close to that of M_1 in that type isomer or in native metMbH$_2$O, while the unique M_2 shift of protoheme-XIII is very close to that of M_3 in that type isomer or in native metMbH$_2$O. Thus the asymmetric spin distribution solely within the prosthetic group clearly differentiates the environments of 1,4-CH$_3$ versus 2,3-CH$_3$ and largely accounts for the M_1, m_3 shift difference in native metMbH$_2$O. We now note that m_1 (and m_3) in orientation I of native protoheme has the protein environment, and hence chemical shift, of M_4 (and M_2) of protoheme III (protoheme XIII). This clearly establishes that the different shift patterns in the two heme orientations can be quantitatively interpreted solely on the basis of the interchange of environments of individual methyls in the two heme orientations. The reason that the in-plane asymmetry in high-spin iron(III) is relatively insensitive to protein environment is due to the fact that the ground state is necessarily orbitally nondegenerate,[6,7] i.e., ^{6}A.

Low-spin iron(III) displays dominant contact shifts from delocalization of the lone metal π spin,[6,17,18] and there exist excellent model compounds for the proteins.[18,51] The models exhibit pairwise essentially degenerate shifts for 5,8-CH$_3$ and 1,3-CH$_3$, with a small difference between the pairs;[18,51] the fractional rhombic asymmetry for 5-CH$_3$, 8-CH$_3$ is small, $\Delta\delta(M_{5,8})/\bar{\delta}(M_{5,8}) \sim 0.1$. Incorporation into the protein matrix strongly conserves $\bar{\delta}(M_{5,8})$,$\bar{\delta}(m_{5,8})$, but the rhombic asymmetry is significantly increased, i.e., $\Delta\delta(M_{5,8})/\bar{\delta}(M_{5,8}) \sim \Delta\delta(m_{5,8})/\bar{\delta}(m_{5,8}) \sim 1.1$, which dictates that the protein induced rhombic perturbation is much larger than the "intrinsic" asymmetry. The large protein induced rhombicity is due to the fact that low-spin iron(III) possesses an orbitally degenerate ground state (^{2}E), and raising the degeneracy places the lone spin into d_{xz} or d_{yz}, which delocalizes π spin into trans pyrroles A and B or C and D, leading to large shifts for 5-CH$_3$, 1-CH$_3$ or 8-CH$_3$, 3-CH$_3$.[18] The observed pairing of M_5,m_1 and m_8,M_3 with contact shift extremes in low-spin ferric hemoproteins[5,10,11,13] is direct confirmation for π bonding with a d orbital derived from a orbitally degenerate ground state.

Table II. Comparison of Heme Hyperfine Shifts for the Two Protoheme Orientations in Various Oxidation/Spin States[a]

	metMbCN		metMbH$_2$O		deoxyMb	
shifts	A[a,d]	B[b,e]	A[a,f]	B[b]	A[a]	B[b]
8-CH$_3$	13.0	27.4	91.8	86.1	7.8	18.5
5-CH$_3$	27.2	13.0	85.1	89.1	16.5	10.1
3-CH$_3$	4.9	17.7	73.4	65.6	11.2	14.0
1-CH$_3$	18.6	7.9	53.3	54.3	7.3	16.5
$\bar{\delta}$(5,8-CH$_3$)[g]	15.9	16.5	75.9	73.8	10.6	14.8
$\Delta\delta$(5,8-CH$_3$)[h]	14.2	14.4	6.7	3.0	8.7	8.4
$\bar{\delta}$(1,3,5,8-CH$_3$)[g]	20.1	20.2	88.4	87.6	12.2	14.2
$\Delta\delta$(1,3,5,8-CH$_3$)[h]	22.3	19.5	38.5	35.8	9.2	7.4
2-H[i]	−20.6	−13.5			42.0	67.0
4-H[i]	−15.0	−21.4			61.4	45.8
$\bar{\delta}$(2,4-H)[g]	−17.8	−17.5			51.7	56.4

[a] Shifts in ppm from DSS at 25 °C in ^{2}H$_2$O solution at pH 6.2 for metMbH$_2$O, pH 8.6 for metMbCN, and pH 8.3 for deoxy Mb. [b] Heme orientation I (Figure 1) for native protoheme (R$_1$ = R$_3$ = CH$_3$, R$_2$ = R$_4$ = vinyl). [c] Heme orientation II (Figure 1) for native protohemin. [d] Data taken from ref 10 for 8-CH$_3$, 5-CH$_3$, and 1-CH$_3$ and from ref 44 for 3-CH$_3$. [e] Data taken from ref 13 for 8-CH$_3$, 5-CH$_3$, and 3-CH$_3$; 3-CH$_3$ shift determined here. [f] Data taken from ref 12. [g] Mean shift for stated signals. [h] Spread of shifts for stated signals. [i] 2-H, 4-H for Mb reconstituted with deuteroheme.

The foregoing analysis concludes the following: (1) conserved $\bar{\delta}(M_{1,3,5,8})$, $\bar{\delta}(m_{1,3,5,8})$, and $\bar{\delta}(M_{5,8})$,$\bar{\delta}(m_{5,8})$ indicate conserved porphyrin–iron bonding, (2) the contact shift patterns for the alternate orientations in both low-spin and high-spin metMb can be interpreted solely on the basis of the interchange of methyl environments, and (3) the sensitivity and pattern of the spin delocalizations into the different pyrroles for the two heme orientations provide information on the orbital degeneracy of the ground state of the iron. The first two conclusions are in direct support of conserved electronic and molecular structural properties that influence ligation for the alternate heme orientations in metMbCN and metMbH$_2$O.

Structural Comparison in DeoxyMb. The dominant contact shift pattern in high-spin ferrous hemes results from a combination of σ and π spin delocalization.[6,7,27,50,53] There are no adequate model compounds for the ^{1}H NMR properties of deoxyMb that allow an assessment of the rhombic influences of the protein matrix on the methyl contact shift pattern.[53] However, comparison of the contact shift pattern solely for the two heme orientations in deoxyMb provides direct information on the nature of the protein rhombic influences. The relevant data on individual shifts, mean shifts, and shift spreads for the two orientations of protoheme in deoxyMb are included in Table II. Inspection reveals that the 5-CH$_3$, 8-CH$_3$ contact shift spread, $\Delta\delta(M_{5,8})$, is very large and comparable to the mean shift, $\bar{\delta}(M_{5,8})$, as in low-spin metMbCN. Moreover, the shift pattern for the two heme orientations differs in the same characteristic manner as for metMbCN, i.e., the two heme orientations interchange the relative shift magnitudes (and, by inference, the environments), M_8,m_5 with m_5,M_8 and M_1,m_3 with m_3,M_1, as expected from π spin delocalization via primarily d_{xz} or d_{yz} (but not simultaneously d_{xz} and d_{yz}). Hence both the sensitivity of the shift asymmetry to

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heme orientation as well as the distinctive pattern of interchange of contact shift magnitudes within the pairs 5-CH_3, 8-CH_3 and 1-CH_3, 3-CH_3 directly support π spin delocalization in an orbital ground state that is degenerate in 4-fold symmetry. This demands that the ground state in deoxyMb is derived largely from the configuration $(d_{xz})^2(d_{yz})(d_{xy})(d_{z^2})(d_{x^2-y^2})$ or $(d_{yz})^2(d_{xz})(d_{xy})(d_{z^2})(d_{x^2-y^2})$ (derived from ^5E), as opposed to $(d_{xy})^2(d_{xz})(d_{yz})(d_{z^2})(d_{x^2-y^2})$ (derived from $^5B_2$). A ground state derived from the 4-fold symmetry ^5E rather than $^5B_2$ is in agreement with several theoretical treatments.[54-56]

Detailed inspection of the ^1H NMR data in Tables I and II, however, indicates a more complicated picture for the electronic ground state of deoxyMb. The upfield methyl peaks, M_z,m_z, both assigned to a Val E11 methyl, differ substantially for the two orientations, with the dipolar shift significantly larger for orientation II than I (by ~50% for M_z,m_z). Since the E11 residue is considered important in modulating ligation properties, the invariant ligand affinity and ligation rates for reduced Mb[37] as well as the conserved E11 Val CH_3 shift in MbCO[35] make it unlikely that the E11 Val positions differ significantly for the two heme orientations. Interestingly, the heme contact shift patterns for the two heme orientations also exhibit important difference not observed in metMbH_2O, metMbCN (Table II). The heme mean methyl shift $\bar{\delta}(M_{1,3,5,8})$,$\bar{\delta}(m_{1,3,5,8})$ as well as the more important mean for the interchanged 5-CH_3,8-CH_3, $\bar{\delta}(M_{5,8})$,$\bar{\delta}(m_{58})$ is some ~50% larger in orientation II than I (Table II). Hence, it *appears* as if equatorial bonding or iron–porphyrin covalency are not the same in the two heme orientations. It is also noted that the 5-CH_3, 8-CH_3 shift asymmetry is smaller in orientation II than I. Inspection of the assigned 2H, 4H shift (Tables I and II) in deoxy deuteroheme Mb similarly shows a larger $\bar{\delta}$(2,4-H) in orientation II than I, while the mean for 2H,4H shifts remains invariant for the two orientations in metMbCN.

The failure to conserve $\bar{\delta}(M_{1,3,5,8})$, $\bar{\delta}(m_{1,3,5,8})$, $(\bar{\delta}(M_{5,8})$, $\bar{\delta}(m_{5,8}))$, and the altered M_z,m_z shifts for deoxyMb, in spite of conserved function,[37] brings into question the interpretation of hyperfine shifts in high-spin ferrous hemoproteins in terms of structural properties relevant to function. Additional support for a lack of a simple correlation between NMR parameters and functionally relevant structural changes is provided by earlier ^1H studies[20] of the unassigned ^1H NMR spectrum of deoxyMb as a function of pH. This study had identified[20] a single pK ~5.3 which has a major influence on both resolved heme methyl shifts, M_a (5-CH_3), M_b (3-CH_3), with the two signals in the acidic form changing shifts in opposite direction by 2–3 ppm. This very substantial fractional change in the contact shift for two methyls (as well as all single protons) is contrasted to the relatively minimal shift changes observed[57] during the same titration (pK ~5.3) in metMbCN. The origin of the pK in both forms has been traced[57] to the titration of the 7-propionate which is involved[34] in a hydrogen bond with His FG3. Moreover, oxygen binding was found to be minimally perturbed[20] by this pK in deoxyMb. Hence, rather substantial shift changes in deoxyMb appear to have relatively minor structural implications. Preliminary ^1H NMR assignment of methyl signals that are resolved from the diamagnetic envelope in several high-spin ferrous hemoproteins reveal surprising variability in the identity of the resolved signals in spite of a series of interpretable similarities among the ^1H NMR properties of cyanide ligated ferric derivatives.[58-60]

A likely origin for the extreme sensitivity of the hyperfine shifts to such a small structural perturbation as alternate heme orientation is the contribution of more than one electronic ground state with differential contributions for the two heme orientations. It is noted that the various theoretical calculations of the electronic structure of high-spin ferrous hemes do not all agree on the ground state,[54-56,61,62] but all agree, and the temperature dependence of both the Mössbauer quadrupolar splitting and susceptibility confirm[55,56] that there are several electronic states, the split components of ^5E as well as $^5B_2$ very close in energy. For equal covalency, a component of the degenerate ^5E, i.e., $(d_{xz})^2(d_{yz})(d_{xy})(d_{z^2})(d_{x^2-y^2})$, will delocalize the lone π spin in d_{yz} into either pyrroles A,C or B,D. On the other hand, the nondegenerate $^5B_2$ state, i.e., $(d_{xy})^2(d_{xz})(d_{yz})(d_{z^2})(d_{x^2-y^2})$, will delocalize twice as much spin density and will do this equally to all pyrroles because there are π spins in both d_{xz} and d_{yz}. The differential dipolar shifts for M_z,m_z could result from very different zero-field splittings for the two states.[6,7] An increased contribution of the B_2 configuration in the heme orientation **II** would lead to an increase in $\bar{\delta}$(1,3,5,8-CH_3), because there are two spins to delocalize, and to a decrease in $\Delta\bar{\delta}$(1,3,5,8-CH_3) because they are delocalized similarly to all four pyrroles. Small differences in the contribution from the B_2 state could markedly alter the NMR properties of deoxyMb without strongly manifesting itself in functional properties. The thermal population of different electronic states with significantly different hyperfine shift patterns is usually characterized by significant deviations from Curie behavior. Two variable temperature studies have been reported on native deoxyMb,[20,24] which report different magnitudes of the deviations from Curie behavior. One basis for this discrepancy is the presence of a pK at 5.3 which results in very large shift changes,[20] and interference from this pK in analyzing the true temperature dependence is larger in the more recent study[24] performed at a lower pH. The studies agree qualitatively that M_a and M_b deviate from Curie behavior, in that one has a steeper and the other a much less steep slope, than predicted by the Curie law. Nevertheless, the variable temperature studies are not inconsistent with the participation of two electronic states.

The present ^1H NMR data provide strong evidence for a dominant, but not exclusive, contribution of an orbital ground state derived from ^5E. However, considerably more work will be required to quantitate the interpretation of the hyperfine shifts and their relationship to molecular structure in high-spin ferrous hemes. Whatever their origin, *it is clear that the detailed hyperfine shifts are much more sensitive to environment in deoxyMb than either of the metMb forms.* This conclusion suggests that considerable caution should be exercised in using heme shifts of the deoxy form of any Mb, Hb as interpretable indicators of functionally significant structural changes. A more detailed understanding of the origin of the contact shift pattern may emerge when complete methyl ^1H and ^{13}C assignments become available in a variety of structurally characterized deoxyMb, Hb natural genetic variants or synthetic point mutants. Several such studies are planned.

Acknowledgment. The authors are indebted to Dr. V. Thanabal for experimental assistance in recording ^2H NMR spectra. This research was supported by grants from the National Institutes of Health, HL-16087, HL-22252.

Supplementary Material Available: Two figures (deuterium labeling of heme methyls in native deoxy Mb; ^1H NMR spectra of native and freshly reconstituted deoxyMb in ^1H_2O) (1 page). Ordering information is given on any current masthead page.

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