Proton Nuclear Magnetic Resonance Investigation of the Nature of Solution Conformational Equilibria of Monomeric Insect Deoxyhemoglobins†

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ABSTRACT: The proton nuclear magnetic resonance spectra of the three monomeric deoxyhemoglobins of the insect larva *Chironomus thummi thummi* have been recorded, assigned, and analyzed. In the two allosteric hemoglobins, the heme methyls and vinyl protons were assigned by specific deuterium labeling. The hyperfine-shifted proximal histidyl imidazole exchangeable protons for the three native and two deuteroheme-reconstituted hemoglobins were assigned by comparison of spectra in H_2O and ^2H_2O. Both native and reconstituted allosteric hemoglobins exhibit two sets of interconvertible resonances indicative of two heme orientations differing by a 180° rotation about the α–γ–meso axis, as previously found for the met-cyano analogues [La Mar, G. N., Smith, K. M., Gersonde, K., Sick, H., & Overkamp, M. (1980) *J. Biol. Chem. 255*, 66]. The relative pH sensitivities of the heme resonance hyperfine shifts for the two allosteric hemoglobins and the apparent p$K \sim 8$ indicate that the t $\rightleftharpoons$ r allosteric transition, as modulated by the Bohr proton, is being observed. For the native hemoglobins, the t $\rightleftharpoons$ r conformational transition was found to be centered at the heme periphery, with the proximal histidyl imidazole environment insensitive to both pH and the rotational position of the heme, consistent with the absence of a pH influence on the ligation on-rate. For the deuteroheme-reconstituted allosteric hemoglobins, both the heme and axial imidazole environments sense the t $\rightleftharpoons$ r transition, and the histidine environments for the two components for each hemoglobin can be clearly distinguished, suggesting that the ligation on-rates may depend on both pH and heme orientation.

The series of three monomeric hemoglobins from the larva of the insect *Chironomus thummi thummi*, CTT[1] I, III, and IV, which are functionally different with respect to the Bohr effect (Gersonde et al., 1972; Wollmer et al., 1972; Sick & Gersonde, 1974), represents idealized models for investigating the tertiary structural changes involved in the control of oxygen affinity within a subunit of tetrameric hemoglobin. The much smaller size (~15000 daltons) (Gersonde et al., 1972) of these allosteric CTT hemoglobins also renders them as much better candidates for high-resolution solid-state X-ray and solution nuclear magnetic resonance (NMR) investigation. One complicating feature of these molecules is that the solution structures are characterized by two nonequivalent orientations of the protoheme (A in Figure 1) within the heme pocket, at least in the metHbCN form (La Mar et al., 1978a,b, 1980a).

These three monomeric hemoglobins, CTT I, III, and IV, possess no, a moderate, and a sizable Bohr effect, respectively, for both CO and O_2 binding (Gersonde et al., 1972; Wollmer et al., 1972; Sick & Gersonde, 1974; Gersonde et al., 1976). Kinetic measurements have revealed that the altered affinity with pH results from changes exclusively in the ligand off-rate (H. Sick, A. I. Raap, and K. Gersonde, unpublished results), indicating that the t $\rightleftharpoons$ r structural change in the ligated form of the protein is primarily responsible for the Bohr effect. In agreement with this conclusion, proton NMR studies of metHbCN's, which are excellent models for HbCO, have shown that while metHbCN I exhibits pH-independent shifts, CTT III and IV metHbCN's exhibit pH-dependent shifts modulated by a single proton with p$K \sim 7.4$ and that the relative magnitudes of the shift changes reflected the relative amplitudes of the Bohr effect (La Mar et al., 1978a,b). More recent investigations have also revealed that the environment of the proximal histidyl imidazole, the most likely origin of control of iron reactivity, is also significantly altered by pH (G. N. La Mar, R. R. Anderson, H. Sick, and K. Gersonde, unpublished results).

The insensitivity of the ligand on-rate to pH for CTT III and IV Hb's suggests the possibility that the t $\rightleftharpoons$ r structural transition may not occur in the unligated form. Alternatively, the t $\rightleftharpoons$ r structural change may still take place in the deoxy-Hb's but is localized in a manner so as not to influence the proximal histidyl imidazole bonding. Evidence against the pH-modulated electronic change of the central atom in the unligated form of the proteins can also be drawn from electron spin resonance (ESR) studies of Co(II)-substituted CTT Hb's, where deoxy-Co^{II}Hb's exhibited pH-insensitive g values as well as ^{59}Co and ^{14}N hyperfine splittings, while $Co^{II}HbO_2$'s revealed significant changes in these properties with pH (K. Gersonde, H. Twilfer, and M. Overkamp, unpublished results). Further evidence of an invariant iron electronic structure in deoxy-Hb's is given by Mössbauer effect experiments (F. Parak, C. Hermes, and S. Formanek, unpublished results), where the temperature dependence of the quadrupole splitting is not influenced by pH and hence does not reflect the Bohr effect.

While the hyperfine shifted heme resonances have served as valuable empirical indicators of changes in quaternary structure of deoxyhemoglobins (Morrow & Gurd, 1975), so far no satisfactory analysis of the shifts in terms of structure and/or bonding has been possible for high-spin ferrous hemes because of the likely presence of multiple spin transfer mechanisms whose hyperfine contributions appear to largely

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[1] Abbreviations used: CTT, *Chironomus thummi thummi*; NMR, nuclear magnetic resonance; ESR, electron spin resonance; DSS, sodium salt of 4,4-dimethyl-4-silapentane-1-sulfonate; Hb, hemoglobin; Mb, myoglobin.

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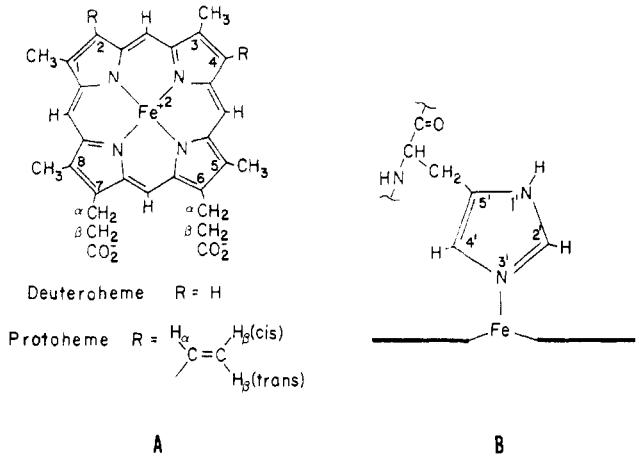

FIGURE 1: (A) Structure of protoheme (R = vinyl) and deuteroheme (R = H). (B) Peak designation for coordinated histidyl imidazole.

cancel at the heme periphery (La Mar et al., 1977; La Mar & Walker-Jensen, 1979; La Mar, 1979). Thus the heme resonances are unlikely to lead to structural information, although their shifts with pH may yield information on the presence of the t ⇌ r structural transition. The hyperfine shifts for the ubiquitous axial histidyl imidazole in deoxy proteins, on the other hand, have been shown to be consistent with dominant σ spin transfer such that the shift magnitudes can be taken as a measure of the Fe–N covalency (La Mar et al., 1977; Goff & La Mar, 1977). Particularly useful in this regard is the readily assignable exchangeable N_1H of the proximal histidyl imidazole (B in Figure 2) which resonates in a unique portion of the low-field NMR spectra of deoxy hemoproteins (La Mar et al., 1977, 1978b, 1980c). It has been shown by ESR that compression of the Fe–N_3 bond is characteristic of the r → t transition which is accompanied by a decrease in ligand affinity (Overkamp et al., 1976).

The assignment of individual heme resonances via deuterium labeling (Mayer et al., 1974; La Mar et al., 1978c, 1980a,b,c,d) may shed additional light on the origin of the heme rotational disorder found for the met-cyano form of both the CTT III and IV proteins (La Mar et al., 1978a, 1980a). Although the single crystal X-ray structures of CTT Hb III in various oxidation/ligation forms failed to provide any confirmation of the heme rotational disorder, the structure of metHbCN was unique in that it indicated that histidine-E7 was disordered, existing both in the normal distal position in the heme pocket as well as turned out toward the exterior (Steigemann & Weber, 1979). Establishing such heme rotational disorder in the deoxy-Hb's will argue strongly against any correlation between the heme orientation and the position in the distal histidine since in deoxy-Hb histidine-E7 exhibits a unique location turned out of the heme pocket.

Materials and Methods

Preparation of Hemoglobins. The monomeric hemoglobins I, III, and IV were purified and the three components separated as described previously (Sick et al., 1972; La Mar et al., 1978a). The preparation of the deuteroheme-reconstituted Hb III and Hb IV has also been described in detail (Overkamp et al., 1976; La Mar et al., 1978a). Reconstitution with deuterium-labeled protohemes was carried out in the identical fashion using the specifically deuterium-labeled protohemes used previously (Budd et al., 1979; La Mar et al., 1980a). The reconstituted proteins yielded NMR spectra identical with those of the native material except for the absence or decreased intensity of the labeled resonances. The interconversion of the two forms of metHbCN was carried out in the manner described before (La Mar et al., 1978a). The relative intensities of the two sets of resonances indicated that there were present comparable amounts of the two components after incubating the metHbCN's at pH 10.5 and 40 °C for 24 h.

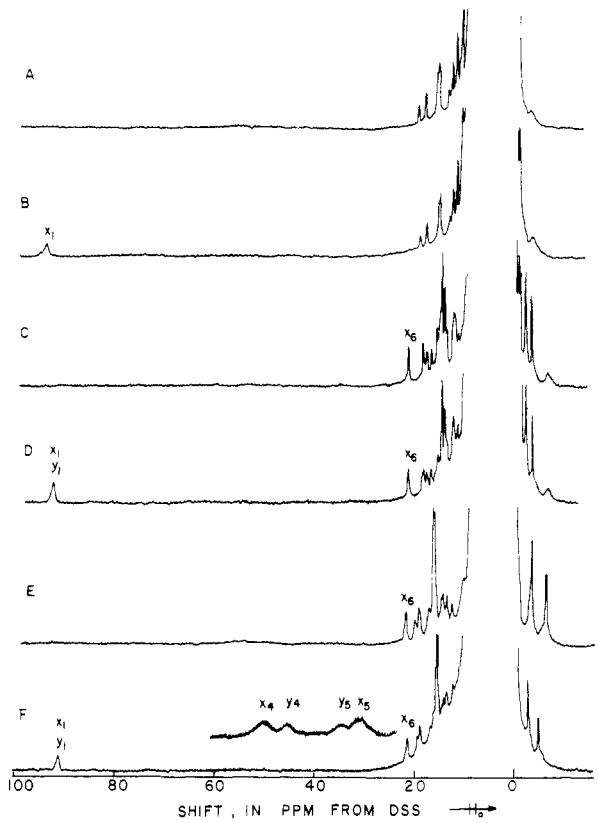

FIGURE 2: Proton NMR spectrum (200 MHz) of the hyperfine-shifted region of the native deoxy-Hb's at 25 °C, "pH" 7.0, of Hb I in ^2H_2O (A) and in 90% H_2O (B), Hb III in ^2H_2O (C) and in 90% H_2O (D), and Hb IV in ^2H_2O (E) and in 90% H_2O (F); chemical shifts are referenced to DSS. The insert in (F) is at 45 °C and illustrates two pairs of nonexchangeable resonances observed at higher amplification. Peaks from the major and minor components are designated x_i and y_i, respectively, with the details of the crowded region 20–10 ppm expanded in (A) of Figures 4 and 5.

Preparation of Samples. Approximately 15 mg of lyophilized methemoglobin was dissolved in 0.5 mL of 0.2 M NaCl in 90% H_2O/10% ^2H_2O or 99.8% ^2H_2O. After standing for a few minutes, the nondissolved material was removed by centrifugation. the deoxy-Hb's were prepared by flushing the solutions with N_2 and either adding directly a small excess of solid $Na_2S_2O_4$ or first adding a 2-fold excess of KCN to form the metHbCN to which $Na_2S_2O_4$ is later added to yield deoxy-Hb's. The proton NMR spectra of samples prepared by the two methods were identical. the reduction of the metHbCN samples permitted direct comparison of the relative amounts of the two components in the two oxidation states. The pH of the sample was adjusted to the acid region by addition of 0.1 M ^2HCl, and pH titrations were carried out by subsequent addition of 0.1 M NaO^2H. The pH was measured within the NMR tube by using an Ingold microcombination electrode and a Beckman 3550 pH meter. The pH readings were not corrected for the isotope effect and are referred to as "pH" whether in pure ^2H_2O or 10% ^2H_2O/90% H_2O. Since we had shown previously that 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) intercalated in the protein interior (La Mar et al., 1978a), the internal calibrant used was *tert*-butyl alcohol. Calibrations using both DSS and *tert*-butyl alcohol indicated that their shift separation was independent of "pH" and temperature to <0.01 ppm. Hence, all shifts are given in parts per million (ppm) referenced to DSS.

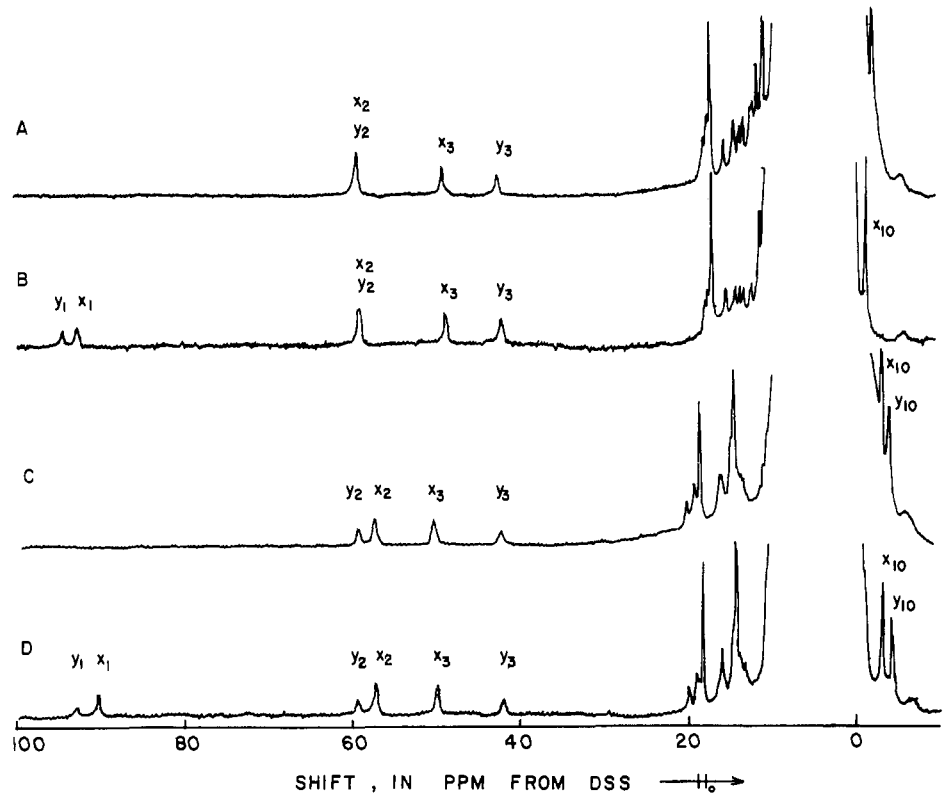

FIGURE 3: Hyperfine-shifted region of the 200-MHz proton NMR spectrum of the deuteroheme-reconstituted deoxy-Hb's at 25 °C and "pH" 6.0. Deuteroheme-Hb III in ^2H_2O (A) and in 90% H_2O (B) and deuteroheme-Hb IV in ^2H_2O (C) and 90% H_2O (D). Chemical shifts are referenced to DSS. Peaks for the major and minor components are designated x_i and y_i, respectively.

Proton NMR Spectra. ^1H NMR spectra of the deoxyhemoglobins were recorded at 25 °C with a 200-MHz FT NMR spectrometer (Type NT-200, Nicolet Technology Corp.), operating in the quadrature detection mode. For samples in ^2H_2O, ~4000 transients were collected by using a bandwidth of 4 kHz, 2K data points, and a 7-μs 90° pulse. For studies performed in 90% H_2O, ~80000 transients were accumulated by using a bandwidth of 20 kHz, 4K data points, and a 7-μs 90° pulse. The solvent resonance was suppressed with a 30-ms presaturation pulse. Chemical shifts are referenced to DSS and are given in parts per million at 25 °C.

Results

The 200-MHz proton NMR traces of native deoxy-Hb's CTT I, III, and IV in H_2O and ^2H_2O are compared in Figure 2. Similar traces for deoxydeuteroheme-Hb's III and IV in H_2O and ^2H_2O are shown in Figure 3. For each protein, the spectrum in H_2O exhibits one additional peak, at ~90–95 ppm, not found in ^2H_2O; for the deuteroheme-Hb spectra, this exchangeable peak is actually split into two peaks. Since we shall show below that both deoxy-Hb's III and IV exist in two forms, we designate peaks from the major component x_i and those of the minor component y_i; peaks which cannot be attributed unambiguously to either component are labeled a–i. For the native Hb's, the exchangeable 1′-H's are coincidentally degenerate, so that the peaks near 90 ppm arise from both x_1 and y_1. The insert in F of Figure 2 has considerably improved signal-to-noise at 45 °C, revealing four additional peaks, x_4, x_5, y_4, and y_5. The former two peaks have the same area as x_6, while the latter two have the same intensity as y_6 (see Figure 5).

The expanded regions of the proton NMR spectra of the deoxy-Hb's which contain the hyperfine-shifted heme resonances are illustrated in Figures 4 and 5. In Figure 4 we compare the trace for the native deoxy-Hb III (A) to that reconstituted with [1,3-(C^2H_3)$_2$]protoheme (C), [1,5-(C^2H_3)$_2$]protoheme (D), and [5,8-(C^2H_3)$_2$]protoheme (E), leading to the assignment of all resolved heme methyls. Trace B exhibits the spectrum of the native protein after incubating the protein at 40 °C, pH 10.5, for 24 h as the met-cyano form (La Mar et al., 1978a) prior to lowering the pH and reducing with dithionite. Figure 5 presents the 200-MHz traces for deoxy-Hb IV (A) compared with those of the proteins reconstituted with [1,3-(C^2H_3)$_2$]protoheme (C), [1,5-(C^2H_3)$_2$]protoheme (D), [2,4-(C^2H_α)$_2$]protoheme (E), and [2,4-($C(^2H_\beta)_2$)$_2$]protoheme (F), respectively. Trace B is that of the native protein which has been incubated at 40 °C, pH 10.5, for 24 h as the met-cyano form prior to lowering the pH and reducing with dithionite.

The resolved resonances in the region 10 to 100 ppm and 0 to −10 ppm all experience considerable hyperfine shifts, as indicated by their strong temperature dependence (Jesson, 1973) illustrated in Figure 6 for deoxy-Hb's III and IV. For straight lines, the apparent intercepts at $T^{-1} = 0$ are listed in Table I. The resonances for deoxy-Hb I do not exhibit a detectable pH dependence (not shown). The pH dependence of these resolved resonances at 25 °C for deoxy-Hb's III and IV are given in Figure 7 for the native protein. Solid lines represent fits to the Henderson-Hasselbalch equation, which yields pK ~8 for Hb IV. In Figure 8 we present the pH dependence of the exchangeable 1′-H peaks (B in Figure 1) and the methyl peaks analogous to y_{10} and x_{10} in native Hb's III and IV for deuteroheme-Hb's III and IV. The shifts for deoxy-Hb's III and IV at "pH" 6.0 and 25 °C are listed in Table I; also included are the changes in shift upon raising the "pH" to 9.5 and the intercepts in the Curie plots (Figure 4) at $T^{-1} = 0$. The shifts of selected resonances for deuteroheme-Hb's III and IV are given in Table II.

Discussion

Resonance Assignments. Comparison of the H_2O and ^2H_2O proton NMR spectra of the native deoxy-Hb's I, III, and IV

Table I: Observed Shifts for Native Deoxyhemoglobins

assignment	peak designation	Hb III shift[a]	Hb III Δ[b]	Hb III intercept[c]	Hb IV shift[a]	Hb IV Δ[b]	Hb IV intercept[c]
1′-H	x_1	92.2	~0	~0	91.0	~0	~0
	y_1						
2′-H	x_4	*d*			32[e]	*f*	
	y_4	*d*			36[e]	*f*	
4′-H	x_5	*d*			51[e]	*f*	
	y_5	*f*			56[e]	*f*	
2,4-H_α	x_6	21.11		~2	21.59	0.56	~1
	y_6	16.40	~0	~7	16.95	0.65	~2
6,7-H_α(?)	x_7	18.15	~0	*g*	18.95	0.45	*g*
	y_7	17.45	~0	*g*	20.05	1.90	*g*
1-CH_3	x_8	14.30	−0.37	~3	16.2 (?)	0.6	~1
3-CH_3	y_8	13.64	−0.28	~6	15.9 (?)	1.3	~1
Ile-E11(?)	x_9	−1.09	0	~1	*h*		
	y_9	−1.32	0	~1	*h*		
5-CH_3	x_{10}	−2.35	~0	~18	−3.58	1.39	~20
8-CH_3	y_{10}	−3.75	~0	~18	−6.25	2.83	~24
	a	15.4	−0.1	~−5	*h*		
	b	14.9	~0	~−5	*h*		
	c	14.6	~0	~10	*h*		
	d	~13.3		~10	~14.3	~1.0	~−5
2,4-H_β(?)	*e*	~12.2		~14	~13.9	~0.7	~7
	f	~11.8			~13.3	~0.3	~10
	g	~11.1		~9	~12.5	~0.8	~8

[a] Shift in parts per million at 25 °C (except when noted otherwise), "pH" 6.0, and references against DSS. Downfield shifts are positive. [b] Change in shift on raising "pH" from 6.0 to 9.5. The downfield bias is positive. [c] Intercept, in parts per million relative to DSS, extrapolated to $T^{-1} = 0$ in Figure 6. [d] Insufficient signal-to-noise to resolve. [e] Shifts at 45 °C where resolution is considerably improved. [f] "pH" dependence not determined. [g] The Curie plot curved, so the intercept was not determinable. [h] Not resolved.

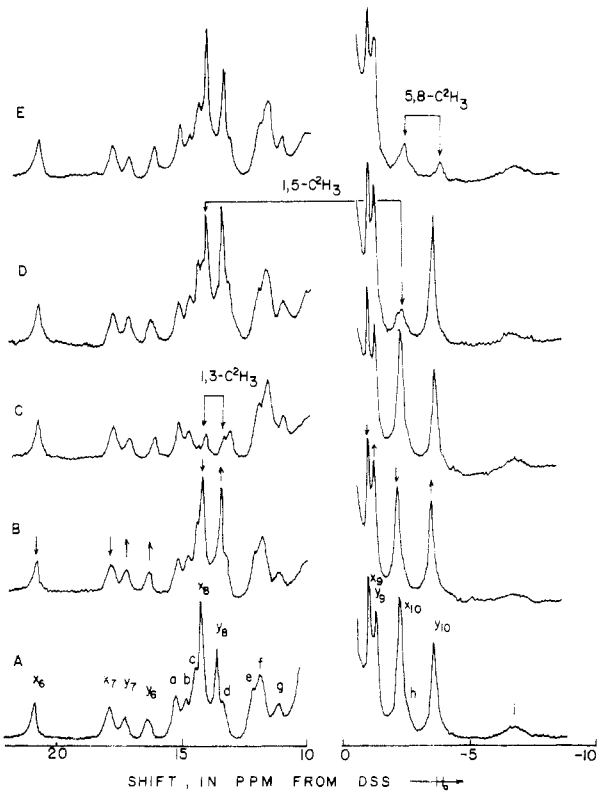

FIGURE 4: Expanded portions of the hyperfine-shifted proton NMR spectra of Hb III in ^2H_2O at "pH" 7.0 and 25 °C. (A) Native protein; (B) native protein equilibrated to yield comparable amounts of the two components, with the vertical arrows indicating which resonances increase (↑) or decrease (↓) during the equilibration; (C) Hb III reconstituted with [1,5-$(C^2H_3)_2$]protoheme (approximately 50% 1-C^2H_3 and 90% 5-C^2H_3); (E) Hb III reconstituted with [5,8-$(C^2H_3)_2$]protoheme (approximately 90% deuteration at each position). All shifts are referenced to DSS, and positions of deuteration in (C–E) are indicated by arrows. The peaks are designated x_i and y_i for the major and minor components, respectively; the designations a–i are used for partially resolved resonances which cannot be attributed unambiguously to either component.

Table II: Selected Observed Shifts for Deuteroheme-Reconstituted Deoxyhemoglobins

assignment	peak designation	deuteroheme-Hb III shift[a]	deuteroheme-Hb III Δ[b]	deuteroheme-Hb IV shift[a]	deuteroheme-Hb IV Δ[b]
1′-H	x_1	92.4	0.8	90.4	1.2
	y_1	94.0	0.9	92.8	1.7
2,4-H	x_2	58.8	0.9	57.2	1.3
	y_2	58.8	0.2	59.1	0.6
	x_3	48.5	0.5	49.9	2.8
	y_3	41.9	0.2	42.1	0.5
5-CH_3(?)[c]	x_{10}	*d*		−3.00	⩾2[e]
8-CH_3(?)[c]	y_{10}	−1.63	~0	−4.71	3.62

[a] Shift in ppm at 25 °C, "pH" 6.0, and referenced against DSS. Downfield shifts are positive. [b] Change in shift on raising "pH" from 6.0 to 9.5. The downfield bias is positive. [c] Assignment by analogy to native Hb's. [d] Not resolved from diamagnetic envelope at any "pH". [e] Peak disappears under diamagnetic envelope at high "pH".

(Figure 2) and those of the deoxy deuteroheme-Hb's III and IV (Figure 3) reveals that the prominent downfield peaks in the region 90–95 ppm originate from exchangeable 1′-H of the proximal histidyl imidazole, as previously found for high-spin ferrous model compounds, deoxymyoglobins, and tetrameric hemoglobins (Goff & La Mar, 1977; La Mar et al., 1977, 1978d, 1980e). For the native Hb's a single exchangeable peak is observed corresponding to one proton per molecule. The second minor peak in Hb I is due to the presence of impurities, as also found for the metHbCN complex (La Mar et al., 1978a). In the case of deuteroheme-Hb's III and IV, two such exchangeable NH peaks, x_1 and y_1, are observed. Moreover, in the region 40–60 ppm, in which only 2,4-H's yield narrow lines, two sets of peaks (x_2, x_3 and y_2, y_3) are resolved in the same ratio of intensities as the two 1′-H's, clearly indicating that the protein is present in two forms in solution.

The existence of two sets of resonances originating from two interconvertible species is demonstrated for the native Hb's

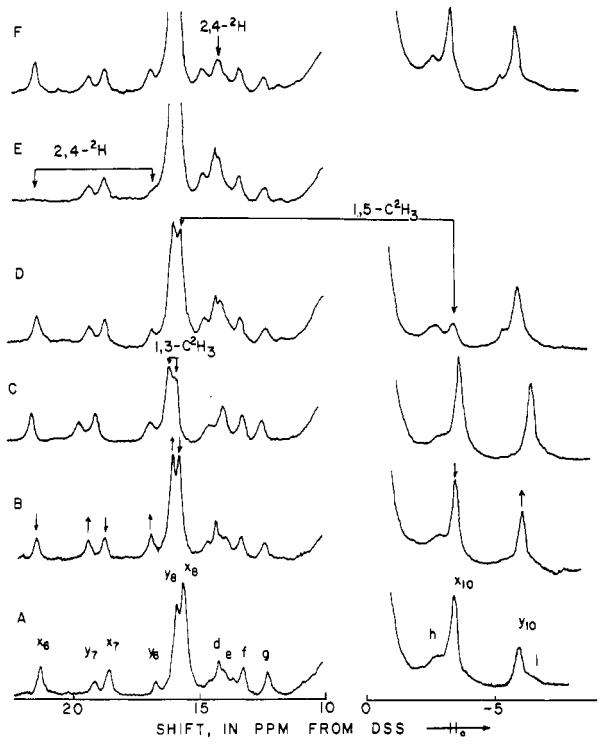

FIGURE 5: Expanded portions of the hyperfine-shifted proton NMR spectra of Hb IV in ^2H_2O at "pH" 7.0 and 25 °C. (A) Native Hb IV; (B) native protein equilibrated to yield comparable amounts of the two components, with the vertical arrows indicating which resonances increase (↑) or decrease (↓) during the equilibration; (C) Hb IV reconstituted with [1,3-(C^2H_3)$_2$]protoheme (approximately 50% deuteration at each position); (D) Hb IV reconstituted with [1,5-(C^2H_3)$_2$]protoheme (approximately 50% 1-C_2H_3 and 90% 5-C^2H_3); (E) Hb IV reconstituted with [2,4-(α-C^2H)$_2$]protoheme (approximately 95% ^2H); (F) Hb IV reconstituted with [2,4-(β-C^2H_2)$_2$]protoheme (approximately 90% ^2H). All shifts are referenced to DSS and positions of deuteration in (C–F) are indicated by arrows. The peaks are designated x_i and y_i for the major and minor components, respectively; the designations d–i are used for partially resolved resonances which cannot be attributed unambiguously to either component.

III and IV in traces A and B of Figures 4 and 5. Upon incubating the native protein at pH 10.5 and 40 °C for 24 h, we had shown previously (La Mar et al., 1978a) that the two forms of metHbCN can be equilibrated with no damage to the proteins. When these solutions possessing comparable amounts of the two forms of metHbCN are reduced to yield deoxy-Hb's (B in Figures 4 and 5), it is noted that one set of resonances decreases in intensity (x_i) while another set increases (y_i). Hence the deoxy-Hb's, as for the metHbCN's, exist in two forms in solution. The two exchangeable 1′-H peaks must have essentially the same shifts for the two components in native proteins (Figure 2).

The expanded regions 23 to 10 and 0 to −10 ppm for Hb's III and IV are quite similar, as illustrated in trace A of Figures 4 and 5, although the region 14 to 15 ppm is better resolved for the former protein. Reconstitution of deoxy-Hb III with [1,3-(C^2H_3)$_2$]- (C), [1,5-(C^2H_3)$_2$]- (D), and [5,8-(C^2H_3)$_2$]-protoheme (E in Figure 4) assigns the four resolved methyls, two from each component, x_8 (1-CH_3), y_8 (3-CH_3), x_{10} (5-CH_3), and y_{10} (8-CH_3). In Hb IV, the composite at ~14 ppm has sufficient intensity to contain a methyl from each component. Reconstitution with [1,3-(C^2H_3)$_2$]protoheme leads to significant reduction in intensity, but the lack of resolution makes the assignment of 3-CH_3 (y_8) and 1-CH_3 (x_8) ambiguous. The identical assignment for 5-CH_3 in both Hb's and their very similar assignments in the metHbCN forms (La Mar et al., 1980a) strongly support the same pattern of heme methyl assignments in deoxy-Hb III and Hb IV.

The four resonances x_6, x_7, y_6, and y_7 correspond to single protons, two for each component. In Hb IV, trace E in Figure 5 assigns x_6 and y_6 to vinyl H_α's. The other two vinyl peaks are probably unresolved in the diamagnetic envelope 10–0 ppm, where the other methyl peaks must also originate. The two single proton peaks, x_7 and y_7, probably arise from propionic acid H_α's on the basis of the appearance of an additional pH influence below pH 6 (Figure 7) and the curvature of their Curie plots (Figure 6) (see below). Although deuterium labeling of vinyl H_α's was not carried out for Hb III, x_7, and y_7 shifts again show the low pH influence (Figure 4) and the curved Curie plot (Figure 6), strongly pointing to x_6 and y_6 as the vinyl H_α's. Deuterium labeling of vinyl H_β's for Hb IV (F in Figure 5) reduces the intensity of the composite peak at ~12 ppm, suggesting that at least one H_β resonates in this region. The remaining single proton resonances in the range 10–14 ppm probably arise from the other propionic acid H_α's and possibly the proximal histidine β-CH, as suggested by studies on model compounds (Goff & La Mar, 1977).

In addition to the 1′-H peaks arising from the proximal histidyl imidazole, two other axial resonances are resolved for each component. The broad resonances x_4, x_5 and y_4, y_5 (insert in F of Figure 2) occur outside the region where high-spin ferrous protoporphyrins are known to yield heme peaks. However, it has been shown in these model compounds that the axial imidazole 2′-H and 4′-H (B in Figure 1) resonate at 50 and 70 ppm, respectively, exhibiting 6–8 times the line width of 1′-H (Goff & La Mar, 1977). Thus we tentatively assign x_4 and y_4 to 4′-H and x_5 and y_5 to 2′-H in the major and minor components.

The upfield methyl peaks (x_9 and y_9) in Hb III cannot arise from the heme and hence must originate from a protein side chain in the vicinity of the heme. We have suggested elsewhere (La Mar et al., 1980b) that such upfield nonheme resonances may arise from nearby distal residues, leading to the possibility that these peaks arise from a methyl of the distal isoleucine-E11. The upfield proton peaks could also arise from meso-H's, which are found near 0 ppm in models (Goff & La Mar, 1977).

Heme Rotational Disorders. The origin of the two set of interconvertible resonances can be attributed to the presence of two heme orientations in the heme pocket which differ by 180° rotation about the α–γ–meso axis (La Mar et al., 1978a, 1980a,c; La Mar, 1979). This can be based solely on the fact that reduction of samples of metHbCN's, which had been previously demonstrated to exhibit the heme rotational disorder in solution (La Mar et al., 1980a), yields deoxy-Hb's which display two sets of resonances with unaltered relative intensities. This view is independently confirmed by the present Hb III methyl assignments, where the major and minor compounds again differ in that the similarly shifted methyls exhibit the 5-CH_3 ⇄ 8-CH_3 and 1-CH_3 ⇄ 3-CH_3 interchange characteristic of the 180° rotation about the α–γ–meso axis in a uniquely folded protein. These assignments suggest that such heme rotational disorder can be independently characterized in other high-spin ferrous hemoproteins exhibiting multiple sets of resonances.

The unaltered ratio of the two heme orientations in deoxy-Hb's III and IV eliminates the possibility that its presence in the metHbCN's can be linked to the crystallographically characterized disorder in the position of the "distal" histidine E7. The crystal structure of deoxy-Hb III was found to have histidine-E7 exclusively outside the heme pocket (Steigemann

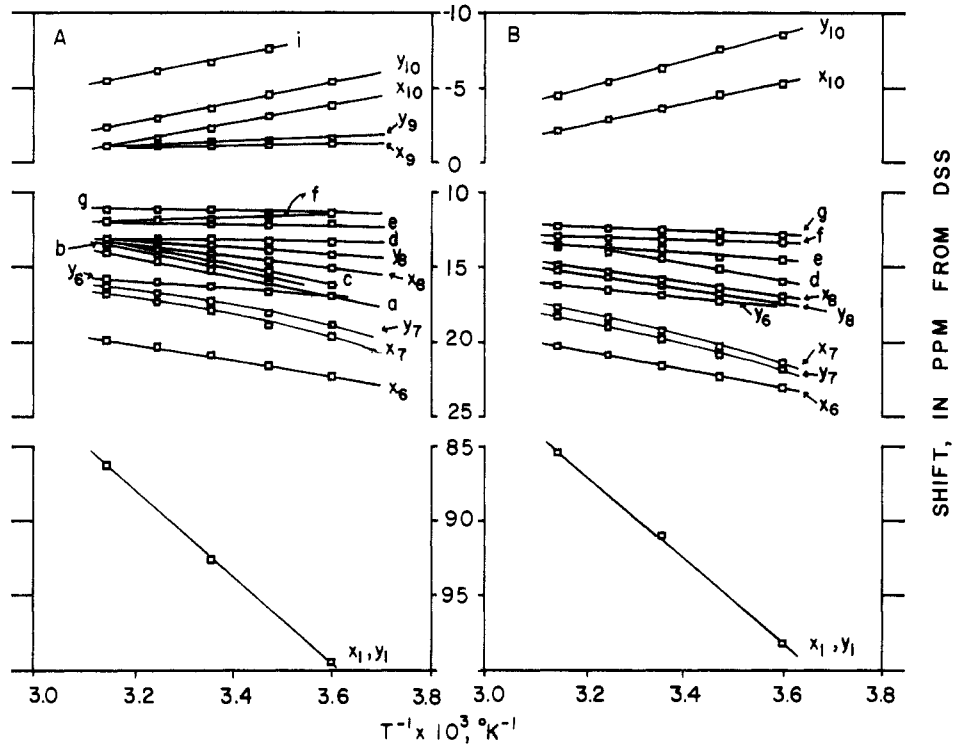

FIGURE 6: Plot of the observed shift, referenced against DSS, vs. the reciprocal temperature (Curie plot) for the resonances of (A) Hb III and (B) Hb IV. All resonances wer observed in ^2H_2O, except for x_i and y_i in 90% H_2O. Peak designations are as outlined in captions to Figures 2, 4, and 5.

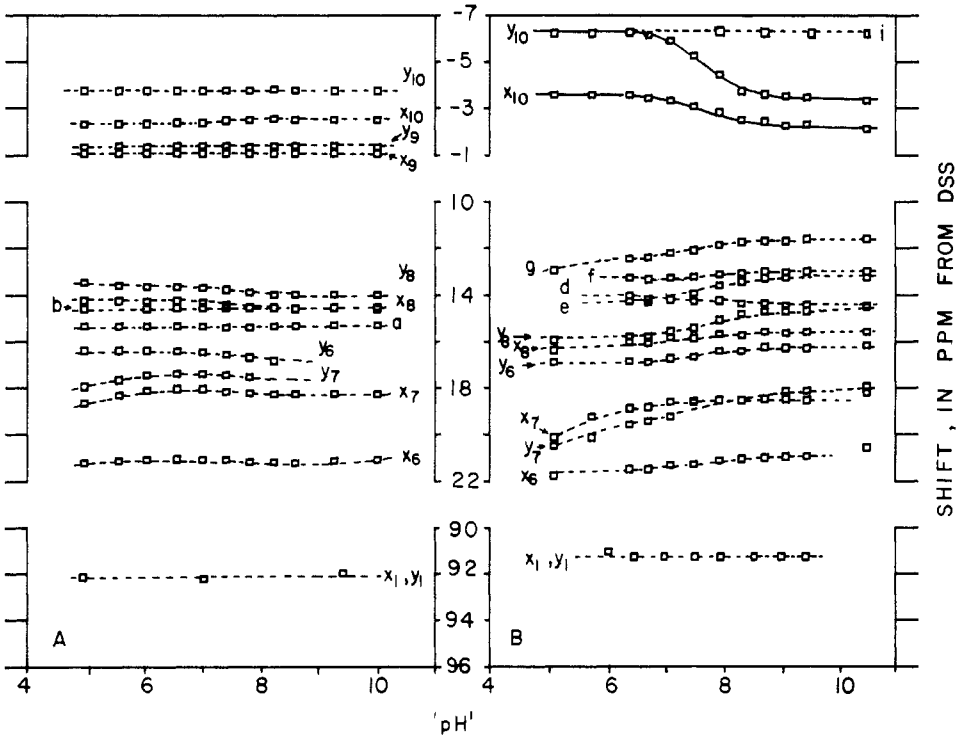

FIGURE 7: Plot of observed shift at 25 °C, referenced to DSS, vs. "pH" of resonances in (A) Hb III and (B) Hb IV. All resonances were observed in ^2H_2O except x_1 and y_1 in H_2O; the nonexchangeable peaks yielded essentially superimposable "pH" profiles in ^2H_2O and H_2O. Peak designations are as indicated in the captions to Figures 2, 4, and 5. Solid lines correspond to fits to the Henderson–Hasselbalch equation; dotted lines only indicate continuity.

& Weber, 1979). Thus we conclude that heme rotational disorder is characteristic of both the unligated and ligated states of the CTT Hb's. The lack of evidence for such rotational disorder in the X-ray data suggests that only one form of the protein crystallizes. This illustrates that hemoglobin conformation in solution must not necessarily be identical with that in crystals.

Electronic Structure and Metal–Ligand Bonding. The heme methyl hyperfine shifts show much larger spreads than those of models (Goff & La Mar, 1977), indicative of the much larger in-plane or rhombic asymmetry found in protein environments for all paramagnetic oxidation/spin states of iron (La Mar, 1979). However, unlike the metHbCN's (La Mar et al., 1980a) or metMbCN (Mayer et al., 1974; La Mar et

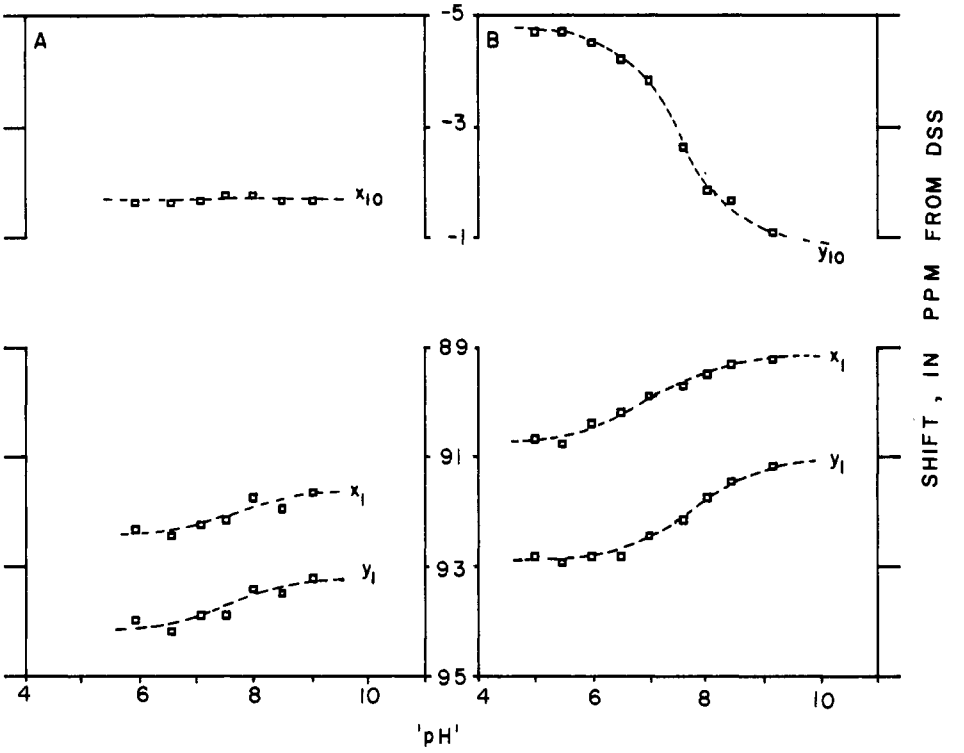

FIGURE 8: Plot of observed shifts at 25 °C, referenced to DSS, vs. "pH" of selected resonances in (A) deuteroheme-Hb III, and (B) deuteroheme-Hb IV. Peaks x_{10} and y_{10} correspond to the presumed 5-CH_3 and 8-CH_3 of the major and minor component, in analogy to the native proteins; all resonances were observed in H_2O.

al., 1978a), where the methyl shift pattern can be correlated with the nature of possible rhombic perturbation (Traylor & Berzinis, 1980) and where similar protein tertiary structures yield similar shift patterns, the shifts in deoxy-Hb's defy interpretation at this time. This is largely due to the fact that the exact origins of the contact shifts in terms of relevant spin transfer mechanisms are still largely obscure (Goff & La Mar, 1977; La Mar & Walker-Jensen, 1979). Thus, while deoxy-Mb exhibits two heme methyls downfield (5-CH_3 and 3-CH_3) (D. L. Budd and G. N. La Mar, unpublished data), deoxy-Hb III displays 1-CH_3 (or 3-CH_3) downfield and 5-CH_3 (or 8-CH_3) upfield. A more definitive discussion of possible origins of the rhombic perturbation must await analysis of both better model compounds and other structurally characterized proteins.

The variable temperature data in Figure 6 indicate approximate adherence to the Curie law in that a number of the downfield resonances yield staight lines which extrapolate well into the diamagnetic region for $T^{-1} = 0$ (Jesson, 1973). The curved Curie plots for x_7 and y_7 strongly suggest that these groups exhibit temperature-dependent conformational changes previously found for both propionic and vinyl H_α's (La Mar et al., 1978b, 1980b). Since the vinyl H_α's are assigned by deuterium labeling and x_6 and y_6 also exhibit an additional apparent $pK \leq 5$, the most likely assignments are propionic acid H_α's (see above).

The nature of the state of the trans ligand, generally imidazole, is considered critical in modulating the reactivity of the iron in hemoproteins (Braunitzer et al., 1974; Overkamp et al., 1976). The exchangeable 1′-H hyperfine shift from the proximal histidyl imidazole has been shown to arise primarily from iron–imidazole σ bonding (Goff & La Mar, 1977), so that its magnitude can be taken as a direct measure of the Fe–N covalency. The CTT deoxy-Hb 1′-H shifts of 90–95 ppm are larger than those observed in either high-spin ferrous model compounds, deoxy-Mb's, or tetrameric deoxy-Hb's (Goff & La Mar, 1977; La Mar et al., 1977; La Mar, 1979), suggesting a stronger iron–imidazole bond in CTT Hb's. Both CTT Hb's are characterized by a pronounced immobility of the proximal imidazole because of a unique stacking with the Phe-F4 (Huber et al., 1971). The large hyperfine shift indicates that slight compression of the Fe–N bond distance is possible. Such a compression of the Fe–N bond is suggested to also account for the unusually high on-rate for both O_2 and CO ligation compared to other monomeric hemoglobins. Although the 1′-H imidazole shifts for the two components are degenerate for both native Hb's III and IV, the influence of the heme disorder on the histidine environment is manifested in different hyperfine shifts for the 2′-H and 4′-H. The average shifts in the major and minor components, however, are essentially unchanged, arguing against significant differences in Fe–N covalency in the two components of the native Hb's. For deuteroheme-Hb III and IV, however, the 1′-H shift is larger for the minor compnents, indicating a slightly greater Fe–N covalency and thus suggesting the possibility that the ligation on-rates in the two components of the reconstituted proteins may differ.

The observation of what appears to be different environments (x_9 and y_9) due to the heme disorder for a nonheme methyl resonance indicates that the heme orientation affects the protein conformation to some degree in the heme cavity. Similar influences of the orientation of deuterohemin (La Mar et al., 1978a) on some amino acid peaks in sperm whale metMbCN have been observed (G. N. La Mar and N. L. Davis, unpublished observations).

pH-Induced Structural Transition. The pH-independent shifts found for Hb I are consistent with the protein exhibiting neither a Bohr effect nor a t $\rightleftharpoons$ r structural transition. This protein also failed to give evidence for a structural change in the metHbCN form (La Mar et al., 1978a). Both native Hb's III and IV exhibit inflection points in the plot of shift vs. p^2H (Figure 7). The amplitudes of the shift changes are larger for Hb IV than for Hb III, as also found for the metHbCN's (La Mar et al., 1978a), and the apparent $pK \sim 8.0$ for Hb IV

is consistent with the known presence of an alkaline Bohr effect (Gersonde et al., 1972, 1976; Sick & Gersonde, 1974). The amplitude of the O_2 Bohr effect curve of Hb IV, $\log p_{1/2}(O_2)_{max} - \log p_{1/2}(O_2)_{min} = 0.8$, and the inflection point at pH 7.3 are consistent with the apparent pK of 8 found for the pH-dependent shift changes in D_2O. Although the small shift changes with "pH" and the extensive overlap of resonances preclude determination of an accurate pK for deoxy-Hb III, the apparent inflection points are also consistent with a pK near 8. The parallel of the magnitude of the pH-induced structural change in deoxy-Hb's and metHbCN's and the correspondence with the pK's calculated from the Bohr effect curve indicate that the structural change monitored in the deoxy-Hb's is also modulated by the Bohr proton and, hence, involves a t $\rightleftharpoons$ r transition in the unligated state.

However, inspection of Figure 7 reveals that the nature of the t $\rightleftharpoons$ r transition localizes the perturbation primarily on the heme resonances, although the shift changes cannot be interpreted in terms of changes in structure at this time. The proximal histidine environment, especially the iron–imidazole covalency thought to control the iron reactivity (Gersonde et al., 1972; Sick et al., 1972; Braunitzer et al., 1974; Overkamp et al., 1976), is essentially unaffected by the t $\rightleftharpoons$ r transition in the deoxy state, as evidenced by the pH-insensitive 1′-H shift in *native* Hb's III and IV. We therefore conclude that while the t $\rightleftharpoons$ r transition takes place in deoxy-Hb's, the iron is little affected, accounting for the pH-independent ligation on-rates (H. Sick, A. I. Raap, and K. Gersonde, unpublished results), the pH-insensitive Co^{II}Hb ESR parameters (K. Gersonde, H. Twilfer, and M. Overkamp, unpublished results), and the pH-insensitive energy levels of the heme iron as determined by the temperature dependence of the quadrupole splitting (F. Parak, C. Hermes, and S. Formanek, unpublished results).

In the case of deuteroheme-Hb's III and IV, we are able to resolve the 1′-H peaks for the two protein components. Moreover, the pH dependence for the hyperfine shifted resonance, although similar to the native Hb's for the heme resonances, is not negligible for the proximal histidyl imidazole 1′-H's in these reconstituted proteins, as depicted in Figure 8. In these proteins, the data suggest that the axial bond compression is less at alkaline (r) than acidic (t) pH, although the shifts in either region are still larger than for deoxy-Mb or models. Thus reconstitution with deuteroheme leads to CTT Hb's in which the electronic state of the central iron can be controlled by the Bohr proton binding site even in the deoxy state. While it is known that deuteroheme-Hb's III and IV exhibit O_2 Bohr effects similar to those of the native proteins (La Mar et al., 1978a), the individual on- and off-rates are not yet known. Hence our studies suggest the possibility that the on-rates for the reconstituted proteins may exhibit some pH dependence. It can also be anticipated that the ESR parameters of deoxy-Co^{II}-deuteroheme-Hb's may exhibit a measureable pH effect. Such studies on Co^{II}-deuteroheme-Hb's are planned.

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