

# Assignment of $^1\text{H}$ NMR Resonances of Histidine and Other Aromatic Residues in Met-, Cyano-, Oxy-, and (Carbon monoxy)myoglobins<sup>†</sup>

John A. Carver<sup>†</sup> and J. Howard Bradbury\*

**ABSTRACT:** The resolved  $^1\text{H}$  NMR resonances of the aromatic region in the 270-MHz NMR spectrum of sperm whale, horse, and pig metmyoglobin (metMb) have been assigned, including the observable H-2 and H-4 histidine resonances, the tryptophan H-2 resonances, and upfield-shifted resonances from one tyrosine residue. The use of different Mb species, carboxymethylation, and matching of pK values allows the assignment of the H-4 resonances, which agree in only three cases out of seven with scalar-correlated two-dimensional NMR spectroscopy assignments by others. The conversion to hydroxymyoglobin at high pH involves rearrangements throughout the molecule and is observed by many assigned residues. In sperm whale ferric cyanomyoglobin, nine H-2 and eight H-4 histidine resonances have been assigned, including the His-97 H-2 resonance and tyrosine resonances from residues 103 and 146. The hyperfine-shifted resonances from heme and near-heme protons observe a shift with a pK =  $5.3 \pm 0.3$  (probably due

to deprotonation of His-97, pK = 5.6) and another shift at pK =  $10.8 \pm 0.3$ . The spectrum of high-spin ferrous sperm whale deoxymyoglobin is very similar to that of metMb, which allows the assignment of seven surface histidine H-2 and H-4 resonances and also resonances from the two tryptophan residues and one tyrosine. In diamagnetic sperm whale (carbon monoxy)myoglobin (COMb), 10 His H-2 and 11 His H-4 resonances are observed, and 8 H-2 and 9 H-4 resonances are assigned, including His-64 H-4, the distal histidine. This important resonance is not observed in sperm whale oxymyoglobin, which in general shows very similar titration curves to COMb. Histidine-36 shows unusual titration behavior in the paramagnetic derivatives but normal behavior in the diamagnetic derivatives, which is discussed in the accompanying paper [Bradbury, J. H., & Carver, J. A. (1984) *Biochemistry* (following paper in this issue)].

**M**yoglobin is a monomeric heme protein from vertebrate muscle that is involved in oxygen storage and transport. The crystal structure of metMb<sup>1</sup> was the first protein structure to be determined by X-ray crystallography (Kendrew et al., 1958, 1960). The molecule consists of 153 amino acid residues folded into eight right-handed  $\alpha$ -helical regions (A-H) with the heme group located in a hydrophobic crevice between the E and F helices. The iron atom is octahedrally coordinated to four nitrogen atoms of the porphyrin ring, a nitrogen atom of the invariant proximal histidine at position-93(F8), and a ligand that is oxygen in the case of the physiologically important diamagnetic ferrous oxyMb. DeoxyMb has no ligand. The iron atom in the paramagnetic ferric state can also bind a number of ligands of various spin states.

Most of the early  $^1\text{H}$  NMR studies on Mb were on low-spin ferric cyanomyoglobin (CNMb). In this case, resonances of protons located near the paramagnetic center are shifted to low and high field and, hence, are readily observed (Wuthrich et al., 1968; Shulman et al., 1969; Sheard et al., 1970). The NH protons of oxyMb were observed in water (Patel et al., 1970), and the titration of nine histidines in diamagnetic COMb was followed (Thompson et al., 1971). Subsequently, assignment was made of the resonances of seven surface histidines in sperm whale ferric metMb (Cohen et al., 1972; Hayes et al., 1975; Botelho & Gurd, 1978; Botelho et al., 1978). Ohms et al. (1979) observed nine H-2 resonances that shifted with pH in oxyMb, and we observed the H-2 and H-4 resonances of the titrating histidines in COMb and oxyMb (Bradbury et al., 1979, 1981); the proximal histidine residue (His-93) does not titrate. The aromatic region of the  $^{13}\text{C}$

NMR spectra of paramagnetic myoglobins has been studied by Wilbur & Allerhand (1976, 1977).

In this paper we assign the histidine H-2 and H-4 resonances and other resonances in the aromatic region in the  $^1\text{H}$  NMR spectrum of metMb, hydroxymMb, CNMb, COMb, deoxyMb, and oxyMb. In the accompanying paper (Bradbury & Carver, 1984) we give additional data on near-heme resonances [assigned by Bradbury et al. (1982)] and examine the nature of the changes undergone by Mb as a result of the binding of different ligands.

## Experimental Procedures

**Materials.** Sperm whale and horse Mb were obtained from Sigma Chemical Co. Pig Mb was prepared from pig hearts by the method of Hapner et al. (1968). Bromoacetic acid (Pflatz and Bauer) was recrystallized from petroleum ether. Deuterium oxide (>99.7%  $^2\text{H}_2\text{O}$ ) was obtained from the Australian Atomic Energy Commission.

**Preparation of Samples.** Salt-free metMb was lyophilized 2 or 3 times from  $^2\text{H}_2\text{O}$  to remove exchangeable protons. A 10% w/v solution was prepared in 0.1 M NaCl/ $^2\text{H}_2\text{O}$ , and the pH was adjusted with  $^2\text{HCl}$  or  $\text{NaO}^2\text{H}$ . A Beckman Model 4500 pH meter was used, fitted with an Ingold combination electrode. The pH was measured before and after the NMR measurement, and all pH values are uncorrected for deuterium isotope effects. CNMb solutions were prepared by adding a 5 molar excess of KCN to metMb solutions. COMb solutions were prepared by reduction of 10% MetMb solutions with

<sup>†</sup> From the Department of Chemistry, Australian National University, Canberra, A.C.T. 2600, Australia. Received August 25, 1983; revised manuscript received March 14, 1984. This work was supported by the Australian Research Grants Scheme.

\* Present address: Department of Biochemistry, University of Oxford, Oxford, England.

<sup>1</sup> Abbreviations: NMR, nuclear magnetic resonance; 2D COSY, scalar-correlated two-dimensional NMR spectroscopy; Mb, myoglobin; metMb, ferric aquomyoglobin; hydroxymMb, ferric hydroxymyoglobin; CNMb, ferric cyanomyoglobin; N<sub>3</sub>Mb, ferric azidomyoglobin; COMb, ferrous (carbon monoxy)myoglobin; deoxyMb, ferrous deoxymyoglobin; oxyMb, ferrous oxymyoglobin; Hb, hemoglobin; deoxyHb, ferrous deoxyhemoglobin; COHb, ferrous (carbon monoxy)hemoglobin; EDTA, ethylenediaminetetraacetic acid.

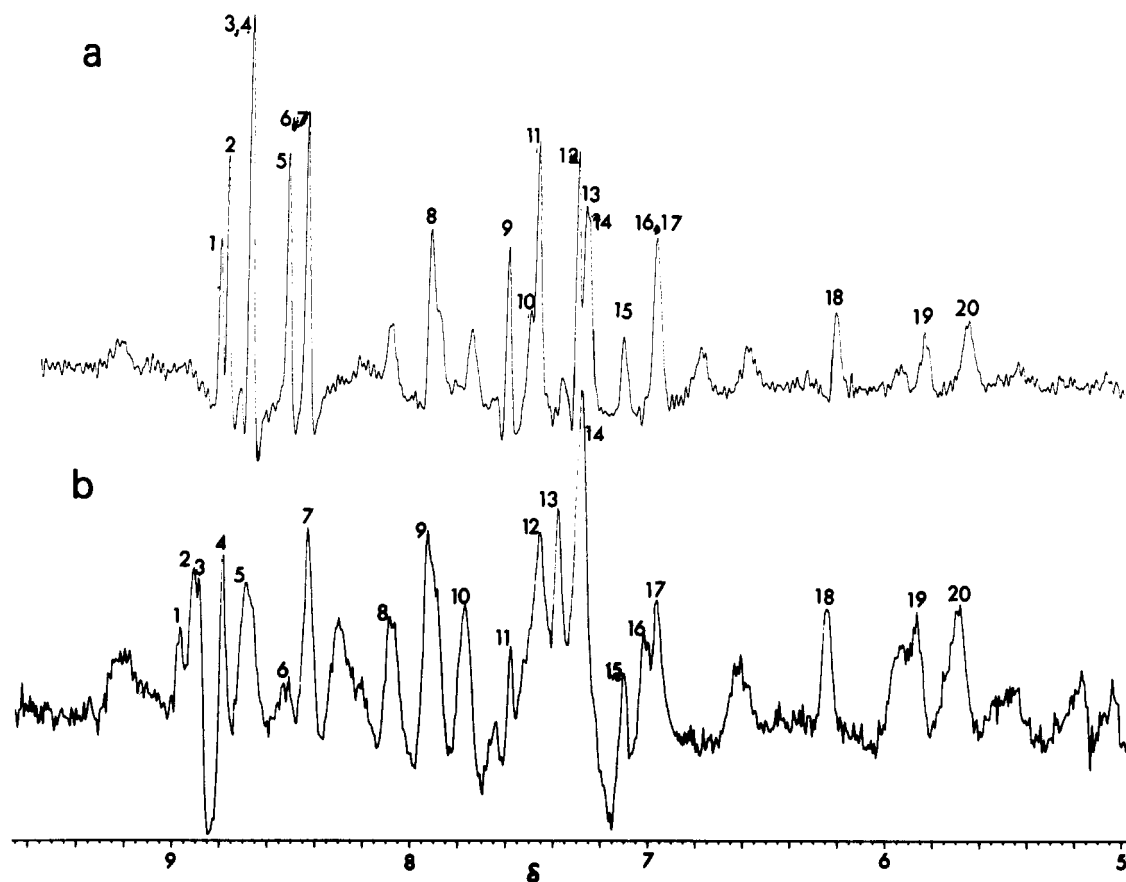


FIGURE 1: Aromatic region of the  $^1\text{H}$  NMR spectra at 270 MHz, pH 5.24, and 20 °C of (a) sperm whale metMb and (b) sperm whale metMb after carboxymethylation at pH 6.8 for 10 days.

dithionite ( $\sim 10$  mg for 200 mg of Mb) and bubbling of CO for several minutes. The solution was loaded onto a column ( $0.5 \times 8$  cm) of Sephadex G-25 preequilibrated with CO-saturated 0.1 M NaCl/ $^2\text{H}_2\text{O}$  and eluted with this solution, and the eluted band of COMb free of dithionite was collected. For oxyMb, a solution of metMb was prepared in 10 mM potassium deuteriophosphate, pH 7, in  $^2\text{H}_2\text{O}$ , containing 0.1 mM EDTA. The solution was reduced with dithionite and loaded onto a column (see above) preequilibrated with air-saturated buffer. The red solution was eluted and completely oxygenated by gentle shaking and exposure to air. The solution was stored in ice prior to use. For deoxyMb, the solution of metMb in the above buffer was bubbled with oxygen-free nitrogen for 1 h; handling and preparations of solutions were done in a glove box under nitrogen. The deoxygenated solution was reduced with dithionite and applied to a column (see above) preequilibrated with nitrogen-saturated buffer. The eluent was collected and stored in ice under nitrogen. The oxyMb and deoxyMb solutions were used soon after preparation. To check that sample degradation had not occurred, a 30-fold dilution of the sample was made after the NMR experiment and the visible absorption spectrum measured on a Cary 219 spectrometer.

**Reaction of Sperm Whale Myoglobin with Bromoacetic Acid.** The carboxymethylation procedure of Hugli & Gurd (1970a) and Nigen & Gurd (1973) was used and the extent of reaction monitored by amino acid analysis. All values agreed with those of Botelho & Gurd (1978).

**NMR Spectroscopy.** NMR spectra were recorded on a Bruker HX-270 NMR spectrometer with an Oxford Instrument Co. superconducting magnet, and some spectra were obtained on a Bruker WM-400 spectrometer. Spectra were accumulated (usually 1024 scans) in 4K or 8K data points with

a Nicolet 1180 computer and transformed over 4K or 8K data points. Resolution enhancement was by trapezoidal multiplication (Gassner et al., 1978). The  $^2\text{HHO}$  resonance was suppressed by selective gated irradiation (Campbell et al., 1975a), and dioxane was the internal reference ( $\delta$  3.74). A Carr-Purcell pulse sequence ( $90^\circ - \tau - 180^\circ - \tau$ ) was used to separate resonances on the basis of differences in their  $T_2$  values ( $\tau = 30$ –60 ms removes resonances with short  $T_2$  values) and differences in multiplet structure ( $\tau = 60$  ms inverts doublets) (Campbell et al., 1975b). Titration data were analyzed by a linear version of the Henderson-Hasselbalch equation, with a computer program to determine titration parameters. When both the acidic,  $\delta_A$ , and basic,  $\delta_B$ , limiting chemical shifts were obtainable and data were collected at pH intervals of 0.2–0.3, the program gave a pK with an error of  $\pm 0.05$ . Larger errors occurred if  $\delta_A$  was not obtainable (due to low stability of Mb derivatives at pH  $< 5$ ) and also if there were problems due to overlapping resonances.

## Results and Discussion

**MetMb H-2 Histidine Resonances.** The pH variation of the resolved histidine H-2 resonances in sperm whale metMb (1–7 in Figure 1a) and the corresponding resonances in horse and pig metMb are given in Figure 2. The agreement between the titration parameters in Table I for different workers is good for sperm whale and horse metMb. New data are presented on pig metMb. The assignments are due to Botelho & Gurd (1978). The H-2 resonance of His-36 (7 in both spectra of Figure 1) was the only H-2 resonance that titrated in the carboxymethylated sample [in agreement with Botelho & Gurd (1978)] but broadened considerably at higher pH values as the resonance shifted upfield. In Figure 1b, resonances 1–4 did not move with pH and disappeared progressively above

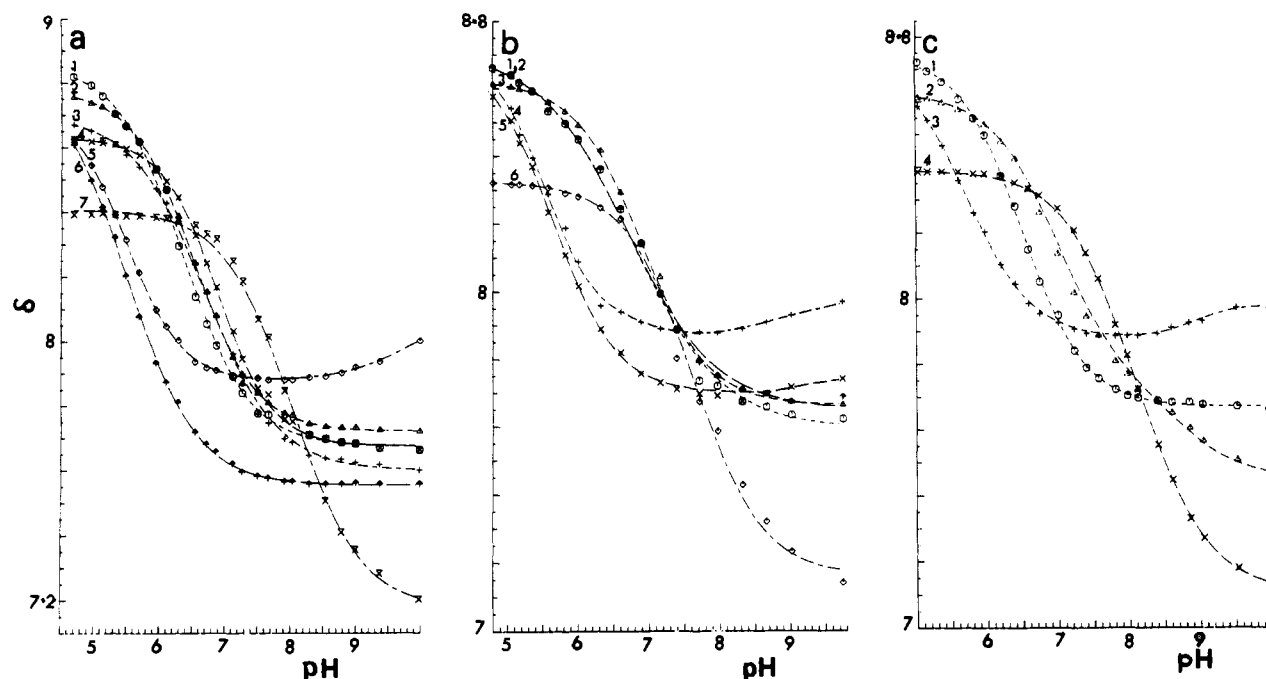


FIGURE 2:  $^1\text{H}$  NMR titration curves at 20 °C for the histidine H-2 resonances of (a) sperm whale, (b) horse, and (c) pig metMb.

Table I:  $^1\text{H}$  NMR Titration Parameters for the Histidine H-2 Resonances of Sperm Whale, Horse, and Pig MetMb at 20 °C<sup>a</sup>

Sperm Whale																					
His-81			His-12			His-48			His-116			His-119			His-113			His-36			
	this work	b	c	this work	b	c	this work	b	c	this work	b	c	this work	b	c	this work	b	c	this work	b	c
resonance	1			2			3			4			5 <sup>e</sup>			6			7		
pK value	6.36	6.17	6.34	6.52	6.82	6.44	6.70	6.73	6.55	6.88	6.55	6.83	5.48	5.46	5.41	5.50	5.49	5.37	8.03	7.97	8.05
$\delta_B$	7.68	7.74	7.77	7.72	7.71	7.80	7.60	7.63	7.71	7.68	7.63	7.75	7.89	7.89	7.93	7.56	7.58	7.70	7.18	7.16	7.31
$\Delta$	1.18	1.02	1.06	1.05	1.00	0.98	1.09	0.99	0.95	0.95	0.97	0.90	0.83	0.81	0.83	1.08	1.00	1.05	1.28	1.18	1.09
Horse																					
His-81			His-48			His-116			His-119			His-113			His-36						
	this work	c	d	this work	c	d	this work	c	d	this work	c	d	this work	c	d	this work	c	d			
resonance	1			2			3			4 <sup>e</sup>			5 <sup>e</sup>			6					
pK value	6.54	6.63	6.5	6.80	6.90	6.8	6.85	7.02	6.9	5.46	5.68	5.5	5.58	6.03	5.8	7.67	7.62	7.6			
$\delta_B$	7.66	7.63		7.65	7.66		7.61	7.61		7.90	7.90		7.70	7.63		7.12	7.21				
$\Delta$	1.11	1.08		1.00	0.96		1.04	1.00		0.76	0.74		1.02	1.01		1.23	1.12				
Pig																					
His-81 (this work)			His-48 (this work)			His-119 (this work)			His-36 (this work)												
resonance	1			2			3 <sup>e</sup>			4											
pK value	6.51			7.02			5.72			8.11											
$\delta_B$	7.67			7.46			7.88			7.11											
$\Delta$	1.07			1.17			0.86			1.28											

<sup>a</sup>  $\delta_B$  is the chemical shift of the resonance in the alkaline titration limit, and  $\Delta$  is the total chemical shift change on deprotonation. The results of other workers are presented for comparison. <sup>b</sup> Results of Botelho & Gurd (1978). <sup>c</sup> Results of Cohen et al. (1972) and Hayes et al. (1975). <sup>d</sup> Results of Botelho et al. (1976). <sup>e</sup> Curve exhibiting a second inflexion at high pH with a pK  $\sim$  9 due to the hemic acid dissociation (see text).

pH 8 (Bradbury & Norton, 1975) and therefore arise from the C-2 protons of the bis(carboxymethyl) derivatives of the surface histidines. Resonances 5 and 6 belong to the mono-(carboxymethyl)histidine H-2 resonances of His-48 and His-119 (Botelho & Gurd, 1978).

**MetMb H-4 Histidine Resonances.** Figure 3 shows the titration curves for the H-4 histidine resonances of sperm whale (Figure 1), horse, and pig metMb, and Table II gives the assignments and titration parameters. Various resonances, 12, 14, 16, and 17 in Figure 3a, 10, 11, and 13 in Figure 3b, and 7, 8, and 10 in Figure 3c, moved slightly at high pH with pK  $\sim$  9 and are therefore observing conformational changes associated with the hemic acid dissociation (the replacement of the water ligand by hydroxyl at pH 9; Brunori et al., 1968) rather than undergoing a titration shift. In the Carr-Purcell

spectrum (Figure 4b), the resonances observed in the H-4 histidine region are all singlets. They belong to the seven surface histidine C-4 protons, Trp-7, and 14 C-2 protons (resonances 14 and 15, see below), because they lack the normal titration shift of H-4 histidine resonances.

The assignment of His-12, -113, and -116 H-4 resonances is achieved because of the absence of His-12 in horse Mb and of His-12, -113, and -116 in pig Mb. Thus, the absence of a titration curve similar to resonance 11 in Figure 3a from Figure 3b (horse metMb) and also from Figure 3c shows that resonance 11 arises from His-12 H-4. His-113 and -116 show pK values of  $\sim$  5.5 and  $\sim$  6.9, respectively (Table I), in sperm whale and horse metMb, and one titration curve in Figure 3a,b with a low pK and one with a normal pK are absent in Figure 3c for pig metMb. Clearly in sperm whale metMb, resonances

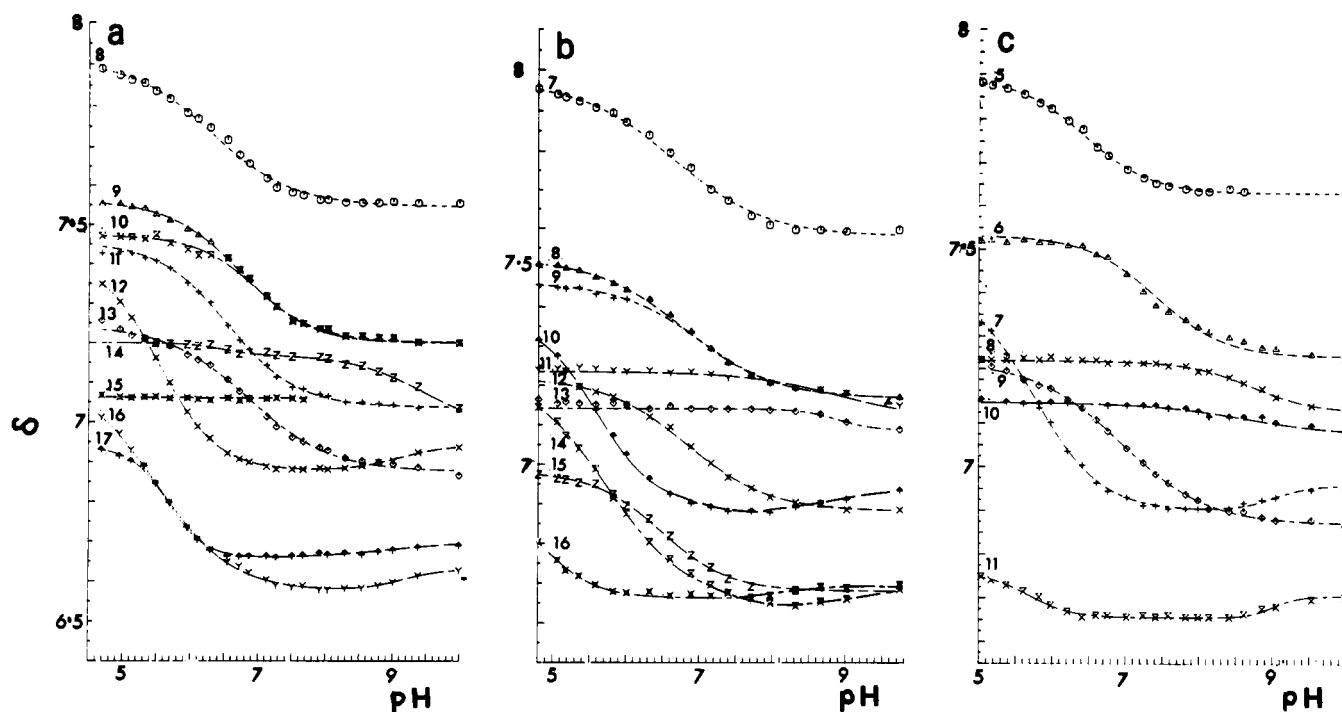


FIGURE 3:  $^1\text{H}$  NMR titration curves at 20 °C for the resonances observed in the H-4 histidine region of the spectrum of (a) sperm whale, (b) horse, and (c) pig metMb. Numbers correspond with those given in Figure 1.

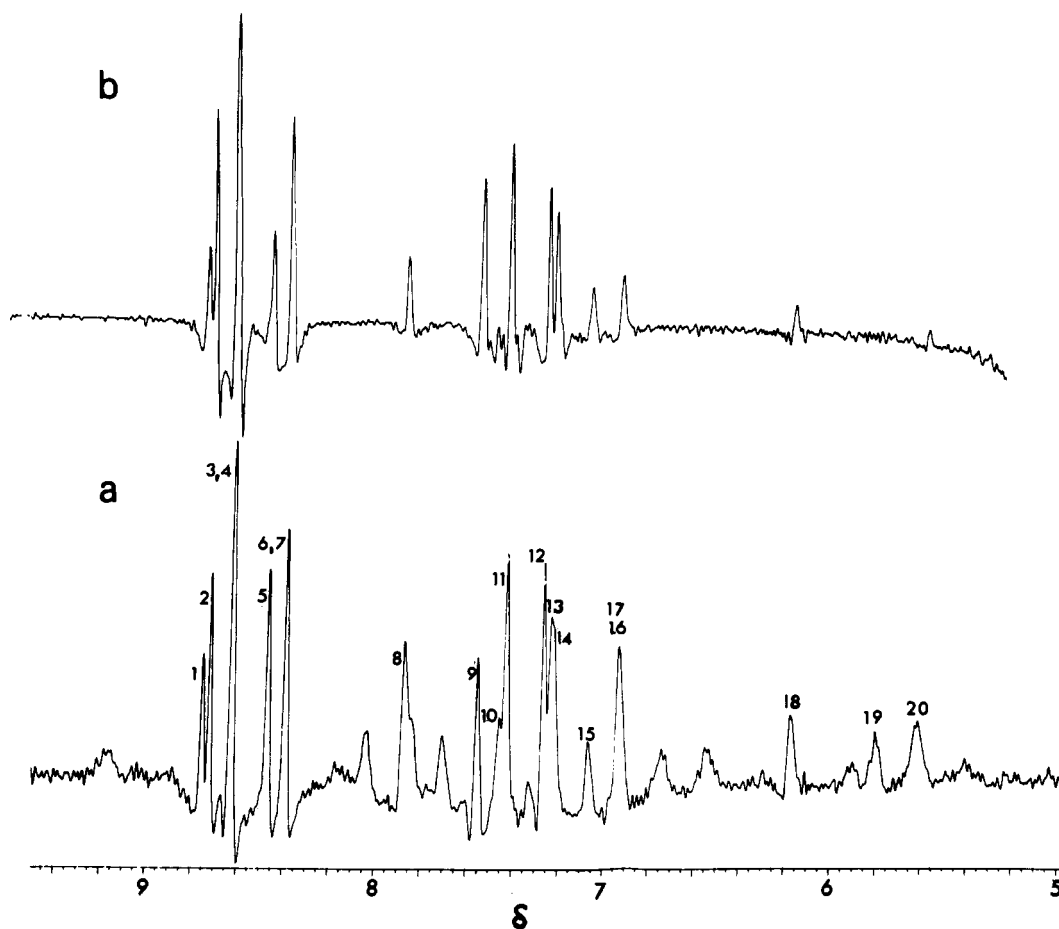


FIGURE 4: Aromatic region of the  $^1\text{H}$  NMR spectrum of sperm whale metMb at 20 °C and pH meter reading of 5.16: (a) resolution-enhanced spectrum; (b) spectrum from Carr-Purcell pulse sequence with  $\tau = 30$  ms.

9 and 16 are assigned to His-116 and -113 H-4, respectively (Table II). Resonances 8 and 13 in sperm whale metMb have  $pK$ 's of 6.34 and 6.85, respectively, and resonance 8 is found downfield of resonance 13. These  $pK$  values agree well with those of His-81 (6.36) and His-48 (6.70) H-2 resonances, and

from Figure 2a, the H-2 curve of His-81 falls downfield from that of His-48.

Hugli & Gurd (1970a) and Nigen & Gurd (1973) found that His-36 is unaffected by carboxymethylation at pH 6.8, whereas His-119 forms the 1-(carboxymethyl) derivative. Only

Table II: <sup>1</sup>H NMR Titration Parameters for the Resonances Observed in the H-4 Histidine Region of Sperm Whale, Horse, and Pig MetMb at 20 °C

	His-81	His-12	His-48	His-116	His-119	His-113	His-36	His-82 <sup>a</sup>	Trp-14 H-2 <sup>a</sup>	Trp-7 H-2 <sup>a</sup>
Sperm Whale										
resonance	8	11	13	9	12 <sup>b</sup>	16 <sup>b</sup>	17 <sup>b</sup>	10	14	15
pK value	6.34	6.54	6.85	6.71	5.53	5.50	5.67	6.97	9.42	
δ <sub>B</sub> <sup>c</sup>	7.55	7.04	6.88	7.21	6.89	6.59	6.66	7.20	6.99	7.06
Δ <sup>c</sup>	0.36	0.41	0.37	0.36	0.52	0.49	0.27	0.27	0.21	
Horse										
resonance	7		12	8	10 <sup>b</sup>	14 <sup>b</sup>	16 <sup>b</sup>	9	11	13
pK value	6.67		6.83	6.80	5.59	5.75	4.83	7.02	8.92	8.90
δ <sub>B</sub>	7.58		6.88	7.17	6.89	6.65	6.66	7.17	7.12	7.09
Δ	0.39		0.34	0.35	0.48	0.58	0.27	0.29	0.12	0.06
Pig										
resonance	5		9		7 <sup>b</sup>		11 <sup>b</sup>	6	8	10
pK value	6.44		6.94		5.76		5.68	7.41	8.72	8.64
δ <sub>B</sub>	7.63		6.87		6.91		6.65	7.25	7.13	7.08
Δ	0.27		0.37		0.51		0.11	0.28	0.12	0.07

<sup>a</sup> Tentative assignments. <sup>b</sup> Curve exhibiting a second inflexion at high pH with a pK ~ 9 due to the hemic acid dissociation. <sup>c</sup> Definitions of δ<sub>B</sub> and Δ are given in Table I. <sup>d</sup> Resonance was smaller in area than the other resonances and was not observed in sperm whale or pig metMb.

one H-4 resonance in carboxymethylated metMb (17 in Figure 1b) moves with pH, and its titration characteristics are identical with those of resonance 17 in sperm whale metMb (Figure 1a), and it is therefore assigned to His-36 H-4. Thus, His-36 H-2 and H-4 resonances exhibit very different titration characteristics with the H-2 showing a high pK, whereas the H-4 exhibits a low pK and an inflexion at high pH in the opposite direction to that observed for the H-2. This behavior is explained in the accompanying paper (Bradbury & Carver, 1984). The remaining unassigned H-4 resonance in Figure 4b is resonance 12, and this therefore is assigned to the remaining surface H-4 histidine (His-119). The pK value of resonance 12 (5.53) agrees well with that of the His-119 H-2 resonance (5.48), and both resonances show a similar inflexion at high pH due to the hemic acid dissociation. Tentative assignments have been made of the H-4 resonances in Figure 1b of metMb carboxymethylated at pH 6.8 (Carver, 1982).

King & Wright (1982) have used 2D COSY spectroscopy to assign the H-4 resonances of sperm whale metMb by using the H-2 resonance assignments of Botelho & Gurd (1978). Their assignments for the H-4 resonances of His-12, -36, and -119 agree with those presented here. Their assignments for His-48 and -116 are incorrectly inverted, presumably due to the overlap of the H-2 resonances of His-48 and -116 at the pH at which their experiment was undertaken. Our assignment of His-116 is based on deletion of His-116 and its H-4 resonance in pig metMb (Table II). The assignment of His-113 H-4 resonance by King & Wright (1982) to resonance 10 of Figures 1a and 3a (pK = 7.0) is compatible with neither the pK value of the His-113 H-2 resonance (5.5) nor the presence of this resonance in the spectrum of pig metMb (resonance 6 in Table II and Figure 3c), since His-113 is deleted from pig metMb. Their assignment of resonance 16 (pK value of 5.5) to the His-81 H-4 resonance is not compatible with the pK value of 6.4 exhibited by the H-2 resonance and the absence of resonance 16 in pig metMb (Table II), since His-81 is retained in pig metMb. The very small intensity of the cross-peak associated with the His-81 H-4 resonance in their COSY spectrum may explain this incorrect assignment. The poor agreement between the COSY assignments and those given here on the basis of species deletions, carboxymethylation, and pK value correlations shows the need for COSY spectra to involve cross-peaks of reasonable intensities and perhaps to be checked at different pH values.

#### Assignment of Other Resonances in MetMb Spectrum.

Resonance 10 (Figures 1a and 4a) is absent in the Carr-

Table III: <sup>1</sup>H NMR Titration Parameters for the Upfield Resonances Observed in the Aromatic Region of Sperm Whale, Horse, and Pig MetMb at 20 °C<sup>a</sup>

	tentative assignments		
	His-24 H-4	Tyr-103 2,6 or 3,5	Tyr-103 2,6 or 3,5
Sperm Whale			
resonance	18	19	20
pK value	9.11	9.38	9.08
δ <sub>A</sub>	6.17	5.76	5.57
Δ	0.17	0.58	0.66
Horse			
resonance	17	18	19
pK value	9.09	9.01	8.91
δ <sub>A</sub>	6.18	5.84	5.69
Δ	0.12	0.56	0.69
Pig			
resonance	12	13	14
pK value	8.90	8.91	8.84
δ <sub>A</sub>	6.13	5.87	5.72
Δ	0.12	0.59	0.72

<sup>a</sup> δ<sub>A</sub> is chemical shift at the acid titration limit; Δ is the total downfield shift in ppm with increasing pH.

Purcell spectrum (Figure 4b), showing that it has a short T<sub>2</sub>, although its titration curve (Figure 3a) is consistent with a H-4 histidine. It is tentatively assigned to His-82 H-4, which is buried (Takano, 1977). Resonances 14 and 15 (Figure 1) show only a small alkaline-pH shift due to the hemic acid dissociation, and a COSY spectrum shows that they do not arise from histidine C-4 protons (King & Wright, 1982). They are singlets (Figure 4b) and therefore arise from the H-2 protons of Trp-14 and Trp-7, respectively, since Trp-14 is near the GH corner, a region of the molecule that undergoes changes at high pH (Schoenborn, 1969). Resonances 18–20 undergo shifts due to the hemic acid dissociation (Carver, 1982), and the titration parameters are given in Table III. Resonance 18 is present in the Carr-Purcell spectrum of Figure 4b but is absent from another spectrum (not shown) with τ = 60 ms in which surface His H-4 resonances remained visible. It probably arises from a buried histidine that is remote from the heme group and responds to the hemic acid dissociation. His-24, which stabilizes the GH corner (Schoenborn, 1969), is the most likely assignment.

Resonances 19 and 20 show large pH shifts (Table III) and also have a large temperature dependence, compared with that of the histidine resonances (Figure 5). The pH and tem-

Table IV: <sup>1</sup>H NMR Titration Parameters and Assignments for Resonances Observed in the H-2 Histidine Region of Sperm Whale CNMb

	His-81	His-12	His-48	His-116	His-119	His-113	His-36	His-24 <sup>a</sup>	His-97		
resonance	2	3	4	5	6	7	11	12	15	1	9 <sup>b</sup>
pK value	6.35	6.44	6.54	6.96	5.15	5.56	8.11	5.82	5.54	5.50	5.31
δ <sub>B</sub> <sup>c</sup>	7.82	7.71	7.73	7.66	8.32	7.71	7.02	7.71	6.76	8.80	8.11
Δ <sup>c</sup>	1.14	1.21	1.02	1.04	0.53	1.18	1.05	0.13	0.91	0.22	0.19

<sup>a</sup> Tentative assignment. <sup>b</sup> Curve exhibits a second inflexion at high pH with a pK value of 10.30 and Δ = 0.08. <sup>c</sup> δ<sub>B</sub> and Δ are defined in Table I.

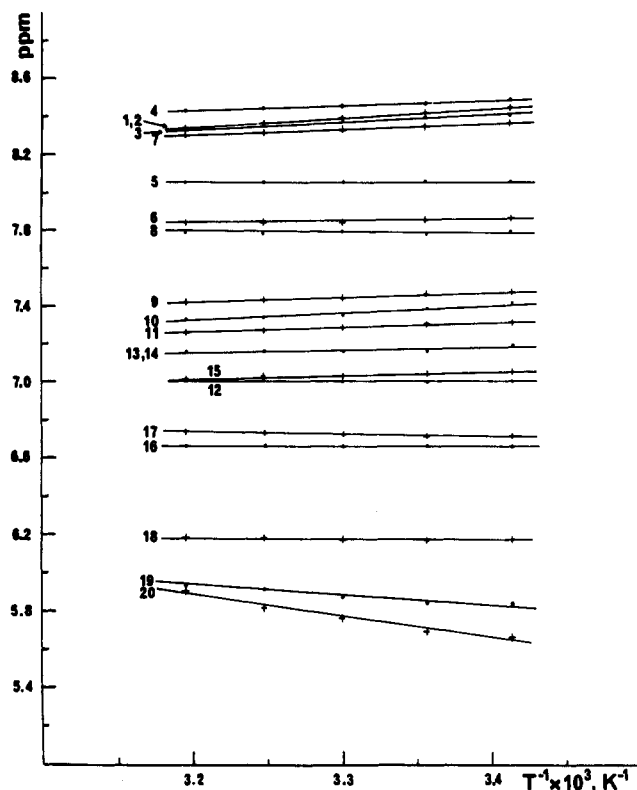


FIGURE 5: Chemical shift (ppm) against  $T^{-1}$  for the aromatic resonances in the <sup>1</sup>H NMR spectrum of sperm whale metMb, pH 6.11 (measured at 20 °C).

perature variation shows that in lower spin species (such as the hydroxy species at high pH) resonances 19 and 20 occur at much lower field. Their areas of about two protons and incipient splitting into doublets observed in some spectra (not shown) imply that they arise from the 2,6 and 3,5 protons of a tyrosine residue. Sperm whale Mb has three tyrosines at positions 103, 146, and 151. The resonances are observed in spectra from horse and pig metMb and hence cannot arise from Tyr-151, which is absent in these species. They undergo a small shift at about pH 5 (Carver, 1982), which is probably due to the titration of a nearby carboxyl group. There are no carboxyl groups near Tyr-146, whereas Glu-38 is close to Tyr-103 (Takano, 1977), implying that Tyr-103 is the most likely assignment for resonances 19 and 20.

**Histidine H-2 Resonances of CNMb.** The similarities between the titration curves for sperm whale CNMb (Figure 6) and those of metMb (Figure 2a) enable assignments to be made in CNMb (Table IV) of the seven H-2 resonances that are observed in metMb. There are, however, several chemical shift changes between titration curves in CNMb and metMb. Most notably, since the transition to hydroxyMb at high pH in metMb is abolished with strong ligands such as cyanide, the inflexions observed in metMb for His-119 H-2 and the upfield shift of His-36 H-2 titration curves are not present in CNMb.

The large chemical shift change with pH of resonance 15 indicates that it belongs to a C-2 proton. Furthermore, it

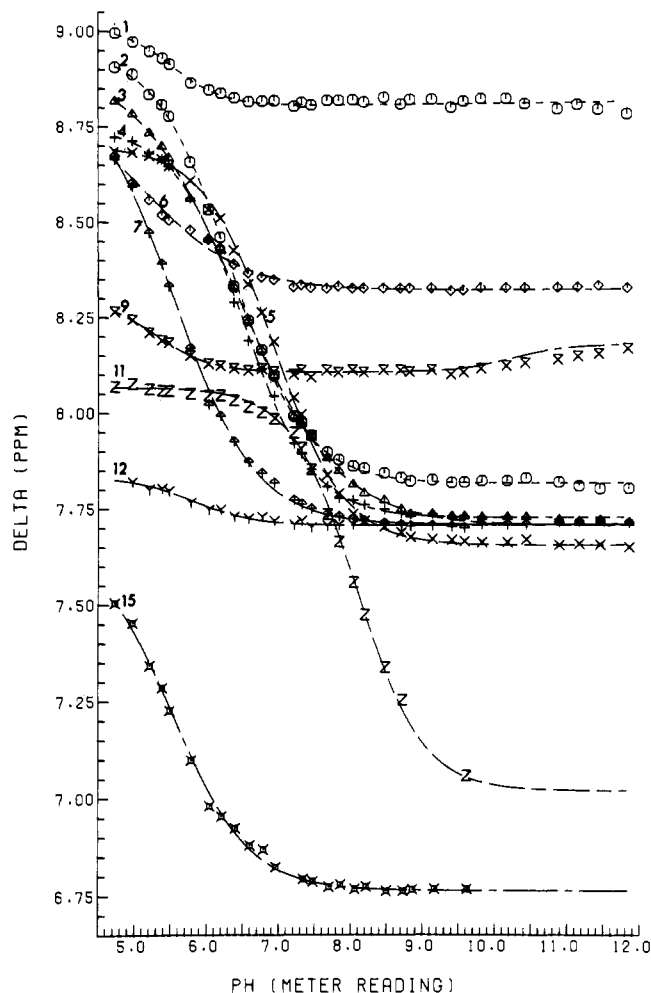


FIGURE 6: <sup>1</sup>H NMR titration curves for the resonances observed in the histidine H-2 region of the spectrum of sperm whale CNMb at 20 °C.

moves rapidly downfield with increasing temperature so that at  $1/T \rightarrow 0$  the shift in a diamagnetic species (Jesson, 1973)  $\delta_A = 9.13$  ppm, which is close to  $\delta_A$  for an unperturbed His H-2. In low-spin CNMb, resonance 15 therefore arises from a His H-2 that is near the paramagnetic center (and therefore not observed in high-spin metMb). An H-2 is observed in diamagnetic COMb and oxyMb with a normal  $\delta_B$  value and a low pK of 5.7 (see below). A hydrogen bond occurs between His-97 (on the proximal side of the heme) and the carboxyl side chain at position 7 of the heme ring in all Mb derivatives, whereas the other near-heme titrating histidine (His-64) has a hydrogen bond with the ligand in metMb (Takano, 1977) and oxyMb (Phillips & Schoenborn, 1981) but no hydrogen bond in COMb (Hanson & Schoenborn, 1981). Thus, the consistent presence of a H-2 resonance with a pK of about 5.7 in oxyMb, COMb, and CNMb suggests that this resonance belongs to His-97, rather than His-64.

The position of resonance 12 (Figure 7), its low pK and small Δ value (Figure 6), and its broadening at low pH at 20 °C but lack of broadening at 40 °C (Carver, 1982) are similar

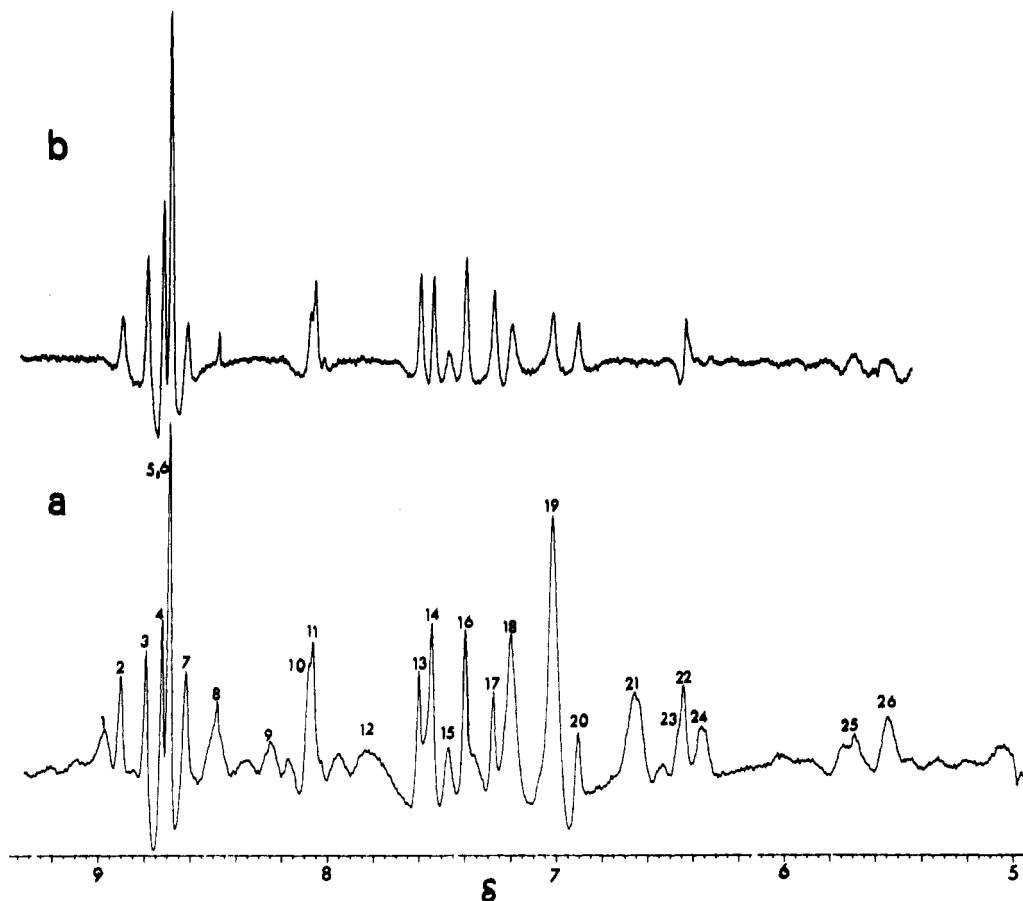


FIGURE 7: Aromatic region of the  $^1\text{H}$  NMR spectrum at 270 MHz of sperm whale CNMb in  $^2\text{H}_2\text{O}$  at 20 °C and pH 4.97: (a) resolution-enhanced spectrum; (b) Carr-Purcell spectrum with  $\tau = 30$  ms.

to those observed for a resonance in COMb and oxyMb [resonance 11; Bradbury et al. (1981) and see below]. The latter had previously been assigned in COMb to His-64 H-2, but recent 2D NMR studies on COMb have shown that this resonance is not connected to the H-4 His-64 resonance (P. E. Wright, personal communication). The broadening at 20 °C may be due to exchange broadening between two different sites for the histidine (Markley, 1975) and/or to moderately slow exchange between the nonprotonated and protonated forms of the residue (Sudmeier et al., 1980). The rate of exchange would be increased at 40 °C, causing removal of line broadening. Resonance 12 probably arises from a H-2 histidine remote from the paramagnetic center, hence explaining the lack of its temperature dependence (Figure 8), which is partly inaccessible to solvent (consistent with the line broadening of resonance 12 at low pH and its small  $\Delta$  value). It must also be sensitive to the bound ligand since there is a change in  $pK$  from 5.8 for CNMb (resonance 12) to 4.5 for COMb and 6.7 for oxyMb (see below). His-24 fulfills this latter condition, whereas the other possibility His-82 is adjacent to His-81, which is insensitive to bound ligand. Resonance 12 is therefore tentatively assigned to His-24 H-2, and its upfield position may be due to closeness of the proton to the ring current of His-119. Hayes et al. (1975) in a study of low-spin  $\text{N}_3\text{Mb}$  observed a resonance with similar titration characteristics and  $pK$  to resonance 12, which broadened at low pH and was assigned to His-24 H-2.

The other additional resonance observed by Hayes et al. (1975), compared with metMb, and assigned to His-82 H-2 has a small titration shift of  $\sim 0.3$  ppm and a  $pK$  value of 6.38 in sperm whale  $\text{N}_3\text{Mb}$ . This resonance has similar chemical shift and titration characteristics to that of His-81 H-4 in metMb, CNMb, COMb, and oxyMb (see below). In metMb,

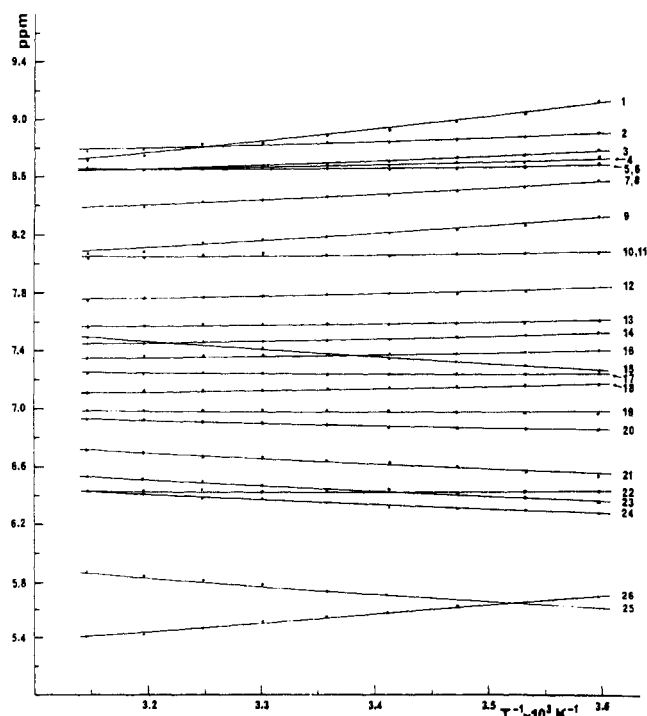


FIGURE 8: Chemical shift vs.  $T^{-1}$  for the aromatic  $^1\text{H}$  NMR resonances of sperm whale CNMb, pH 5.13 (measured at 20 °C).

this resonance is not removed by deuteration, as are those that arise from C-2 protons, hence showing that it is not from a C-2 proton (Carver, 1982). Thus, this resonance in  $\text{N}_3\text{Mb}$  is reassigned to His-81 C-4 proton.

**Histidine H-4 Resonances in CNMb.** Six of the resonances in Figure 7a (10, 13, 14, and 16–18) were assigned to histidine

Table V: <sup>1</sup>H NMR Titration Parameters for the Histidine H-4 Resonances and Other Resonances in the Upfield Aromatic Region of Sperm Whale CNMb at 20 °C

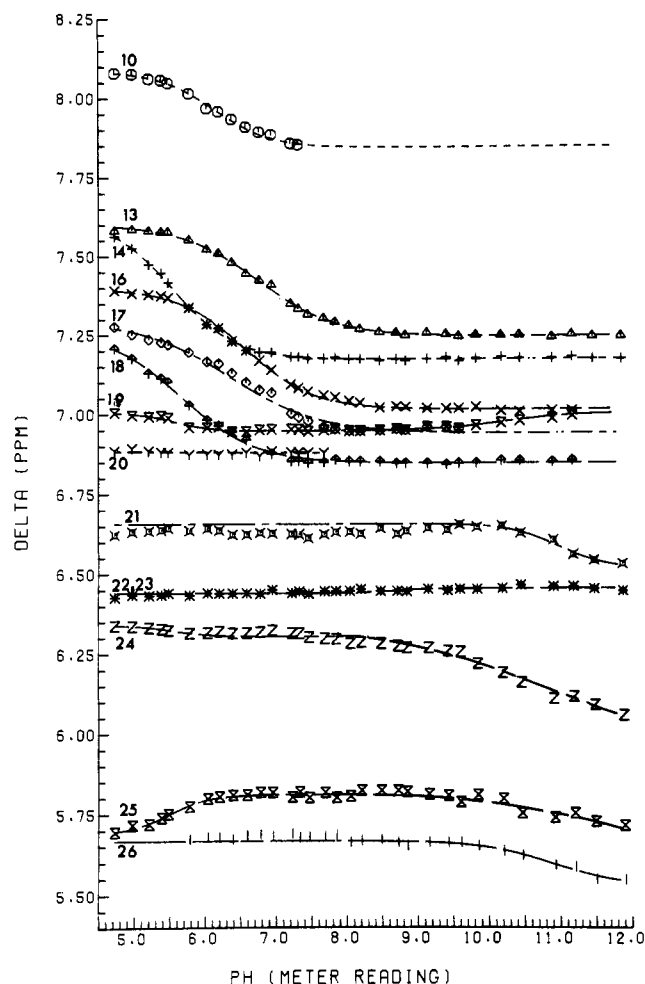
	His-81	His-12	His-48	His-116	His-119	His-113	His-36	Trp H-2 <sup>a</sup>	Tyr-103 3,5	His-24 <sup>a</sup>	Tyr-103 2,6	Tyr-146 2,6 or 3,5	Tyr-146 2,6 or 3,5
resonance	10	16	17	13	14	18	19 <sup>b</sup>	20	21	22	23	24 <sup>c</sup>	25 <sup>d</sup>
pK value	6.17	6.59	6.38	6.92	5.56	5.68	5.58	6.88	11.01	6.46	6.46	5.56	5.49
δ <sub>B</sub> <sup>e</sup>	7.84	7.02	6.94	7.24	7.17	6.85	6.95	6.88	6.52	6.46	6.46	6.31	5.81
Δ <sup>e</sup>	0.24	0.38	0.36	0.35	0.45	0.42	0.05		0.14			0.04	0.11

<sup>a</sup> Tentative assignments. <sup>b</sup> This resonance is a composite that includes the sharp H-4 resonance; the titration curve exhibits a second inflexion at high pH with a pK value of 10.3 and Δ = 0.04. <sup>c</sup> Curve shows a second inflexion at pH with a pK value of 10.39 and Δ = 0.23. <sup>d</sup> Curve exhibits a second inflexion at high pH with a pK value of 10.39 and Δ = 0.10. <sup>e</sup> δ<sub>B</sub> and Δ are defined in Table I.

C-4 protons by their titration shifts of about 0.4 ppm (Figure 9), their small temperature dependences (Figure 3), and their presence in the Carr-Purcell spectrum of Figure 7b. Their assignment to specific H-4 protons was facilitated by the observation that both the H-2 and H-4 resonances in CNMb are shifted upfield or downfield by similar amounts compared with metMb, although their pK values are not affected significantly; e.g., His-81 and -119 H-2 resonances are ~0.1 and ~0.45 ppm downfield, respectively, from their positions in metMb, and similar shifts are observed for the H-4 resonances. The assignments are given in Table V. In the Carr-Purcell spectrum of CNMb (Figure 7b), it was found that, apart from the above resonances, resonances 19, 20, and 22 were also present. These latter resonances were also observed in the spectrum of carboxymethylated CNMb (that had been carboxymethylated as metMb at pH 6.8 for 10 days) and, like His-36 H-2, exhibit little chemical shift variation with temperature. Resonance 19 is known from a 400-MHz spectrum (Carver, 1982) to be a combination of various aromatic resonances. At low pH, its movement is similar to that of His-36 H-4 in metMb and therefore resonance 19 is assigned to His-36 H-4. The assignment of resonances 20 and 22 is discussed below.

**Carboxymethylated Sperm Whale CNMb and Line-Broadening Effects.** The H-2 resonances in the spectrum of a sample of carboxymethylated metMb that had been converted to CNMb show similar features to those for carboxymethylated metMb (Figure 1b). Thus, the H-2 resonance of unmodified His-36 at its δ<sub>A</sub> limit broadened greatly with increasing pH so that it became impossible to follow its movement with pH. Similar problems were encountered with the H-2 resonances of unmodified His-24 and -97 resonances, which probably arises from moderately slow exchange between protonated and nonprotonated forms of these histidines that are not freely accessible to solvent (Sudmeier et al., 1980). Carboxymethylation also causes a significant degree of line broadening for all resonances including those from surface histidine C-2 and C-4 protons that are remote from the heme in addition to hyperfine-shifted heme and near-heme resonances (Carver, 1982). Thus, the general broadening results from an increase in the correlation time of the molecule and is not due to a change in the electronic structure of the heme. The broadening may be due to the bulky negatively charged carboxymethyl groups on the surface of the molecule (Hugli & Gurd, 1970a,b) that cause an increase in both intra- and intermolecular electrostatic interactions. It may also result from the presence of dimeric myoglobin introduced by lyophilization during preparation (Hardman et al., 1966; Van Den Oord et al., 1969).

**Other Well-Resolved Resonances in CNMb.** Resonance 1 (observed at 20 °C in Figure 7 but obscured at 40 °C in Figure 10) and resonance 9 both have large chemical shift variations with temperature (Figure 8) such that at 1/T → 0 their chemical shift values are δ ~6, which implies that they arise

FIGURE 9: <sup>1</sup>H NMR titration curves for the resonances observed in the region upfield of the histidine H-2 resonances of sperm whale CNMb at 20 °C.

from aromatic residues that occupy a perturbed upfield position due to proximity to the heme group (e.g., Phe-43 or Phe-46). Alternatively, they may arise from unassigned heme protons (see below). Resonance 8 is a nontitrating singlet resonance (Figure 7b) whose downfield position is suggestive of protonated His-82 H-2, which is inaccessible to solvent (Hugli & Gurd, 1970a,b). Resonance 20 (Table II) is a singlet and is tentatively assigned to a tryptophan H-2; the sharp resonance at δ 7.14 in Figure 10 may arise from the other tryptophan H-2. Their upfield position in low-spin CNMb relative to the position of the two tryptophan H-2 resonances in sperm whale metMb (Table II) is consistent with their upfield movement with increasing pH in metMb on conversion to lower spin hydroxymMb. Thus, the resonance at δ 7.14 is tentatively assigned to Trp-14 H-2 and resonance 20 to Trp-7 H-2. Resonance 22 is a singlet (Figure 7b), it has no chemical shift variation with temperature (Figure 8) or pH (Figure 9), and



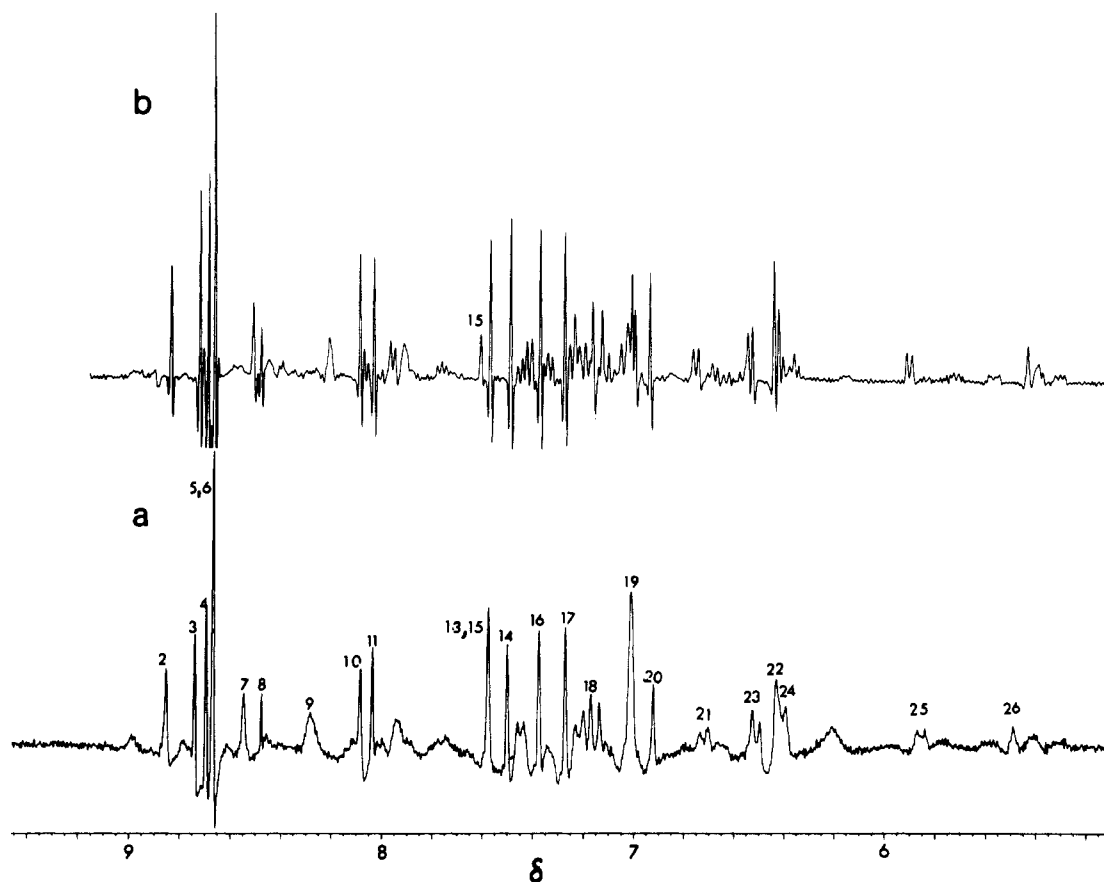


FIGURE 10: Aromatic region of  $^1\text{H}$  NMR spectrum of sperm whale CNMb at  $40^\circ\text{C}$  and pH 5.02 at (a) 270 and (b) 400 MHz. Variations in chemical shift positions of temperature-sensitive resonances may be due to slight differences in probe temperature between the two spectrometers.

it occurs  $\sim 0.3$  ppm downfield of resonance 18 in metMb and is tentatively assigned to His-24 H-4 (Table III). Upon conversion of metMb to lower spin hydroxyMb, resonance 18 moved  $\sim 0.2$  ppm downfield, which is consistent with the assignment of resonance 22 in low-spin CNMb to His-24 H-4.

Resonances 21, 23, and 25 are doublets (Figure 10) and resonance 23 is inverted in the Carr-Purcell spectrum of Figure 7b and completely inverted with  $\tau = 60$  ms (not shown), confirming that it is a doublet (Campbell et al., 1975b). Resonances 21 and 23 are linked by a double-resonance experiment and so are resonances 24 and 25; hence, they arise from the 2,6 and 3,5 protons of two tyrosine residues. A spectrum of horse CNMb (in which Tyr-151 is deleted) showed resonances corresponding to resonances 21 to 25 in sperm whale CNMb. Thus, resonances 21 and 23–25 must arise from Tyr-103 and -146. The upfield shift of resonance 21 at very high pH with a pK value of  $\sim 11$  (Figure 9 and Table V) and the slight upfield shift of resonance 23 are consistent with the high pK value observed for Tyr-103 by  $^{13}\text{C}$  NMR (Wilbur & Allerhand, 1976). The larger shift observed for resonance 21 indicates its assignment to 3,5 protons and, therefore, resonance 23 to 2,6 protons of Tyr-103. Furthermore, the chemical shift positions of Tyr-103 resonances in metMb on conversion from aquo- to hydroxyMb (Table III) are slightly upfield of those observed in completely low-spin CNMb, implying that these resonances in both derivatives are from the same residue, i.e., Tyr-103. Tyr-146 is reasonably close to the heme and is hydrogen bonded to Ile-99 on the proximal side of the heme (Takano, 1977). The pH variation of resonances 24 and 25 at low and high pH (Figure 9) is similar to that observed for hyperfine-shifted heme and near-heme resonances (see below). Wilbur & Allerhand (1976, 1977) observed that Tyr-146 in CNMb did not titrate

but that its C-1 resonance had a small upfield shift with increasing pH above pH  $\sim 10$  that was not due to deprotonation. Thus, resonances 24 and 25 are assigned to Tyr-146, and their pH dependence is due to conformational changes near the heme. Resonance 26 has a large upfield temperature variation with increasing temperature, implying that it may belong to an  $\alpha$ -CH proton of a near-heme residue.

**Hyperfine-Shifted Resonances in CNMb.** Three of the methyl heme resonances (1, 2, and 5; Figure 11) have been assigned by selective deuteration (Mayer et al., 1974) to the methyl protons at the 5-, 1-, and 8-positions, respectively, of the heme ring (Figure 12). Resonances 17 and 18 arise from near-heme protein methyl groups (Sheard et al., 1970) and resonance 19 from a protein single proton (Carver, 1982). The results in Figure 13 agree with the earlier results of Sheard et al. (1970), who followed the downfield resonances only up to pH 9. Most of the resonances that exhibit pH dependence show two distinct pK values of  $5.3 \pm 0.3$  and  $10.8 \pm 0.2$ , which are similar to those of the hyperfine-shifted resonances observed in the aromatic region (Figure 9). In the metMb crystal, the imidazole ring of His-97 is placed below and parallel to pyrrole ring 4 of the heme group (Figure 12). His-64 (distal) is located about equidistant from pyrrole rings 1 and 4 and near the  $\gamma$ -meso proton (Watson, 1969; Takano, 1977). Pyrrole ring 2 is isolated from these two histidine residues, which accounts for the lack of movement of resonance 2 (1- $\text{CH}_3$ ) with pH. The similarity of the pK value of His-97 ( $5.5$ , Table 4) to that of  $5.3 \pm 0.3$  for those hyperfine-shifted resonances that exhibit a pH shift suggests that these resonances are witnessing conformational changes associated with the deprotonation of His-97. The large shift downfield with increasing pH of the 5- $\text{CH}_3$  resonance 1 is consistent with its proximity to His-97. The hydrogen bond between the pro-

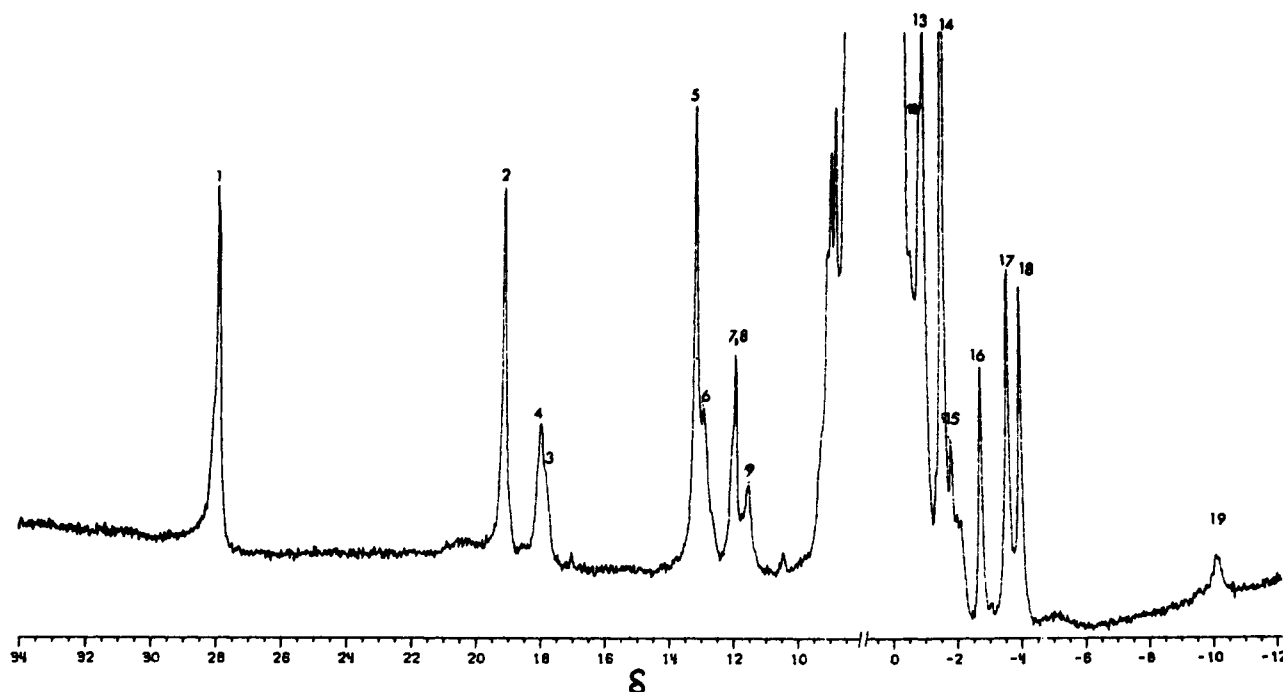


FIGURE 11: Hyperfine-shifted resonances in the <sup>1</sup>H NMR spectrum of sperm whale CNMb at pH 8.17 and 20 °C. The region of the spectrum  $\delta$  0–10 has been deliberately omitted. Assignments (see text) of resonances are 1 (5-CH<sub>3</sub>), 2 (1-CH<sub>3</sub>), 5 (8-CH<sub>3</sub>), and 17 and 18 (protein methyls).

Table VI: <sup>1</sup>H NMR Titration Parameters for the Histidine H-2 Resonances of Sperm Whale DeoxyMb at 20 °C<sup>a</sup>

	His-81		His-12		His-48		His-116		His-119		His-113		His-36	
	this work	<i>b</i>	this work	<i>b</i>	this work	<i>b</i>	this work	<i>b</i>	this work	<i>b</i>	this work	<i>b</i>	this work	<i>b</i>
resonance	1		2		3		4		5		6		7	
pK value	6.56	6.71	6.69	6.60	6.79	7.16	7.09	6.78	5.63	5.79	5.51	5.76	7.94	8.12
$\delta_B$	7.68	7.63	7.68	7.64	7.60	7.57	7.65	7.62	8.04	7.99	7.64	7.59	7.48	7.18
$\Delta$	1.08	0.89	1.03	0.89	1.08	0.94	0.97	0.89	0.63	0.56	1.26	1.00	0.76	1.03

<sup>a</sup> Ohms et al. (1979) observed an extra upfield resonance that they assigned to His-97 H-2. This resonance belongs to His-81 H-4.  $\delta_B$  and  $\Delta$  are defined in Table I. <sup>b</sup> Results of Ohms et al. (1979).

Table VII: <sup>1</sup>H NMR Titration Parameters for the Histidine H-4 Resonances of Sperm Whale DeoxyMb at 20 °C

	His-81	His-12	His-48	His-116	His-119	His-113	His-36
resonance	8	12	14	10	13	15	17
pK value	6.45	6.31	7.10	6.84	5.53	5.32	
$\delta_B^a$	7.60	6.96	6.86	7.15	7.04	6.70	6.74
$\Delta^a$	0.29	0.53	0.29	0.39	0.56	0.52	0.03

<sup>a</sup> Definitions of  $\delta_B$  and  $\Delta$  are given in Table I.

pionic acid at position 7 and His-97 (Watson, 1969; Takano, 1977) will be modified by its deprotonation, and this would affect the magnetic environment of the 8-CH<sub>3</sub> protons (resonance 5).

Since the shift of resonances at high pH in Figure 13 is also observed at low pH (except for resonance 12), it is likely that the conformational changes that are responsible for the two pH variations are concentrated in the vicinity of His-97, i.e., on the proximal side of the heme. The titration of Tyr-103 cannot be the source of this effect because <sup>13</sup>C NMR of kangaroo CNMb, in which Tyr-103 is replaced by Phe, show similar shifts of heme carbon resonances to those of horse and sperm whale CNMb (Wilbur & Allerhand, 1976). The high-pH shifts observed for the proton resonances, for <sup>13</sup>C resonances (Wilbur & Allerhand, 1976), and for the <sup>15</sup>N cyanide resonance in horse C<sup>15</sup>NMb (Morishima & Inubushi, 1977, 1978) may result from the rupturing at high pH of the hydrogen bond between His-97 and the propionic acid side chain at position 7 of the heme ring, as has been observed at

high pH in metMb crystals (Schoenborn, 1969).

**DeoxyMb H-2 Resonances.** The spectrum of high-spin deoxyMb given in Figure 14 is similar to that of high-spin metMb (Figure 1). In both cases there are seven H-2 histidine resonances, and the titration curves are very similar, hence allowing assignments based on metMb. The results of Ohms et al. (1979) for sperm whale deoxyMb are generally in agreement with those presented here (Table VI), although they assigned an extra upfield resonance to His-97 H-2, which we have assigned to His-81 H-4 (see Tables II and IV).

**DeoxyMb H-4 Resonances.** The titration curves for the H-4 resonances of sperm whale deoxyMb are assigned by their similarity to those of metMb (Figure 3a) and, hence, are not shown here (Carver, 1982). Differences arise in the relative chemical shift positions of His-36, -113, and -119 H-4 resonances, but these are consistent with shifts in corresponding H-2 resonances. Titration parameters are given in Table VII. The normal behavior of His-36 resonances is discussed in the accompanying paper (Bradbury & Carver, 1984).

Table VIII:  $^1\text{H}$  NMR Titration Parameters for the Histidine H-2 Resonances of Sperm Whale, Horse, and Pig COMb at 40 °C

	His-81	His-12	His-48	His-116	His-119	His-113	His-36	His-97	His-24 <sup>a</sup>	His-82 <sup>a</sup>
Sperm Whale										
resonance	1	2	3	4	6	7	5	8	11	9
pK value	6.11	6.23	6.42	6.74	5.63	5.93	6.74	5.85	4.53	6.57
$\delta_B^b$	7.71	7.82	7.68	7.62	8.01	7.70	7.62	7.61	7.59	8.01
$\Delta^b$	1.30	1.02	1.04	0.97	0.67	1.06	0.97	1.03	1.13	0.13
Horse										
resonance	1		2	3	5	4		6	9	7
pK value	6.14		6.61	6.80	5.53	5.76		5.91	4.59	6.51
$\delta_B$	7.65		7.62	7.58	8.00	7.88		7.59	7.55	7.82
$\Delta$	1.32		1.18	1.09	0.76	0.87		1.23	1.04	0.29
Pig										
resonance	1		2		3			4	7	5
pK value	6.20		6.80		5.54			5.80	6.01	7.54
$\delta_B$	7.60		7.65		8.03			7.88	7.69	8.14
$\Delta$	1.25		0.96		0.86			0.67	0.24	0.08

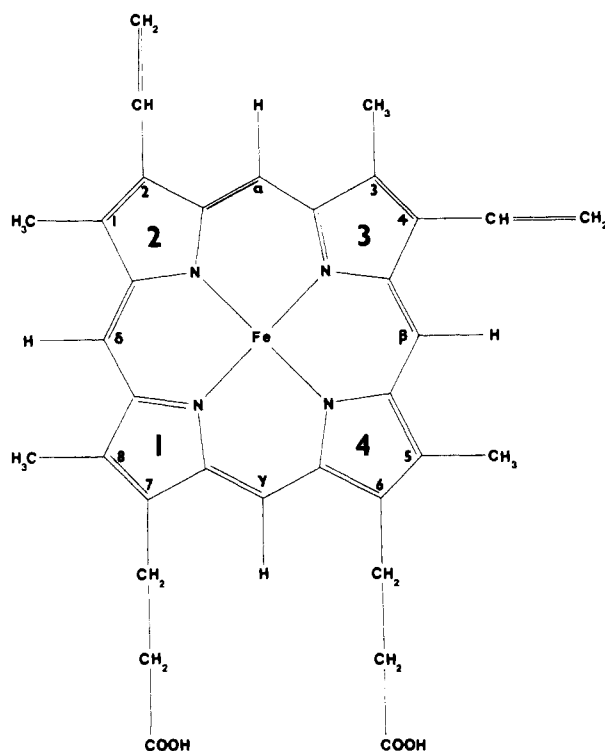
<sup>a</sup> Tentative assignment. <sup>b</sup> These symbols are defined in Table I.

FIGURE 12: Structure of the heme group in myoglobin.

**Other Aromatic Resonances of DeoxyMb.** Assignment of the remaining resonances that do not move with pH depends on their temperature dependence, their positions relative to those for ferric derivatives, and the Carr–Purcell spectrum (Carver, 1982). Resonance 9 in Figure 14 does not arise from a histidine since it is not present in a Carr–Purcell spectrum and may arise from phenylalanine residues. Resonances 11 and 16 are tryptophan H-2 singlets and by analogy with metMb are assigned tentatively to Trp-14 and -7, respectively. Resonances 19 and 22 are both singlets, and one of these could arise from His-24 H-4. Resonances 20 and 21 belong to a tyrosine residue, and by analogy with met Mb, they are assigned to Tyr-103.

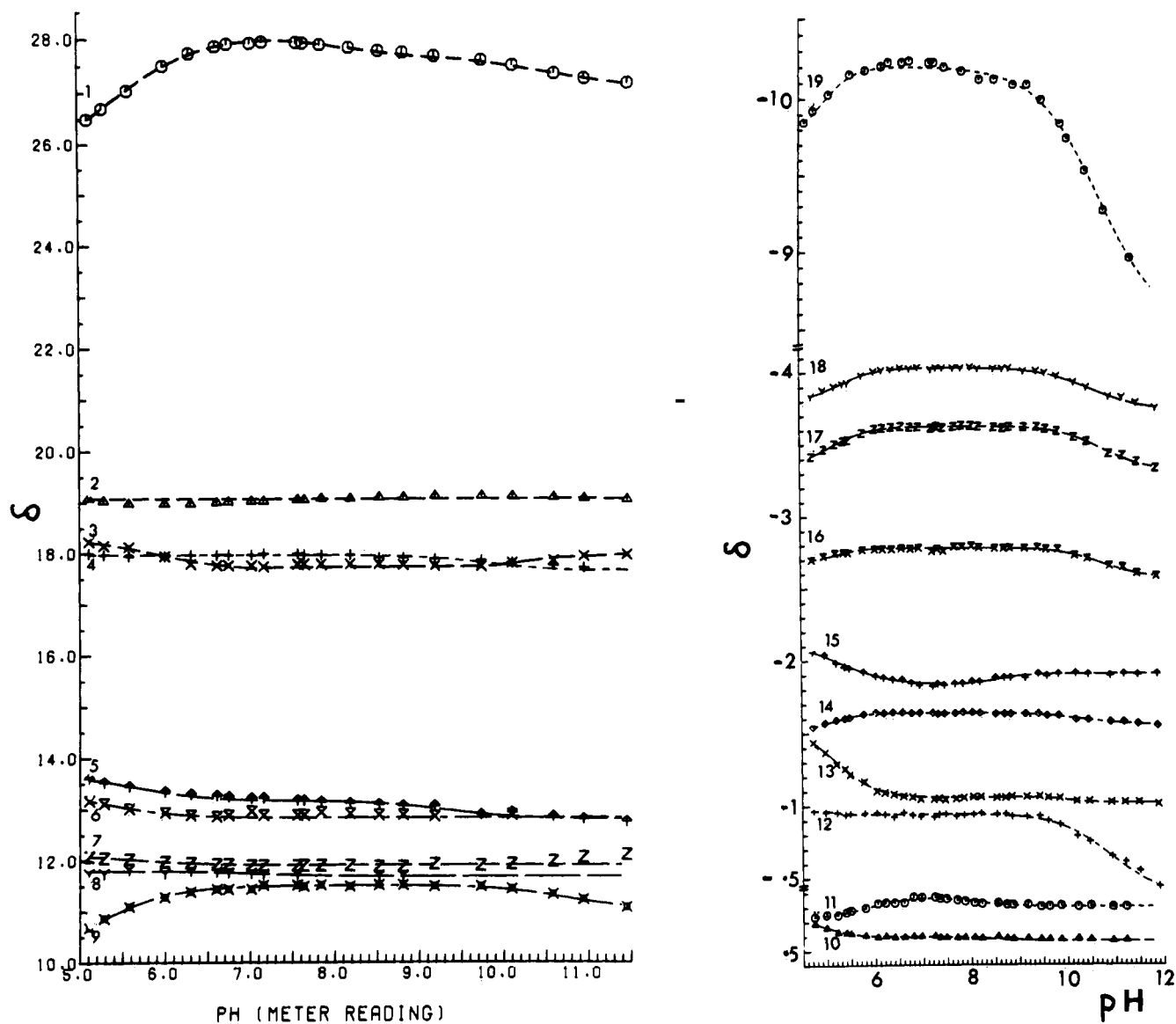
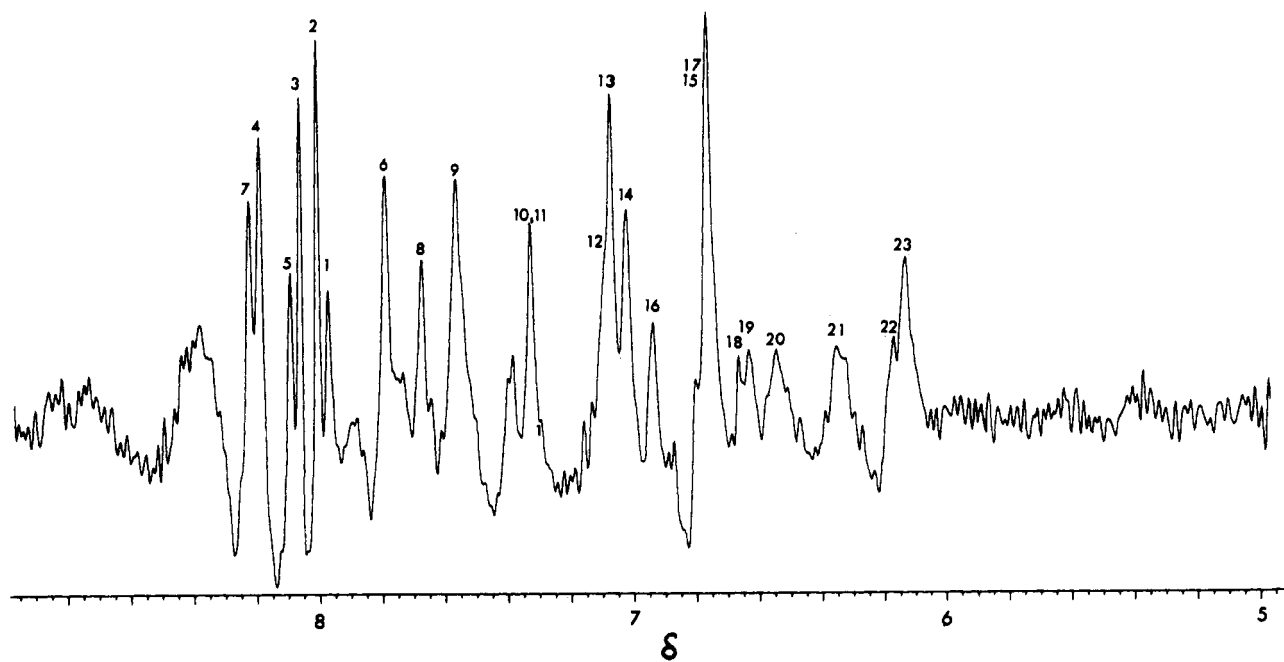
**H-2 Resonances of COMb.** In the spectrum of diamagnetic COMb shown in Figure 15 a greater number of resonances occur than in spectra of paramagnetic metMb, deoxyMb, and CNMb, because of the removal of shifts and/or broadening of resonances from protons near the paramagnetic iron atom. This makes it difficult to follow the movement with pH of some resonances, particularly in the crowded histidine H-4 region.

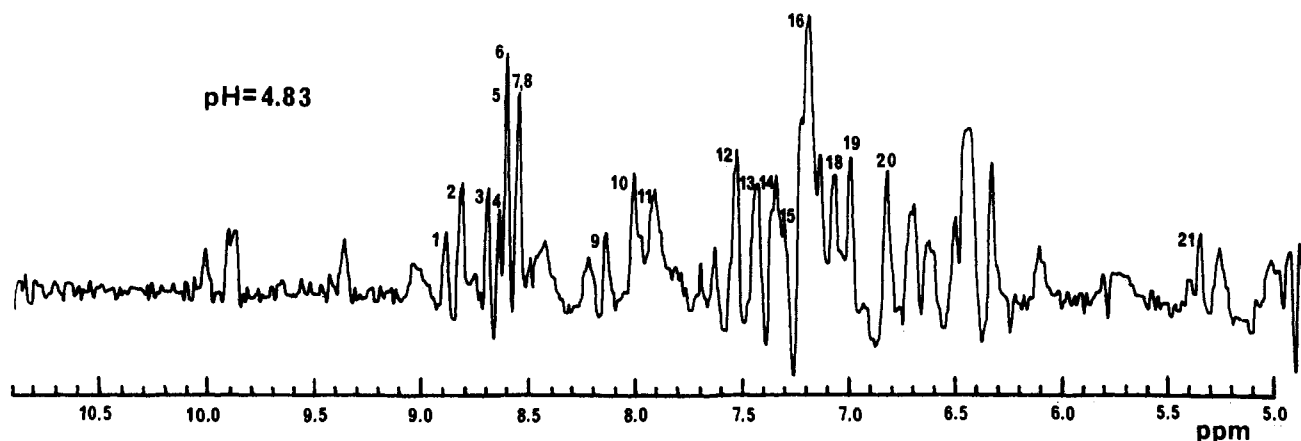
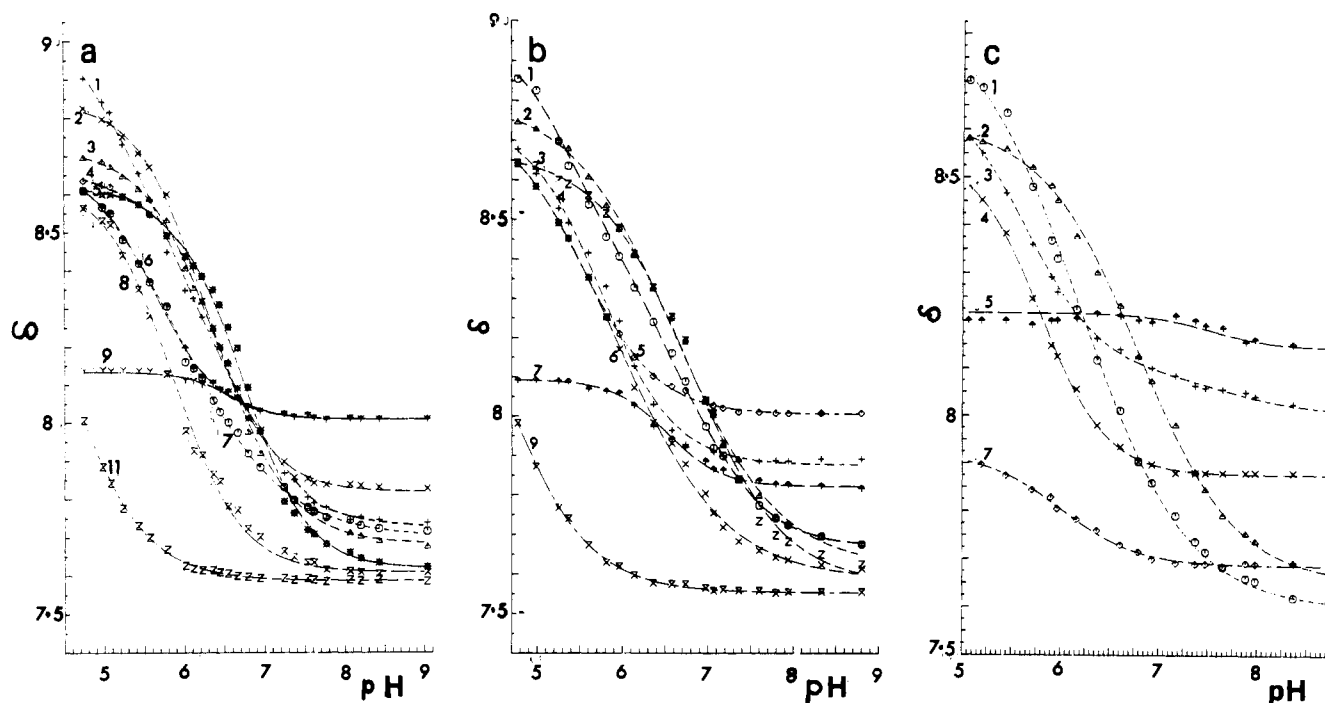
In Figure 16a, 10 resonances (1–9 and 11) were observed to move with pH (resonance 10 is also in this region but belongs to His-81 H-4, see below). Similar titration curves to those of Figure 16a were obtained by titration of sperm whale COMb at 20 °C (Carver, 1982), except that broadening of resonance 11 occurred at 20 °C at low pH and prevented its observation below pH ~6. In general, there was a small decrease in pK of corresponding histidines in sperm whale COMb on increase of temperature from 20 to 40 °C (Table VIII), which agrees with similar studies on the histidine residues of ribonuclease A (Roberts et al., 1969; Westmoreland et al., 1975) and lysozyme (Cohen, 1969).

Resonances 1–4 (Figure 16a) are assigned to His-81, -12, -48, and -116, respectively, because of the similarity of their titration curves in metMb, CNMb, deoxyMb, and COMb. A titration series in which resonance 2 of Figure 16a was absent in horse COMb (Figure 16b) and resonances 2 and 4 (Figure 16a) were both absent in pig COMb (Figure 16c) confirmed the assignments of resonances 2 and 4 to His-12 and His-116. Similarly, resonances 6 and 7 in Figure 16a are assigned to His-119 and -113 because they have similar titration characteristics to resonances in the paramagnetic derivatives and resonance 7 (Figure 16a) is absent in Figure 16c (pig COMb). Resonance 5 (Figure 16a) is assigned to His-36 (Bradbury et al., 1981) and only separates from resonance 4 at low pH.

Resonance 8 was not observed in metMb and deoxyMb, but in CNMb a similar resonance that was hyperfine-shifted into the H-4 region was observed (resonance 15 in Figure 6). Its approximate diamagnetic chemical shift at low pH and its pK value of 5.5 correspond with the pK of resonance 8 (5.85) in COMb. Resonance 8 is therefore assigned to His-97 H-2. Resonance 11 (Figure 16a) shows a large degree of broadening at low pH at 20 °C but not at 40 °C, which is similar to the broadening behavior of resonance 12 in CNMb. We propose that the two resonances in CNMb and COMb probably arise from the same proton and, hence, tentatively assign resonance 11 in COMb to His-24 H-2, rather than as previously to His-64 H-2 (Bradbury et al., 1979, 1981). The argument for the previous assignment, viz., the similarity of pK values (~4.5) between resonance 21 (Figure 15) assigned to His-64 H-4 and resonance 11, is not applicable because of lack of connectivity between resonances 11 and 21 in a COSY 2D NMR spectrum (P. E. Wright, private communication).

The remaining H-2 resonance number 9 (Figure 16a) was not observed in metMb or deoxyMb but is present in CNMb (resonance 8 of Figure 7, possibly His-82 H-2). A Carr–Purcell spectrum (not shown) shows that resonance 9 is a


 FIGURE 13: pH variation of the hyperfine-shifted resonances in the  $^1\text{H}$  NMR spectrum of sperm whale CNMb at 20 °C.

 FIGURE 14: Aromatic region of the  $^1\text{H}$  NMR spectrum at pH 6.95 and 20 °C of sperm whale deoxyMb.

FIGURE 15: Aromatic region of the  $^1\text{H}$  NMR spectrum at pH 4.83 and 40 °C of sperm whale COMb.FIGURE 16:  $^1\text{H}$  NMR titration curves for the histidine H-2 resonances of COMb at 40 °C from (a) sperm whale, (b) horse, and (c) pig.Table IX:  $^1\text{H}$  NMR Titration Parameters for the Histidine H-2 Resonances of Sperm Whale OxyMb at 20 °C

	His-81		His-12		His-48		His-116		His-119		His-113		His-36		His-97		His-24 <sup>a</sup>	His-82 <sup>a</sup>
	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	<sup>b</sup>	this work	this work
resonance <sup>c</sup>	1		2		3		4		6		7		5		8		11	9
pK value	6.32	6.42	6.45	6.07	6.68	6.87	6.88	6.78	5.72	5.30	5.71	5.85	6.85	7.93	5.60	5.69	6.65	6.60
$\delta_B^d$	7.66	7.69	7.72	7.60	7.60	7.54	7.68	7.49	8.01	7.89	7.84	7.46	7.72	7.11	7.62	7.48	7.58	8.03
$\Delta^d$	1.27	0.90	1.07	1.04	1.11	0.95	0.94	1.00	0.66	1.12	0.86	1.03	0.90	0.91	1.20	1.10	0.28	0.14

<sup>a</sup> Tentative assignment, see text. <sup>b</sup> Results of Ohms et al. (1979). Their resonance tabulated here as belonging to His-97 was tentatively assigned by them to His-64. <sup>c</sup> Numbering of resonances is the same as that of Figure 16a, and the curves are those of Figure 2 of Bradbury et al. (1981), which included resonance 10 (now assigned to His-81 H-4) and had the numbering of resonances 6 and 7 reversed. <sup>d</sup>  $\delta_B$  and  $\Delta$  are defined in Table I.

singlet with a fairly long  $T_2$  value, indicative of a histidine H-2 resonance. In agreement with Bradbury et al. (1981), this resonance is tentatively assigned to His-82 H-2, since a similar resonance with similar pK is observed in oxyMb (see below) and we expect the environment of His-82 to be unchanged by change of ligand, in contrast with the situation for His-24 (see above).

**H-2 Resonances of OxyMb.** As shown previously (Bradbury et al., 1981), the titration curves for oxyMb and COMb are very similar, and hence, only the tabulated assignments and

titration parameters are given in Table IX. Comparison of our results with Ohms et al. (1979) shows that they observed a high pK for His-36 H-2 in oxyMb. We, however, only observed a high pK in paramagnetic derivatives (e.g., deoxyMb), whereas in COMb and oxyMb a normal pK is observed. Furthermore, they did not observe our resonances 9 and 11 (Table IX), which are absent in deoxyMb. Ohms et al. (1979) oxygenated their samples after obtaining deoxyMb spectra. If they had incomplete conversion to oxyMb, then this would account for the observed differences. As with COMb, the only

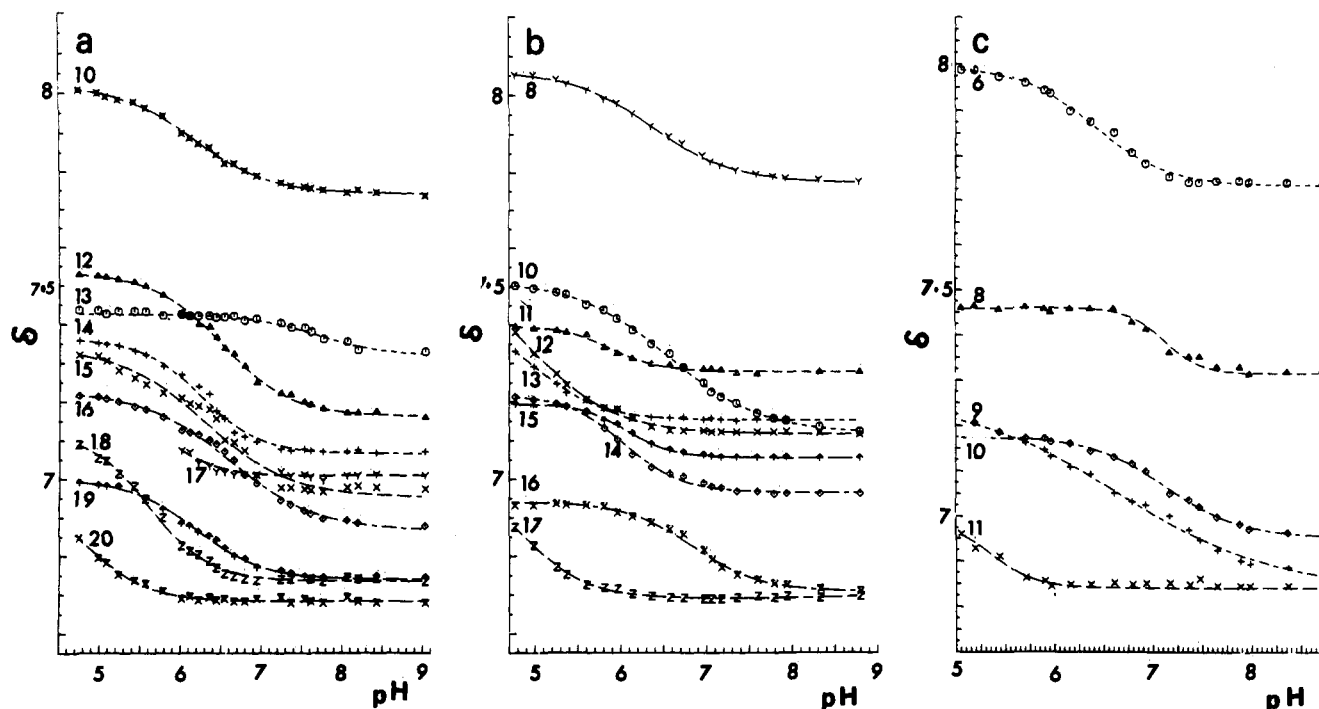


FIGURE 17: <sup>1</sup>H NMR titration curves for the histidine H-4 resonances of COMb at 40 °C from (a) sperm whale, (b) horse, and (c) pig. The numbering of resonances for sperm whale is from Figure 15; the titration curve of resonance 21 (assigned to His-64 H-4) is given in Figure 3 of Bradbury et al. (1979).

Table X: <sup>1</sup>H NMR Titration Parameters for the Histidine H-4 Resonances Observed in the Spectra of Sperm Whale, Horse, and Pig COMb at 40 °C

	His-81	His-12	His-48	His-116	His-119	His-113	His-36	His-97	His-64	His-24 <sup>a</sup>	His-82 <sup>a</sup>
Sperm Whale											
resonance	10	14	16	12	17	19	15	18	21	20	13
pK value	6.20	6.25	6.61	6.51		6.20	6.31	5.61	4.95	4.87	7.68
δ <sub>B</sub> <sup>b</sup>	7.74	7.07	6.87	7.16	7.01	6.74	6.95	6.74	5.00	6.68	7.32
Δ <sup>b</sup>	0.28	0.30	0.36	0.37		0.26	0.39	0.38	0.59	0.27	0.11
Horse											
resonance	8		14	10	13	16	15	12	18	17	11
pK value	6.40		6.04	6.56	4.99	6.85	6.07	4.91	4.96	4.79	5.88
δ <sub>B</sub>	7.77		6.97	7.12	7.15	6.71	7.06	7.12	4.93	6.69	7.28
Δ	0.29		0.25	0.40	0.28	0.23	0.14	0.46	0.55	0.36	0.12
Pig											
resonance	6		9		<sup>c</sup>		10	<sup>c</sup>	12	11	8
pK value	6.42		6.65				7.14		5.43	5.36	7.12
δ <sub>B</sub>	7.73		6.84				6.95		4.90	6.84	7.31
Δ	0.27		0.42				0.22		0.18	0.15	0.15

<sup>a</sup> Tentative assignments. <sup>b</sup> δ<sub>B</sub> and Δ are defined in Table I. <sup>c</sup> Resonances not observed because of large degree of spectral crowding.

assignments open to doubt are those of resonances 9 and 11. The titration curves of resonance 9 are the same in COMb and oxyMb, and therefore, they must arise from the same proton, tentatively assigned to His-82 H-2. Resonance 11 in oxyMb has a similar δ<sub>B</sub> to resonance 11 in COMb (Tables VIII and IX); they both show considerable line broadening at 20 °C and low pH, but their pK's are very different (4.5 in COMb at 40 °C, 6.7 in oxyMb at 20 °C). It is probable that they arise from the same proton, one which is sensitive to the bound ligand, tentatively assigned to His-24 H-2.

**H-4 Resonances of COMb.** Although continuities of some of the H-4 titration curves in Figure 17 are doubtful around 7.0 ppm (due to spectral crowding), it is possible to make assignments given in Table X. Resonances 14, 12, and 19 in Figure 17a belong to His-12, -116, and -113 and are identified by comparison with metMb and the absence of curves for His-12 in horse COMb and His-12, -116, and -113 in pig COMb. Resonances 16 and 10 (Figure 17a) are readily assigned to His-48 and His-81 by comparison with titration

curves in metMb (Figure 3a); similarly, resonance 17 is assigned to His-119, but it is difficult to follow at low pH (Figure 17a). Resonances 15 and 18 are not observed in metMb and are assigned to His-36 and His-97 by matching pK's of H-2 resonances. The upfield resonance 21 was previously assigned to the distal histidine-64 H-4 (Bradbury et al., 1979) on the basis of proximity to the large ring current of the porphyrin and will be discussed in the accompanying paper (Bradbury & Carver, 1984). Resonance 20 is observed as a singlet in a Carr-Purcell spectrum (not shown) and is tentatively assigned to His-24, while resonance 13 may be from His-82.

**H-4 Resonances of OxyMb.** Similar titrating resonances are observed for the H-4 resonances of His-81, -116, -12, -36, -119, and -48 in oxyMb at 20 °C (Figure 18 and Table XI) and COMb at 20 °C (not shown) and at 40 °C (Figure 17a). In oxyMb, resonance 17 from His-97 H-4 is moved downfield by ~0.3 ppm relative to its position in COMb. Resonance 19 has been tentatively assigned to His-24 on the basis of similarity to resonance 20 (Figure 17a). No resonances

Table XI:  $^1\text{H}$  NMR Titration Parameters for the Histidine H-4 Resonances of Sperm Whale OxyMb at 20 °C

	His-81	His-12	His-48	His-116	His-119	His-36	His-97	His-24 <sup>a</sup>	His-82 <sup>a</sup>
resonance	10	14	18	12	16	15	17	19	13
pK value	6.3	6.2	6.8	6.7	5.5	6.6	5.2	6.3	7.6
$\delta_B^b$	7.71	7.14	7.23	7.18	6.97	6.97	7.10	6.68	7.34
$\Delta^b$	0.29	0.28	0.37	0.37	0.45	0.42	0.29	0.25	0.09

<sup>a</sup>Tentative assignments. <sup>b</sup> $\delta_B$  and  $\Delta$  are defined in Table I.

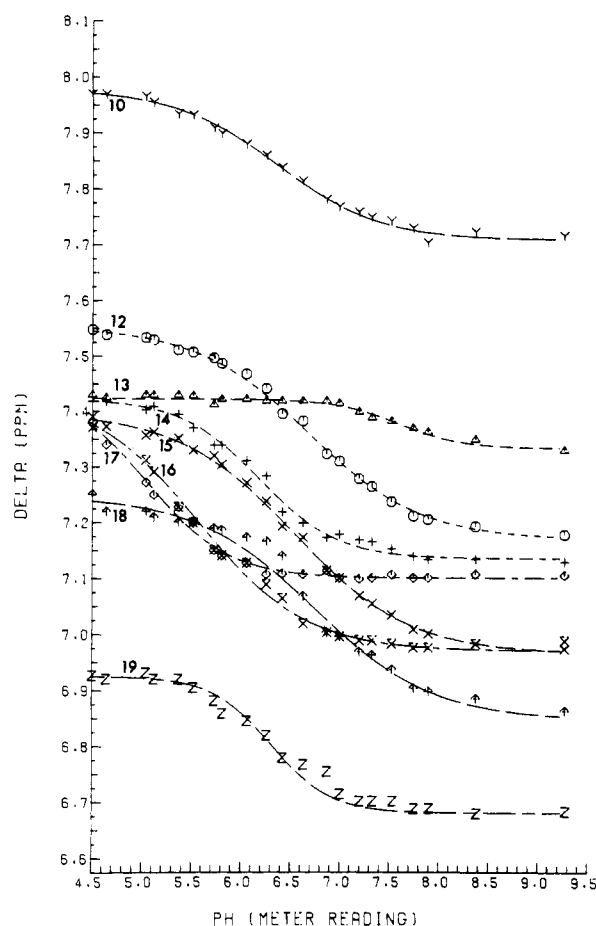


FIGURE 18:  $^1\text{H}$  NMR titration curves for the histidine H-4 resonances of sperm whale oxyMb at 20 °C.

corresponding with resonances 19 and 21 in sperm whale COMb (Table X) were found in oxyMb. Resonance 21 arises from the distal histidine H-4, which adopts different orientations (and correspondingly different heme ring currents) in the heme pocket in COMb and oxyMb as shown by the occurrence of a hydrogen bond between it and the oxygen ligand in oxyMb (Phillips, 1980) and the absence of a hydrogen bond between CO and His-64 in COMb (Hanson & Schoenborn, 1981).

In the accompanying paper (Bradbury & Carver, 1984), we use the above data to analyze the conformational differences between the various Mb derivatives. Particular attention is given to the heme region and the near-heme histidines His-64 and His-97.

Added in Proof

Krishnamoorthi & La Mar (1984) have identified the titration of the near-heme titrating group with  $pK = 5.3$  in sperm whale CNMb and  $pK = 5.6$  in deoxyMb as due to the titration of nearby His-97. These conclusions are in agreement with those presented herein.

## Acknowledgments

We thank M. W. Parker for the data used in Table IX and Figure 18, Dr. A. J. Jones and W. M. Begg of the National NMR Centre and Drs. R. Cassels and G. A. Jenkins for obtaining the NMR spectra, J. R. Reimers and M. D. Ridgway for writing the pK computer program, and Professor W. Moore for access to 400-MHz facilities.

## References

- Botelho, L. H., & Gurd, F. R. N. (1978) *Biochemistry* 17, 5188.
- Botelho, L. H., Hanania, G. I. H., & Gurd, F. R. N. (1976) in *Handbook of Biochemistry and Molecular Biology—Proteins* (Fasman, G. D., Ed.) 3rd ed., Vol. 2, CRC Press, Boca Raton, FL.
- Botelho, L. H., Friend, S. H., Matthew, J. B., Lehman, L. D., Hanania, G. I. H., & Gurd, F. R. N. (1978) *Biochemistry* 17, 5197.
- Bradbury, J. H., & Norton, R. S. (1975) *Eur. J. Biochem.* 53, 387.
- Bradbury, J. H., & Carver, J. A. (1984) *Biochemistry* (following paper in this issue).
- Bradbury, J. H., Deacon, S. L. M., & Ridgway, M. D. (1979) *J. Chem. Soc., Chem. Commun.*, 997.
- Bradbury, J. H., Carver, J. A., & Parker, M. W. (1981) *J. Chem. Soc., Chem. Commun.*, 208.
- Bradbury, J. H., Carver, J. A., & Parker, M. W. (1982) *FEBS Lett.* 146, 297.
- Brunori, M., Amisconi, G., Antonini, E., Wyman, J., Zito, R., & Rossi Fanelli, A. (1968) *Biochim. Biophys. Acta* 154, 315.
- Campbell, I. D., Dobson, C. M., & Williams, R. J. P. (1975a) *Proc. R. Soc. London, Ser. B* 189, 485.
- Campbell, I. D., Dobson, C. M., Williams, R. J. P., & Wright, P. E. (1975b) *FEBS Lett.* 57, 96.
- Carver, J. A. (1982) Ph.D. Thesis, Australian National University.
- Cohen, J. S. (1969) *Nature (London)* 223, 43.
- Cohen, J. S., Hagenmaier, H., Pollard, H., & Schechter, A. N. (1972) *J. Mol. Biol.* 71, 513.
- Gassner, M., Jardetzky, O., & Conover, W. (1978) *J. Magn. Reson.* 30, 141.
- Hanson, J. C., & Schoenborn, B. P. (1981) *J. Mol. Biol.* 153, 117.
- Hapner, K. D., Bradshaw, R. A., Hartzell, C. R., & Gurd, F. R. N. (1968) *J. Biol. Chem.* 243, 683.
- Hardman, K. D., Eylar, E. H., Ray, D. K., Banaszak, L. J., & Gurd, F. R. N. (1966) *J. Biol. Chem.* 241, 432.
- Hayes, M. B., Hagenmaier, H., & Cohen, J. S. (1975) *J. Biol. Chem.* 250, 7461.
- Hugli, T. E., & Gurd, F. R. N. (1970a) *J. Biol. Chem.* 245, 1939.
- Hugli, T. E., & Gurd, F. R. N. (1970b) *J. Biol. Chem.* 245, 1930.
- Jesson, J. P. (1973) in *NMR of Paramagnetic Molecules* (La Mar, G. N., Horrocks, W. D., & Holm, R. H., Eds.) Academic Press, New York.

- Kendrew, J. C., Bodo, G., Dinzis, H. M., Parrish, R. G., Wyckoff, M., & Phillips, D. C. (1958) *Nature (London)* 181, 662.
- Kendrew, J. C., Dickinson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., & Shore, V. C. (1960) *Nature (London)* 185, 422.
- King, G., & Wright, P. E. (1982) *Biochem. Biophys. Res. Commun.* 106, 559.
- Krishnamoorthi, R., & La Mar, G. N. (1984) *Eur. J. Biochem.* 138, 135-140.
- Morishima, I., & Inubushi, T. (1977) *FEBS Lett.* 81, 57.
- Morishima, I., & Inubushi, T. (1978) *J. Am. Chem. Soc.* 100, 3568.
- Nigen, A. M., & Gurd, F. R. N. (1973) *J. Biol. Chem.* 248, 3708.
- Ohms, J. P., Hagenmaier, H., Hayes, M. B., & Cohen, J. S. (1979) *Biochemistry* 18, 1599.
- Patel, D. J., Kampa, L., Shulman, R. G., Yamane, T., & Wyluda, B. J. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 67, 1109.
- Phillips, S. E. V. (1980) *J. Mol. Biol.* 142, 531.
- Phillips, S. E. V., & Schoenborn, B. P. (1981) *Nature (London)* 292, 81.
- Roberts, G. C. K., Meadows, D. H., & Jardetzky, O. (1969) *Biochemistry* 8, 2053.
- Schoenborn, B. P. (1969) *J. Mol. Biol.* 45, 297.
- Sheard, B., Yamane, T., & Shulman, R. G. (1970) *J. Mol. Biol.* 53, 35.
- Shulman, R. G., Wüthrich, K., Yamane, T., Antonini, E., & Brunori, M. (1969) *Proc. Natl. Acad. Sci. U.S.A.* 63, 623.
- Sudmeier, J. L., Evelhoch, J. L., & Jonsson, N. B. H. (1980) *J. Magn. Reson.* 40, 377.
- Takano, T. (1977) *J. Mol. Biol.* 110, 537.
- Thompson, J. C., Haar, W., Maurer, W., Rüterjans, H., Gersonde, K., & Sick, H. (1971) *FEBS Lett.* 16, 262.
- Van Den Oord, A. H. A., Wesdorp, J. J., Van Dam, A. F., & Verheij, J. A. (1969) *Eur. J. Biochem.* 10, 140.
- Watson, H. C. (1969) *Prog. Stereochem.* 4, 299.
- Westmoreland, D. G., Matthews, C. R., Hayes, M. B., & Cohen, J. S. (1975) *J. Biol. Chem.* 250, 7456.
- Wilbur, D. J., & Allerhand, A. (1976) *J. Biol. Chem.* 251, 5187.
- Wilbur, D. J., & Allerhand, A. (1977) *J. Biol. Chem.* 252, 4968.
- Wüthrich, K., Shulman, R. G., & Peisach, J. (1968) *Proc. Natl. Acad. Sci. U.S.A.* 60, 373.

## Conformational Differences between Various Myoglobin Ligated States As Monitored by <sup>1</sup>H NMR Spectroscopy<sup>†</sup>

J. Howard Bradbury\* and John A. Carver<sup>‡</sup>

**ABSTRACT:** In paramagnetic metmyoglobin, cyanomyoglobin (CNMb), and deoxymyoglobin, His-36 has a high pK (~8), and the NMR titration behavior of the H-2 resonance is perturbed, due to the presence at low pH of a hydrogen bond with Glu-38, which is broken at high pH. The His-36 H-4 resonance shows no shift with pK ~ 8 because of two opposing chemical shift effects but monitors the titration of nearby Glu-36 (pK = 5.6). In diamagnetic derivatives [(carbon monoxymyoglobin (COMb) and oxymyoglobin (oxyMb)], the titration behavior of His-36 H-2 and H-4 resonances is normalized (pK ~ 6.8). The very slight alkaline Bohr effect in sperm whale myoglobin (Mb) is interpreted in terms of the pK change of His-36 from deoxyMb to oxyMb and compensating pK changes in the opposite direction of other unspecified groups. In sperm whale COMb at 40 °C, the distal histidine (His-64) and His-97 have pK values of 5.0 and 5.9. The meso proton resonances remote from these groups do not show a titration shift, but the nearby γ-meso proton (pK = 5.3) responds to titration of both histidines, and the upfield Val-68

methyl at -2.3 ppm (pK = 4.7) witnesses the titration of nearby His-64. At 20 °C, the latter resonance is reduced in size, and a second resonance occurs at -2.8 ppm, which is insensitive to pH and, hence, more remote from His-64. Both resonances arise from two conformations of Val-68 in slow equilibrium. In oxyMb at 20 °C, only the latter resonance is observed, presumably because of the steric restrictions imposed by the hydrogen bond between ligand and His-64 in oxyMb, which is not present in COMb. In oxyMb the pK of His-97 (5.6) is similar to that of the meso proton resonances (5.5) and to the pK of other pH-dependent processes, including the very small acid Bohr effect. It is likely that these processes are controlled by the titration of His-97. The differences in the NMR titration behavior between the CO and oxygen complexes of Mb and leghemoglobin (Lb) are explained in terms of the larger and more flexible heme pocket in Lb and the absence in Lb of any titrating histidine other than the distal histidine.

In the preceding paper (Carver & Bradbury, 1984), we assigned many aromatic and hyperfine-shifted resonances in the <sup>1</sup>H NMR spectra of metMb,<sup>1</sup> hydroxyMb, CNMb, deoxyMb, COMb, and oxyMb. We have therefore identified many

protons in various parts of the molecule including the meso heme protons in oxyMb and COMb (Bradbury et al., 1982)

<sup>†</sup> From the Department of Chemistry, Australian National University, Canberra, A.C.T. 2600, Australia. Received August 25, 1983; revised manuscript received March 14, 1984. This work was supported by the Australian Research Grants Scheme.

<sup>‡</sup> Present address: Department of Biochemistry, University of Oxford, Oxford, England.

<sup>1</sup> Abbreviations: NMR, nuclear magnetic resonance; Mb, myoglobin; metMb, ferric aquomyoglobin; hydroxyMb, ferric hydroxymyoglobin; CNMb, ferric cyanomyoglobin; deoxyMb, ferrous deoxymyoglobin; COMb, ferrous (carbon monoxymyoglobin); oxyMb, ferrous oxymyoglobin; N<sub>3</sub>Mb, ferric azidomyoglobin; Hb, hemoglobin; deoxyHb, ferrous deoxyhemoglobin; oxyHb, ferrous oxyhemoglobin; COHb, ferrous (carbon monoxymyoglobin); Lb, leghemoglobin; deoxyLb, ferrous deoxy-leghemoglobin; oxyLb, ferrous oxyleghemoglobin; COLb, ferrous (carbon monoxymyoglobin); leghemoglobin.