Near-Heme Histidine Residues of Deoxy- and Oxymyoglobins†

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ABSTRACT: Proton NMR titration curves of the histidine C^{ϵ}–H resonances of the deoxy and oxy forms of human, horse, and sperm whale myoglobins (Mb) were determined and compared with the results for the met and azide forms. One extra titrating resonance (H-8) was observed for each deoxy-Mb compared with the corresponding met-Mb, and a further extra resonance (H-9) was observed for the oxy-Mb form. These resonances correspond to the two additional resonances previously described for azide–Mb [Hayes, M., Hagenmaier, H., & Cohen, J. S. (1975) *J. Biol. Chem. 250*, 7461–7472]. This new evidence prompts us to reassign these resonances to the near-heme histidine residues.

Histidine residues are important functional groups in heme proteins. Proton nuclear magnetic resonance (NMR) provides an excellent means to monitor individual histidine residues in proteins. Earlier success in observing and analyzing multiple titrating histidine C^{ϵ}–H resonances in proton NMR spectra of met- and azide–myoglobins (Mbs) of three species (Cohen et al., 1972; Hayes et al., 1975) prompted us to extend this study to the deoxy- and oxy-Mb forms.

Since there are 12 histidine residues present in sperm whale Mb, and only 7 titrating resonances were observed for the met form, 5 histidine resonances are not observable. In fact, the same number of resonances were consistently lacking in all three met-Mb species studied (Hayes et al., 1975). This could be explained on two grounds. First, three of the histidine residues (His-64, -93, and -97) are very close to the heme group (Watson, 1970; Takano, 1977a) and their resonances would be expected to be shifted and broadened by the paramagnetic effect of the iron atom. In fact, the proximal His-93 resonance has recently been observed in deoxy-Mb shifted far downfield (LaMar et al., 1977). Second, two of the histidine residues, His-24 and -82, are buried in the X-ray structure (Watson, 1970; Takano, 1977a) and these residues cannot be carboxymethylated (Nigen & Gurd, 1973); consequently their resonances might not be observed due to line broadening as a result of their correlation times being similar to the overall protein correlation time.

On addition of excess azide, two additional titrating histidine resonances (H-8 and H-9) with unusual titration characteristics were previously reported (Hayes et al., 1975). A similar result has been obtained in the present work for the overall transition from met- to oxy-Mb for all three species studied. These resonances would appear to have the same characteristics and hence derive from the same residues in each case. Thus, two histidine residues are apparently altered in the overall conversion of met- to oxy- or azide–Mb. The origin and significance of these two resonances is a central concern of the present study.

Experimental Procedures[1]

Materials were generally obtained as described previously (Hayes et al., 1975).

Preparation of Samples for NMR Studies. Since it was difficult to maintain a sample of Mb in the deoxy form through a pH change and NMR spectrum accumulation cycle, as judged spectrophotometrically by visible absorption, separate samples were prepared in the deoxy form at each pH value and the glass NMR tube was sealed. The samples were prepared in a glove box with strict exclusion of oxygen. All materials and apparatus were placed in the glove box, which was subjected to excess nitrogen gas pressure (50 mm water) for 12 h, followed by a further 12 h using dry nitrogen gas. All solvents to be used had nitrogen gas bubbled through them. Horse and sperm whale (126 μmol each) and human (34 μmol) myoglobins were each dissolved in 0.1 M NaCl solution (50 and 30 mL, respectively) with magnetic stirring, and a tenfold molar excess of sodium dithionite (1.89 and 0.51 mmol, respectively) was added. To remove excess reducing agent, the solution was passed with the aid of a peristaltic pump through a column of Sephadex G-25 (coarse) gel and collected in a lyophilization vessel, and a small sample was extracted, diluted, and checked for contamination of O_2 spectrophotometrically. The samples of deoxy-Mb were lyophilized and then lyophilized three times from D_2O (99.5%). In each case precautions were taken to introduce only D_2O which had been saturated with dry nitrogen using a specially prepared tube with two necks. At each stage the solution was allowed to stand for 2 h at room temperature with a flow of dry nitrogen before freezing and lyophilizing. After the third lyophilization from D_2O, the final sample of nitrogen-saturated D_2O (99.7%) was injected into the lyophilization vessel. The vessel was returned to the glove box, with precautions as before. The solutions of deoxy-Mb were transferred to a vessel with a snap-top, which allowed a constant stream of dry nitrogen gas to be bubbled through. The pH was measured inside the glove box with an electrode which had been thoroughly washed with D_2O. Solutions of NaOD or DCl (1.0 M) were introduced into the Mb solutions to obtain pH values every ca. 0.3 pH unit in the range of pH 5–9.5. At each pH value, 1.2 mL of Mb solution was removed and transferred to a centrifuge tube, centrifuged in a small preparative centrifuge, and transferred to an NMR tube, all within the glove box. The pH was measured in the NMR tube using a long, thin Ingold combination electrode and a sample was removed to check for O_2 contamination. Then five tubes were attached by pressure tubing to a sealed nitrogen-containing apparatus, containing an olive oil trap to preclude entry of oxygen. This was removed

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[1] For a detailed description of all the results of this work and further experimental details, see Ohms, J. P. (1976) Dissertation, Tübingen University. A preliminary report of some of this work was presented at the Xth International Biochemistry Congress, Hamburg, 1976 (Abstract 04-2-413).

Table I: NMR Titration Parameters for Deoxy- and Oxymyoglobins[a]

resonance	residue assignment[b]		human		horse		sperm whale	
			deoxy	oxy	deoxy	oxy	deoxy	oxy
H-1	81	curve	2	2	3	3	3	2
		pK_a	6.75	6.63	6.55	6.84	6.71	6.42
		δ_b	7.47	7.51	7.67	7.59	7.63	7.69
		Δ	1.05	1.14	0.94	0.94	0.89	0.90
H-2	12	curve					4	4
		pK_a					6.60	6.07
		δ_b					7.64	7.60
		Δ					0.89	1.04
H-3	48	curve	1	1	1	1	1	1
		pK_a	7.24	7.13	6.85	7.03	7.16	6.87
		δ_b	7.57	7.51	7.75	7.53	7.57	7.54
		Δ	0.92	0.97	0.86	0.99	0.94	0.95
H-4	116	curve			2	2	2	3
		pK_a			6.81	6.91	6.78	6.78
		δ_b			7.73	7.59	7.62	7.49
		Δ			0.84	0.92	0.89	1.00
H-5	119	curve	3	3	4	4	5	5
		pK_a	5.96	5.96	5.64	5.58	5 79	5.30
		δ_b	7.98	7.87	8.08	7.93	7.99	7.89
		Δ	0.63	0.73	0.63	0.95	0.56	1.12
H-6	113	curve			6	6	7	7
		pK_a			5.99	6.11	5.76	5.85
		δ_b			7.69	7.54	7.59	7.46
		Δ			0.93	1.05	1.00	1.03
H-7	36	curve	4	5	5	7	6	6
		pK_a	7.92	7.74	7.86	7.75	8.12	7.93
		δ_b	7.07	6.87	7.33	7.01	7.18	7.11
		Δ	1.06	1.10	0.87	1.00	1.03	0.91
H-8	97	curve	5	6	7	8	8	9
		pK_a	6.82	6.60	6.57	6.66	6.47	6.80
		δ_b	7.53	7.67	7.67	7.68	7.57	7.51
		Δ	0.31	0.26	0.25	0.28	0.21	0.31
H-9	64	curve		4		5		8
		pK_a		5.80		5.89		5.69
		δ_b		7.61		7.69		7.48
		Δ		1.13		0.96		1.10

[a] Chemical shift values in parts per million downfield from tetramethylsilane at 220 MHz; δ_b is the chemical shift of the unprotonated form, Δ is the chemical shift change on protonation; parameter values determined by curve fitting. Standard errors were usually less than ±0.05 but in three cases were greater than ±0.10. [b] These assignments are taken from Hayes et al. (1975) and were confirmed by Botelho (1975), with the exception of His-119 and -48 which were interchanged and resonances H-8 and H-9 which were not described by her.

from the glove box and the NMR tubes were sealed by melting. The samples were stored at 4 °C, under which conditions deoxy-Mb remained stable for months.

After NMR spectra had been obtained on the sealed deoxy-Mb samples, the seal was broken with a glass file. Under these conditions oxy-Mb is formed in ca. 100 s at room temperature. The pH value was checked, and a small sample was taken for spectrophotometric observation. After the NMR spectra had been run, the pH was again checked, and a further aliquot was taken to check for autoxidation to met-Mb Only two samples were found to have autoxidized, and their spectra were not included in the analysis. (For further details of sample preparation and apparatus used, including measurement of oxygen binding capacity, see Ohms (1976).)

NMR Spectroscopy. Proton NMR spectra were obtained at 220 MHz with a Varian Associates HR220 spectrometer equipped with a pulse-FT accessory and a 620 L computer. A 90° pulse (45 μs) was applied with an aquisition time of 1.6 s for an 8K transform. For several spectra a water-elimination method was applied (Patt & Sykes, 1972). The ambient probe temperature was 20 ± 1 °C, and an external standard of 5% tetramethylsilane in CCl_4 was used to calibrate chemical shift. Spectra were saved on magnetic tape using a Sykes Casette system. Computer curve-fitting of NMR titration data was carried out as described previously (Hayes et al., 1975).

Results and Discussion

In our earlier work various criteria were established for the analysis of multiple titrating histidine resonances (Hayes et al., 1975). We suggested tentative assignments of the seven titrating resonances observed in spectra of sperm whale met-Mb to histidine residues in the amino acid sequence. Three of these can be considered firm assignments since they are based on the absence of a specific peak on comparing spectra of sperm whale with those of the homologous horse and human Mbs (which lack histidine residues 12, and 113 plus 116, respectively). In addition, the independent work of Botelho (1975) agreed with all our assignments (Hayes et al., 1975) except for a transposition of His-119 and His-48 for resonances H-5 and H-3, respectively (Table I).

Our observation of two "extra" titrating resonances with unusual characteristics on the conversion of met- to azide–Mb for all three species was a noteworthy result of that study. Since resonances H-8 and H-9 were "extra" resonances in addition to those present in met-Mb, there was less ambiguity in regard to their continuities in spectra of deoxy-, oxy-, and azide-Mb.

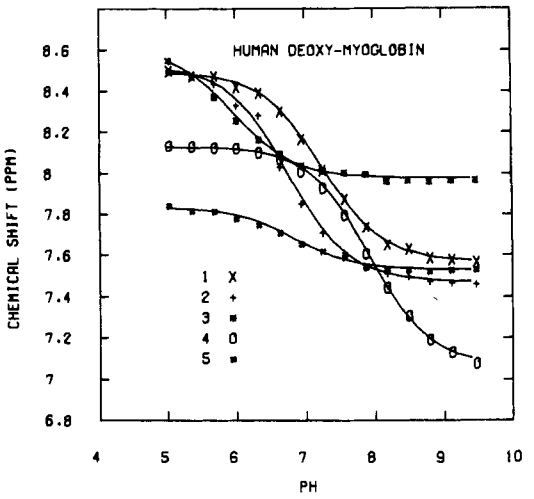

FIGURE 1: Protron NMR titration curves of human deoxymyoglobin at 220 MHz. Samples were between 50 and 100 mg/mL and were prepared under nitrogen in sealed tubes.

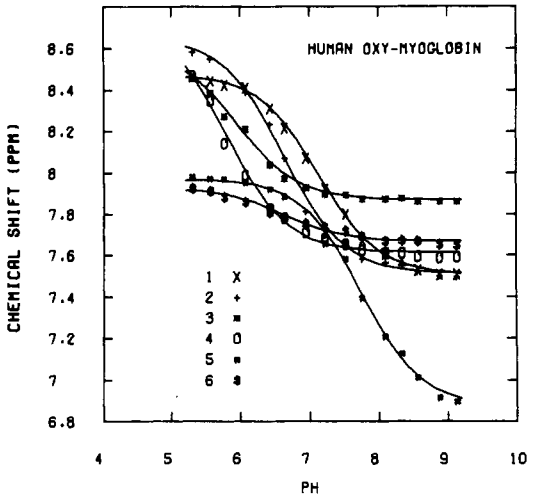

FIGURE 2: Proton NMR titration curves of human oxymyoglobin at 220 MHz. Samples from Figure 1 were unsealed and equilibrated with air for several minutes, and the conversion to the oxy form was checked by optical absorption both before and after the NMR run.

Our initial interpretation was that these extra resonances derived from the two buried histidines (24 and 82) which had become more mobile as a result of a conformational change on azide binding. However, the current results have led us to reconsider this assignment for several reasons. First, the values of the titration parameters of most of the histidine residues are now seen to be very similar comparing the liganded species aquo-met- (Hayes et al., 1975) and oxy-Mb (Table I). Thus, no major conformational differences between aquo-met- and oxy-Mb are likely, and this is supported by the X-ray results (Phillips, 1978). Thus, it would seem unlikely that the two histidines buried in one form would become exposed in the other. Second, His residues 64 and 97 are relatively freely accessible to solvent (Takano, 1977a), are not directly bound to the iron atom (as is His-93), and would thus be expected to titrate. Third, oxy-Mb is diamagnetic, thus removing the main origin of proton line broadening and/or shifting the paramagnetism of the iron atom. Therefore, we currently favor the view that the two extra resonances (H-8 and H-9) present in oxy-Mb derive from the near-heme histidines 64 and 97. Wilbur & Allerhand (1977) have also observed two extra titrating resonances in the ^{13}C NMR spectra of horse cyanoferri-Mb compared with met-Mb which they also attributed to these two near-heme histidine residues. The pK_a values (5.3 and ca. 4.5) were both much lower than the values determined here, for different liganded species.[2]

Resonance H-9 might be assigned to His-64 since the loss of paramagnetism in going from the deoxy to the oxy form is a more cogent basis on which to attribute the observation of this extra resonance than a postulated conformational change at a buried site. The pK_a value (5.8) obtained for resonance H-9 is consistent with the value (5.6) attributed to His-64 by Hayashi et al. (1976), determined spectrophotometrically, and by Saito et al. (1977), by electron spin resonance of oxy-CoMb (5.5), but is lower than the value (7.8) calculated by Shire et al. (1975). A pK_a value of 6.0 has also recently been reported for the distal histidine residue of the heterologous soybean leghemoglobin (Johnson et al., 1978).

An alternative interpretation to that given above is the possibility that one of the two additional resonances observed could derive from a near-heme histidine and one from a buried histidine. This might be considered possible from the fact that only *one* extra resonance (H-8) is observed in deoxy-Mb (Figures 1 and 2) compared with met-Mb. Since deoxy-Mb is paramagnetic, this resonance must arise from a conformational change and not directly as a result of a change in the spin state of the iron atom. A conformational change affecting a histidine residue in deoxy-Mb described by Takano (1977b) is the movement of the side chain of His-64. Specifically, it is observed that the distal histidine-64 is no longer indirectly in contact with the iron atom through a water molecule, and the iron atom moves away from the distal histidine toward the proximal histidine (Takano, 1977b). The very small change in chemical shift (Δ) of resonance H-8 (0.2–0.3 ppm) might also not represent a true protonation, but could possibly arise from a pH-dependent conformational effect. Consequently, resonance H-8 might arise from His-64. A further possible point in favor of this assignment is the fact that the pK_a value of H-8 is quite high (6.5–6.8) and it is reported that His-64 remains hydrogen bonded to a water molecule in the deoxy form (Takano, 1977b). However, the actual increase in the Fe–C^{ϵ}–H distance comparing deoxy- with met-Mb is very small (ca. 0.1–0.2 Å).

At present there is no clear factual basis to distinguish between these two alternative assignments for His-64. The resonances H-8 and H-9 may be observed in the paramagnetic azide form due to the effect of the ligand producing a conformational change reported for the EF portion of the molecule in the azide form (Stryer et al., 1964) which may result in a movement of His-97 and/or His-64.

There is quite a remarkable degree of consistency between the titration parameters determined by NMR for each of the two resonances H-8 and H-9 in the various species studied (Table I). Between the oxy and azide forms, the pK_a values (ca. 5.8) for H-9 differ somewhat (Table I and Hayes et al., 1975), but the largest difference, for sperm whale Mb (lower by ca. 0.8 pH unit), cannot be considered very precise due to a lack of data in this case. Resonance H-8 was observed in all but the met-Mb forms and in all cases the titration parameters showed a remarkable consistency of values (Table I), except for the pK_a (7.5) of H-8 in human azide-Mg (Hayes et al., 1975) compared with deoxy- or oxy-Mb (parameters could not be determined for horse azide–Mb). However, it is perhaps significant that the pK_a values do not change

[2] The fact that Wilbur & Allerhand (1977) did not observe the high pK_a titration curve (H-7) in ^{13}C NMR, while this has been consistently observed in our ^1H NMR studies, indicates either a higher relative degree of mobility for the C^{ϵ} proton compared with the ring C^{γ} atom or an unusual exchange phenomenon.

appreciably between deoxy- and oxy-Mb for this resonance. The small value (0.2–0.3 ppm) of the change in chemical shift (Δ) exhibited by resonance H-8 mainly derives from an unusual upfield shift at low pH. This indicates a strong shielding of the protonated form of this residue which could derive from the close proximity to an aromatic ring, such as Phe-46 if H-8 is His-64 or the porphyrin ring if H-8 is His-97, or to a shielding H-bonding group (water or oxygen) when the ring is protonated. These results indicate a similar degree of conservation of microenvironment in the myoglobin molecule for several species, which might be expected for such important residues which are conserved through evolution.

In the absence of any other information, the similarity of the pK_a value of resonance H-9 to that determined for His-64 (Hayashi et al., 1976; Saito et al., 1977) must be considered the firmest piece of corroborative evidence on which to base an assignment, and hence it is tentatively concluded that His-64 is the preferable of the two possible assignments. In view of the potential importance of these resonances, if they do indeed correspond to the near-heme histidine residues, we are currently investigating approaches to their definitive assignment.

Added in Proof

For further details of assignments for resonances H-1 to H-7, see Botelho & Gurd (1978) and Botelho et al. (1978).

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Interpretation of Nuclear Magnetic Resonance Spectra for *Lactobacillus casei* Dihydrofolate Reductase Based on the X-ray Structure of the Enzyme–Methotrexate–NADPH Complex†

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ABSTRACT: The three-dimensional molecular structure of *Lactobacillus casei* dihydrofolate reductase complexed with NADPH and methotrexate has been used to interpret published magnetic resonance spectra for this enzyme. Proton resonances from histidine residues and ^{19}F resonances from fluorine-labeled fluorotyrosine and fluorotryptophan dihydrofolate reductase have been assigned in several cases to specific amino acids in the primary sequence. Furthermore, the ^{31}P signals from the pyrophosphate moiety of bound NADPH have been assigned and the large upfield shift for ^{13}C-labeled (at the carboxamide carbon) $NADP^+$ upon binding to the reductase has been explained in terms of desolvation effects.

Dihydrofolate reductase (DHFR)[1] is the NADPH-dependent enzyme that reduces dihydrofolate to tetrahydrofolate. Fully reduced folates have an essential role in purine and pyrimidine biosynthesis. DHFR is thought to be the intracellular receptor for drugs such as methotrexate (MTX) and trimethoprim that have proven clinically useful in antineoplastic and antibacterial chemotherapy, respectively. Because of its low molecular weight (~20 000) and the relative ease with which the enzyme can be isolated from a variety of sources, DHFR is being studied by a number of physical and chemical methods. Five DHFRs have been completely sequenced, and spectroscopic studies utilizing fluorescence,

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[1] Abbreviations used: DHFR, dihydrofolate reductase; MTX, methotrexate.

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