PROTON NMR STUDIES OF HEMOGLOBIN IN H_2O

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Summary: Well resolved proton nuclear magnetic resonance peaks have been observed in hemoglobin at low fields. They appear in H_2O solution but not in D_2O solution and therefore come from exchangeable protons. These resonances change considerably going from deoxy- to oxy-hemoglobin and can be used to follow the structural changes upon ligation.

Exchangeable hydrogens in the high resolution proton nmr* spectra of proteins can be identified by comparison of spectra measured in H_2O and D_2O solvent. Glickson, McDonald and Phillips (1) showed that the tryptophan NH resonances can be observed in this way, while recently we (2) have resolved resonances of hydrogen bonded protons in myoglobin, in the low field region between -10 and -15 ppm. In this study we report on the exchangeable NH peaks of the tryptophan residues of hemoglobin and show that they differ between oxy and deoxy hemoglobin. In addition we have observed two other exchangeable proton peaks, between -12 and -14 ppm which also have different positions in oxy and deoxy hemoglobin.

Sample Preparation

Hemoglobin was prepared by the method described previously (3). Oxy PMB-alpha and PMB-beta chains were prepared by the method of Bucci and Fronticelli (4). PMB was removed and the $(\beta)_4$ prepared by the method of Geracci, Parkhurst and Gibson (5).

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* Abbreviations: nmr, nuclear magnetic resonance; ppm, parts per million, given in terms of the reference resonance of DSS (3-(trimethylsilyl)-propane sulfonic acid sodium salt), negative values indicating the resonance is found at lower fields; HbO_2, oxyhemoglobin; Hb, deoxy hemoglobin; met Hb, met hemoglobin.

RESULTS AND DISCUSSION

Chains and Noncooperative $(\beta)_4$ Tetramer

An investigation of the constituent chains preceded our study of hemoglobin. The indole NH exchangeable protons of tryptophans at positions A12 and C3 in the β chain should give resonances between -10 and -11 ppm (1). From a comparison of the nmr spectra of oxy β^{PMB} chains in H_2O and D_2O we have been able to identify exchangeable resonances at -10.2 and -10.5 ppm (Table 1). Their positions remain essentially unchanged in the paramagnetic deoxy form. Since the tryptophans are distant from the heme they are not susceptible to appreciable shifts and broadening by the paramagnetic electrons In contrast to myoglobin (2) where sizeable shifts were observed in the posi-

TABLE 1

(A) EXCHANGEABLE PROTONS OF HUMAN β CHAINS AND TETRAMER

		oxy				deoxy	
β^{PMB}	19°	-10.19	-10.54	β^{PMB}	19°	-10.05	-10.60
$(\beta)_4$	18°	-10.15	-10.59	$(\beta)_4$	18°	-10.02	-10.61

(B) NONEXCHANGEABLE PROTONS OF HUMAN α CHAINS, 18°

oxy		deoxy	
H_2O, pH 7	D_2O, pD 7	H_2O, pH 7	D_2O, pD 7
-10.05	-10.16	-10.27	-10.25
-10.53	-10.50		
-10.70	-10.77		

Table 1(A)

Chemical shifts in ppm from DSS of the exchangeable protons of human β chains and $(\beta)_4$ in the oxy and deoxy form between -10 and -11 ppm.

Table 1(B)

Chemical shifts in ppm from DSS of the nonexchangeable protons of human α chains in the oxy and deoxy form between -10 and -11 ppm.

tions of the tryptophan NH peaks upon oxygenation, very small shifts are observed in the α and β chains, indicating very slight structural changes, if any, in their vicinity on oxygenation. The position of the tryptophan indole NH resonances in the noncooperative $(\beta)_4$ tetramer were also essentially unaffected by oxygenation (Table 1). Here again no appreciable structural variations, as reflected in chemical shift changes between the diamagnetic oxy and paramagnetic deoxy forms of the $(\beta)_4$ tetramer, were detectable. Further, going from the single chain β^{PMB} form to the noncooperative $(\beta)_4$ tetramer had no effect upon the tryptophan resonances. Equally important, no exchangeable resonances were detectable down-field from -10.6 ppm in H_2O in

TABLE 2

(A) EXCHANGEABLE PROTONS OF HUMAN HEMOGLOBINS BETWEEN -10 TO -12 PPM AT pH 7 AND 23°C

oxy		-10.58	-10.06
deoxy	-11.17	-10.21	-9.98

(B) EXCHANGEABLE PROTONS OF HUMAN HEMOGLOBINS BETWEEN -12 TO -14.5 PPM AT pH 7.4 AND 23°C

oxy		-12.90	-12.20
met		-12.92	-12.18
deoxy	-14.14	-13.07	

(C) NONEXCHANGEABLE PROTONS OF DEOXY HUMAN HEMOGLOBIN AT pH 7 AND 23°C

H_2O, pH 7	-12.39	-17.32
D_2O, pD 7	-12.41	-17.20

Table 2

Low field proton NMR peaks in human hemoglobin.

the diamagnetic and paramagnetic forms of β^{PMB} and $(\beta)_4$ in marked contrast to the myoglobin derivatives (2).

The tryptophan at positions A12 in the α-chain should give an exchangeable resonance between -10 and -11 ppm in H_2O. Oxygenated α^{PMB} chains in H_2O has peaks at -10.0, -10.5 and -10.7 ppm which are also present in D_2O. The -10.3 ppm resonance in deoxy α^{PMB} chains is also a nonexchangeable peak (Table 1). We have been unable to observe an exchangeable resonance which could be assigned to the indole NH of tryptophan A12α. There were no exchangeable resonances between -11 to -15 ppm in the diamagnetic and paramagnetic forms of the α^{PMB} chain.

Cooperative $(\alpha\beta)_2$ Tetramer

In contrast to the chains, large differences are observed in the nmr spectra of human HbO_2 and Hb in H_2O in the tryptophan region (Fig. 1). The resonances in HbO_2 at -10.6 ppm are completely exchangeable while those at -10.05 ppm are partially exchangeable with D_2O. The areas under these resonances are difficult to evaluate because of baseline uncertainties but are estimated to be 2-3 exchangeable protons/αβ dimer at -10.6 ppm and approximately 1 exchangeable proton/αβ dimer at -10.05 ppm. In the Hb spectrum there is approximately one exchangeable proton/αβ dimer each at -11.17, -10.21 and -9.98 ppm. Figure 1 shows that the exchangeable resonances at -10.5 ppm in HbO_2 disappear upon deoxygenation and new lines appear at -11.1 ppm and -10.2 ppm. The exchangeable resonances between -10 and -11.5 ppm have not been identified with the specific tryptophans which are at A12α, A12β and C3β in human hemoglobin A. The absence of these shifts in the β subunit and the noncooperative $(\beta)_4$ tetramer suggest that these tryptophan resonances are sensitive probes to structural differences between the oxygenated and deoxygenated forms of the cooperative hemoglobin tetramer.

As stated above, there are no exchangeable resonances in the α and β chains and the $(\beta)_4$ tetramer between -11 to -15 ppm. Figure 2 shows that this region contains two exchangeable resonances in human deoxy and oxyhemoglobin

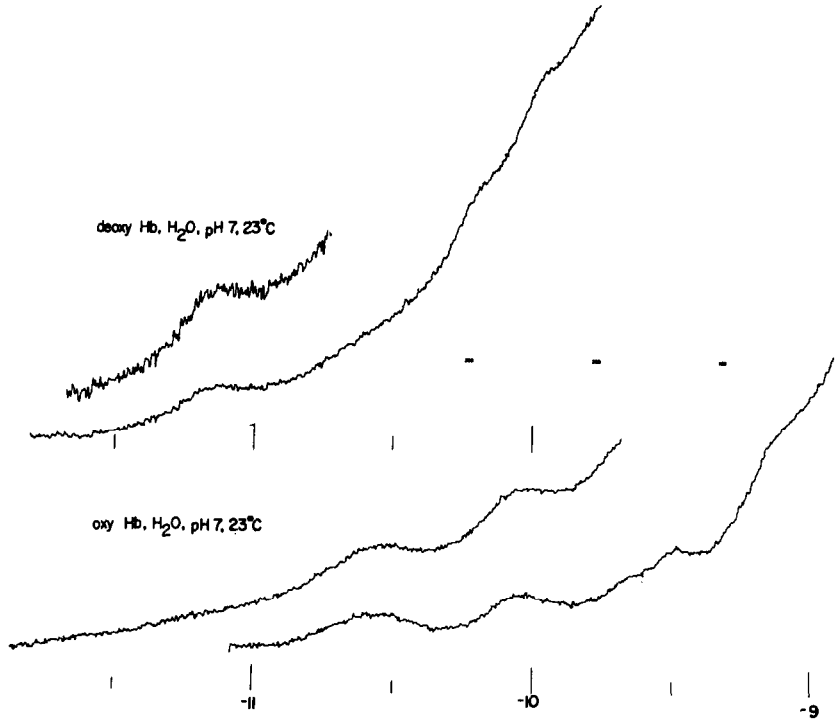

Fig. 1 220 MHz proton nmr spectra between -9 to -12 ppm of human HbO_2 and Hb in H_2O, pH 7 and 23°C.

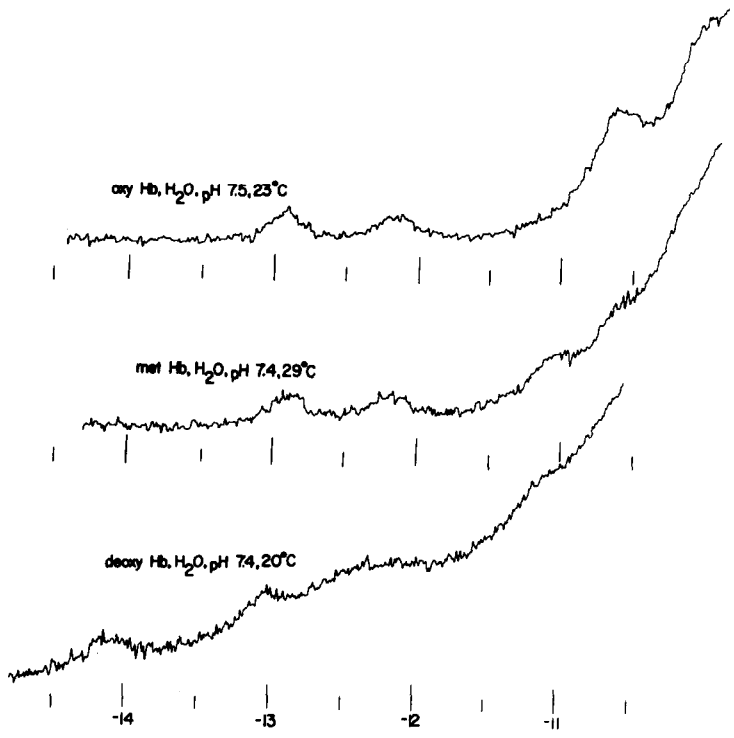

Fig. 2 220 MHz proton nmr spectra between -10 to -15 ppm of human HbO_2, met Hb and Hb in H_2O at pH 7.4. In the Hb spectrum, the broad resonance at -12.4 ppm is nonexchangeable.

at pH 7.4 and that these resonances are different in the two complexes. Relative to the upfield methyl resonance in HbO_2 at +2.8 ppm with an intensity of six protons/$\alpha\beta$ dimer and the downfield resonance of met HbCN in H_2O at -21.5 ppm with an intensity of eight protons/$\alpha\beta$ dimer (6) the exchangeable resonances at -12.18 and -12.92 ppm in HbO_2 each have an area 0.92 protons/$\alpha\beta$ dimer. Figure 2 indicates that these exchangeable resonances have similar line widths, areas and chemical shifts in paramagnetic met Hb and diamagnetic HbO_2. The absence of the effects of iron spin on the chemical shifts and widths of the exchangeable resonances suggests that the resonances are >10 Å from the heme. In contrast to these liganded derivatives of hemoglobin, Hb in H_2O at pH 7.4 shows exchangeable resonances at -13.07 and -14.14 ppm and a nonexchangeable resonance at -12.4 ppm (Fig. 2). In both oxy and deoxy Hb these resonances between -12 and -14 ppm did not change as the pH was raised from 7 to 9 and the temperature from 15 to 30°C. In contrast to this pH range, the spectra at pH 5.3 of human hemoglobin in H_2O

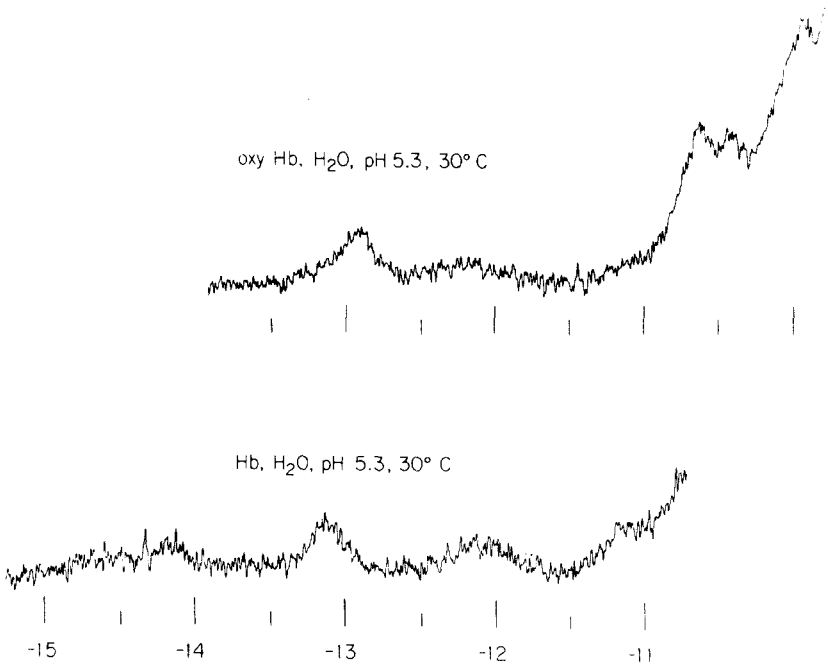

Fig. 3 220 MHz proton nmr spectra between -11 to -15 ppm of human HbO_2 and Hb in H_2O at pH 5.3.

(Fig. 3) show that the -12.18 ppm HbO_2 resonance and the -14.14 ppm Hb resonance have broadened considerably while the exchangeable resonance at approximately -13 ppm has the same unchanged line width in HbO_2 and Hb. The data indicate that the -12.18 ppm HbO_2 resonance is shifted 2 ppm downfield to -14.14 ppm in Hb while the peak near -13 ppm remains unchanged in the two forms. Since these exchangeable resonances are not affected by the magnetic electrons but only by the state of ligation of the heme, the large shifts do not originate from the unpaired iron spin or the porphyrin ring current. Identification of these resonance with specific amino acids must await future experiments. The low field shifts and slow exchange rates in water of these resonances suggest their participation in hydrogen bonds and/or hydrophobic environments. The absence of these resonances in the subunits, their distance from the heme and the large shift on deoxygenation suggest that the one proton/$\alpha\beta$ dimer resonance which moves from -12.18 ppm in HbO_2 to -14.14 ppm in Hb is an indication either of the quaternary structure (7), or of a tertiary structural change which is related to the quaternary structure.

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