A Proton Nuclear Magnetic Resonance Study of the Quaternary Structure of Human Hemoglobins in Water†

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ABSTRACT: Proton nuclear magnetic resonance spectra of human hemoglobins in water reveal several exchangeable protons which are indicators of the quaternary structures of both the liganded and unliganded molecules. A comparison of the spectra of normal human adult hemoglobin with those of mutant hemoglobins Chesapeake (FG4α92 Arg→Leu), Titusville (G1α94 Asp→Asn), M Milwaukee (E11β67 Val→Glu), Malmö (FG4β97 His→Gln), Kempsey (G1β99 Asp→Asn), Yakima (G1β99 Asp→His), and New York (G15β113 Val→Glu), as well as with those of chemically modified hemoglobins Des-Arg(α141), Des-His(β146), NES (on Cys-β93)-Des-Arg(α141), and spin-labeled hemoglobin [Cys-β93 reacted with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide], suggests that the proton in the important hydrogen bond between the tyrosine at C7α42 and the aspartic acid at G1β99, which anchors the $\alpha_1\beta_2$ subunits of deoxyhemoglobin (a characteristic feature of the deoxy quaternary structure), is responsible for the resonance at −9.4 ppm from water at 27°. Another exchangeable proton resonance which occurs at −6.4 ppm from H_2O is a spectroscopic indicator of the deoxy structure. A resonance at −5.8 ppm from H_2O, which is an indicator of the oxy conformation, is believed to originate from the hydrogen bond between the aspartic acid at Glα94 and the asparagine at G4β102 in the $\alpha_1\beta_2$ subunit interface (a characteristic feature of the oxy quaternary structure). In the spectrum of methemoglobin at pH 6.2 both the −6.4- and the −5.8-ppm resonances are present but not the −9.4-ppm resonance. Upon the addition of inositol hexaphosphate to methemoglobin at pH 6.2, the usual resonance at −9.4 ppm is shifted to −10 ppm and the resonance at −6.4 ppm is not observed. In the spectrum of methemoglobin at pH ≥7.6 with or without inositol hexaphosphate, the resonance at −5.8 ppm is present, but not those at −10 and −6.4 ppm, suggesting that methemoglobin at high pH has an oxy-like structure. Two resonances (at −8.2 and −7.3 ppm) which remain invariant in the two quaternary structures could come from exchangeable protons in the $\alpha_1\beta_1$ subunit interface and/or other exchangeable protons in the hemoglobin molecule which undergo no conformational changes during the oxygenation process. These exchangeable proton resonances serve as excellent spectroscopic probes of the quaternary structures of the subunit interfaces in studies of the molecular mechanism of cooperative ligand binding to hemoglobin.

In investigating the molecular mechanism for the cooperative oxygenation of hemoglobin in solution, it is essential to monitor the conformational changes of various parts of the molecule during the ligand binding process. The local conformation of one region of the molecule as detected by a specific spectroscopic probe is, in general, not sufficient to define the quaternary structure of the entire protein molecule in solution. The quaternary structure can, in principle, be described by a set of intersubunit interactions whose properties may be deduced from certain specific spectroscopic signals. Thus, it is desirable to seek spectroscopic probes that could provide such information.

High-resolution proton nuclear magnetic resonance (NMR) spectroscopy has been recognized as a unique technique in the study of structural and functional properties of hemoglobins. It has provided detailed information about the heme environment (Davis et al., 1969, 1971; Ogawa and Shulman, 1972; Lindstrom et al., 1972a,b; Lindstrom and Ho, 1973), the functional properties of individual hemes (Lindstrom and Ho, 1972; Lindstrom et al., 1973; Johnson and Ho, 1974; Wiechelman et al., 1974), the subunit interactions during ligand binding (Davis et al., 1971; Ho et al., 1973), the acid–base properties of a Bohr group (Greenfield and Williams, 1972; Kilmartin et al., 1973), and the conformational properties of several histidine residues of the hemoglobin molecule (Fung and Ho, unpublished results). More recently, NMR spectra of the hyperfine shifted proton resonances have been used to probe the changes in the quaternary structure of the hemoglobin molecule (Ho et al., 1973; Perutz et al., 1974a). Much information has been obtained from these studies, but most of them are conducted in D_2O medium in order to suppress the intense proton signal of the H_2O absorption in aqueous samples. Thus, samples in D_2O have a wider observable spectral region and resonances near the water proton signal have better resolution; but the signals from the NH and/or OH exchangeable protons are sacrificed. Recently, with the development of high-frequency NMR spectrometers, several ^1H NMR studies of proteins have been carried out in H_2O, and exchangeable proton resonances at low fields have been observed (Glickson et al., 1969; Patel et al., 1970; McDonald et al., 1971; Ogawa et al., 1972, 1974; Ho et al., 1973; Mayer et al., 1973; Breen et al., 1974).

X-Ray diffraction studies of hemoglobin crystals have shown that there are large differences in the quaternary

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structure between deoxyhemoglobin (Hb)[1] and oxy-like methemoglobin (Hb^+) (Perutz, 1970; Perutz and Ten Eyck, 1971). In addition to the changes in the tertiary structure of the heme pockets (including the conformational changes of the C-terminal residues), the $\alpha_1\beta_2$ subunit interface undergoes significant changes as the two subunits rotate relative to each other. Perutz and Ten Eyck (1971) have found that there are about 20 residues or 69 atoms in contact in deoxyhemoglobin, compared to 19 and 80 in the oxy-like conformation. The $\alpha_1\beta_2$ subunit interface is dovetailed as has two sets of characteristic hydrogen bonds, one between lysine at C5α40 and histidine at HC3β146 and another between tyrosine at C7α42 and aspartic acid at G1β99 in the deoxy conformation. In the oxy conformation, the characteristic hydrogen bonds are between aspartic acid at G1α94 and asparagine at G4β102 and possibly between threonine at C6α41 and histidine at FG4β97 (Perutz and Ten Eyck, 1971). Furthermore, other studies have shown that single amino acid substitutions in the $\alpha_1\beta_2$ subunit contacts not only can alter the structure of the hemoglobin molecule but also can change the functional properties of the mutants (Perutz and Lehmann, 1968; Davis et al., 1971; Morimoto et al., 1971; Ho et al., 1973; Pulsinelli, 1973; Lindstrom et al., 1973; Wiechelman et al., 1974). On the other hand, the $\alpha_1\beta_1$ subunit interface undergoes only small collective movement upon ligand binding, which involves a loosening rather than a sliding (as in the case of the $\alpha_1\beta_2$ subunit interface) at certain areas of the contacts (Perutz and Ten Eyck, 1971). Hence, a direct method capable of monitoring changes in the quaternary structure in solution, especially in the region of the $\alpha_1\beta_2$ subunit interface, is of paramount importance in the study of the structure–function relationships of both normal and abnormal hemoglobins.

In this communication, we wish to report a 250-MHz ^1H NMR study of the exchangeable proton resonances in human adult hemoglobin (Hb A) as well as in several genetically and chemically modified hemoglobins in H_2O. Among the observable proton resonances due to the NH and/or OH protons, we believe that the resonance at −9.4 ppm from H_2O at 27° is associated with the important $\alpha_1\beta_2$ subunit hydrogen bond between the phenolic hydroxyl of tyrosine at C7α42 and the carboxyl of aspartic acid at G1β99, characteristic of the deoxy quaternary structure (T structure). The resonance at ~−5.8 ppm from H_2O may be associated with the hydrogen bond between the carboxyl of aspartic acid at G1α94 and the amide group of asparagine at G4β102, characteristic of the oxy quaternary structure (R structure). The origin of the resonance at −6.4 ppm from H_2O is not known, but is related to an exchangeable proton in the deoxy conformation. The proton resonances at −8.2 and −7.3 ppm from H_2O, which are independent of ligand binding, may be associated with exchangeable protons in the region of the $\alpha_1\beta_1$ subunit interface and/or in other regions of the hemoglobin molecule which undergo no conformational changes in going from the deoxy to the oxy form.

Experimental Section

Materials. Hb A was prepared from fresh whole blood samples obtained from the local blood bank by the standard procedure used in this laboratory (Lindstrom and Ho, 1972). Mutant hemoglobins were isolated and purified from individual heterozygous hemolysates by standard methods with either Bio-Rex 70, DEAE-Sephadex, or carboxymethylcellulose column chromatography (Davis et al., 1971; Lindstrom et al., 1972a, 1973; Perutz et al., 1974a). Hemoglobins Des-Arg(α141), NES-Des-Arg(α141), and Des-His(β146) were prepared from Hb A according to the procedures of Kilmartin and Hewitt (1971). Methemoglobin A (Hb^+ A) was obtained by oxidizing carbonmonoxyhemoglobin A (HbCO A) with a fourfold molar excess of potassium ferricyanide per Hb tetramer. The spin-labeled hemoglobin was prepared by reacting HbCO A with a twofold molar excess of *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)iodoacetamide (obtained from Synvar) per β chain in bis-tris (obtained from Aldrich) buffer at pH 7.8 for at least 36 hr and at 4° (Ogawa et al., 1968; Baldassare et al., 1970). Hb^+ A and spin-labeled hemoglobin solutions were then passed through a column of G-25 Sephadex to remove excess ferri- and ferrocyanide and excess spin-label, respectively. After the removal of phosphate (Berman et al., 1971), the hemoglobin samples were exchanged with deionized water repeatedly through a ultrafiltration membrane (obtained from Amicon) to remove the remaining ions in the samples and were then adjusted to the desired pH value with a stock bis-tris buffer. A stock solution of sodium inositol hexaphosphate (IHP) (obtained from Sigma as the sodium salt) was added to the hemoglobin samples to a final concentration of four to five molar excess per Hb tetramer when required. L-Ascorbic acid (Fisher) was dissolved in bis-tris buffer at the desired pH and was added to the spin-labeled hemoglobin sample to a final concentration of a sixfold molar excess per Hb tetramer when required. Samples for NMR studies were prepared by the usual methods used in this laboratory (Lindstrom and Ho, 1972). The sample concentration was 10–20 g% hemoglobin. All pH values reported were the direct pH meter readings obtained from a Radiometer Model 4 pH meter equipped with a Beckman Model 39030 frit junction combination electrode.

Methods. ^1H NMR spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer interfaced with a Sigma 5 computer (Dadok et al., 1970). The magnetic sweep field was set for 6000 Hz with 0.6–0.8-sec sweep time. Several hundreds to thousands of scans were accumulated for each spectrum and the signal-to-noise ratio was enhanced by the NMR correlation technique (Dadok and Sprecher, 1974). Proton chemical shifts were referenced with respect to the water signal, which was 4.83 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 27°, the ambient temperature of the probe. Chemical shifts downfield from water were assigned negative values with ±0.2 ppm accuracy.

Results

Figure 1 shows the 250 MHz ^1H NMR spectra of deoxy, oxy, and carbonmonoxy Hb A at pH 6.6 and methemoglobin A at pH 6.2 in H_2O at 27°. In the deoxy Hb A spectrum, the three prominent low field paramagnetically or hyperfine shifted resonances (at approximately −17.6, −12.1, and −8.0 ppm from H_2O) remain the same in H_2O as in D_2O (Ho et al., 1973). Additional resonances at about −9.4 and −6.4 ppm from H_2O as well as the two resonances at −8.3 and −7.6 ppm which are superimposed upon the −8 ppm hyperfine shifted resonance, are from the exchange-

[1] Abbreviations used are: Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; HbCO, carbonmonoxyhemoglobin; Hb^+, methemoglobin; IHP, inositol hexaphosphate; bis-tris, 2,2-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol; NES, *N*-ethylsuccinimide; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

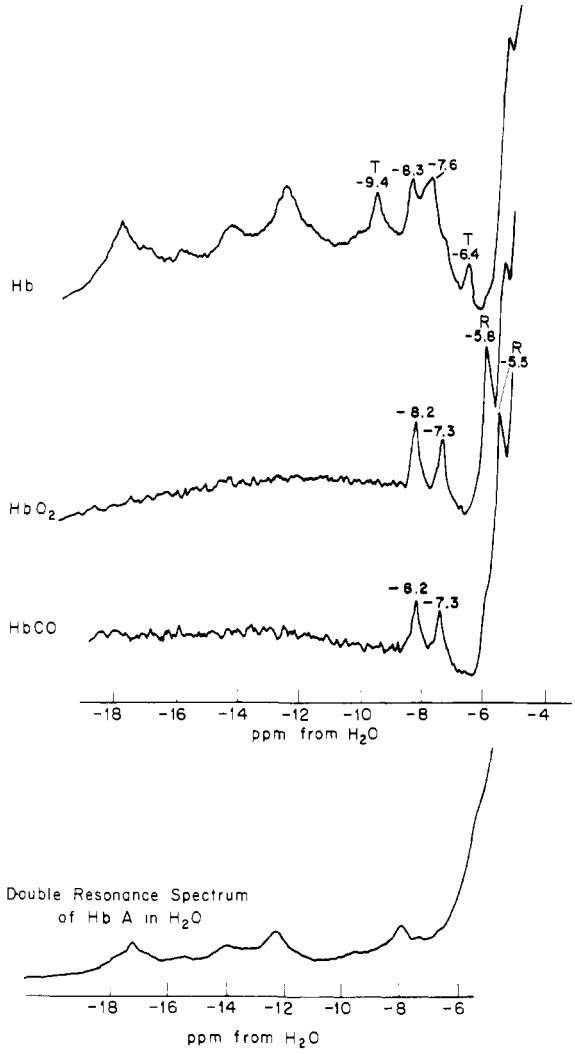

FIGURE 1: The 250-MHz ^{1}H NMR spectra of 15% Hb A in H_2O and 0.1 *M* bis-tris at pH 6.6 and 27°.

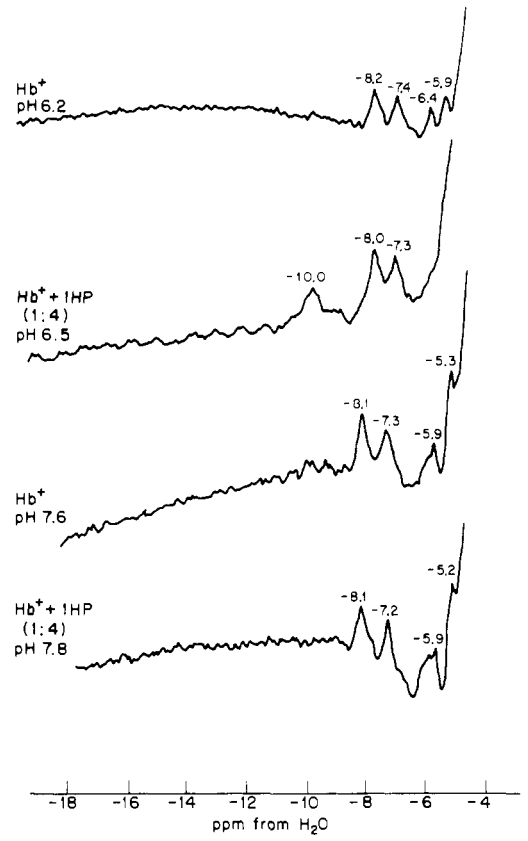

FIGURE 2: The 250-MHz ^{1}H NMR spectra of 12% methemoglobin A in H_2O and 0.1 *M* bis-tris at 27°.

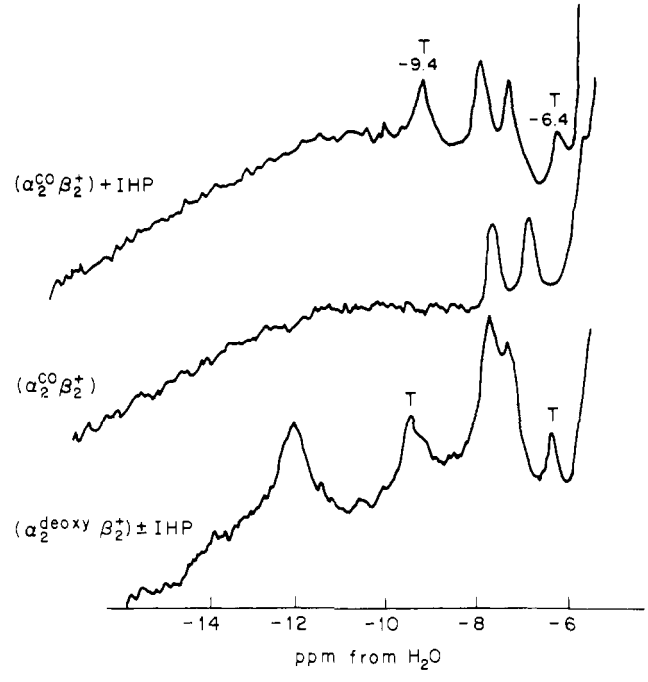

FIGURE 3: The 250-MHz ^{1}H NMR spectra of 15% Hb M Milwaukee in H_2O and 0.1 *M* bis-tris at pH 6.6 and 27°.

able protons. These resonances vanish when the hemoglobin samples are in D_2O or when the proton resonance of water is saturated with a second rf pulse in a double resonance experiment. In the spectra of both oxy and carbonmonoxy samples, the −9.4- and −6.4-ppm resonances are absent; instead, new resonances appear at −5.8 and −5.5 ppm, respectively. The −5.8-ppm (or −5.5 ppm) resonance is observed in the oxy (or carbonmonoxy) conformation of all the modified hemoglobins studied. The addition of a sufficient amount of IHP shifts the hyperfine shifted resonances of the deoxy sample slightly downfield, as is the case with samples in D_2O (Ho et al., 1973), but does not affect the exchangeable proton resonances of the deoxy, oxy, and carbonmonoxy samples of Hb A. The spectrum of methemoglobin A shows that both the −6.4- and 5.8-ppm resonances are present at pH 6.2 but only the −5.8-ppm resonance at pH 7.6. Figure 2 indicates the effects of IHP, a strong allosteric effector, on Hb^+ A at two different pH's. At pH 6.5 in the presence of IHP, the −9.4-ppm resonance appears but has shifted downfield to −10.0 ppm, whereas the −6.4- and −5.8-ppm resonances were not observed. At a higher pH value, pH 7.8, IHP appears to have no effect on the exchangeable proton resonances of Hb^+ A. In Figure 3, we have shown the effect of IHP on the mutant hemoglobin M Milwaukee, a naturally occurring valency hybrid in which the iron atoms in the β chains are in the ferric state and cannot combine with ligands, such as oxygen or carbon monoxide. The −9.4- and −6.4-ppm resonances are present in the deoxy spectrum and accordingly disappear in the spectrum of HbCO M Milwaukee. When IHP is added to the sample, these two resonances reappear as predicted due

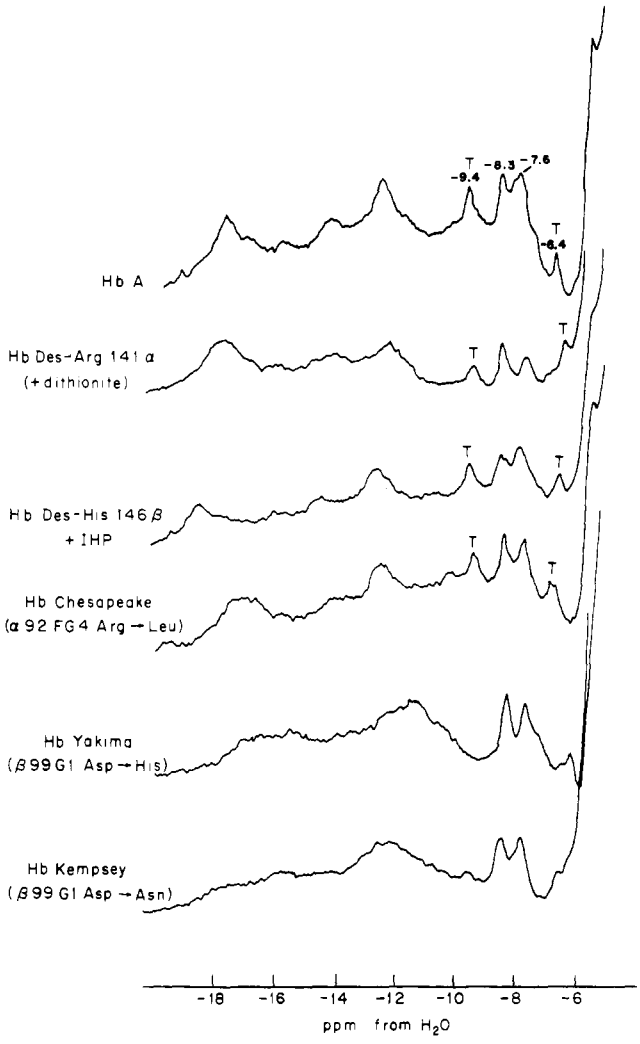

FIGURE 4: The 250-MHz ^1H NMR spectra of 12% deoxyhemoglobins Des-Arg, Des-His, Chesapeake, Yakima, and Kempsey in H_2O and 0.1 *M* bis-tris at pH 6.6 and 27°.

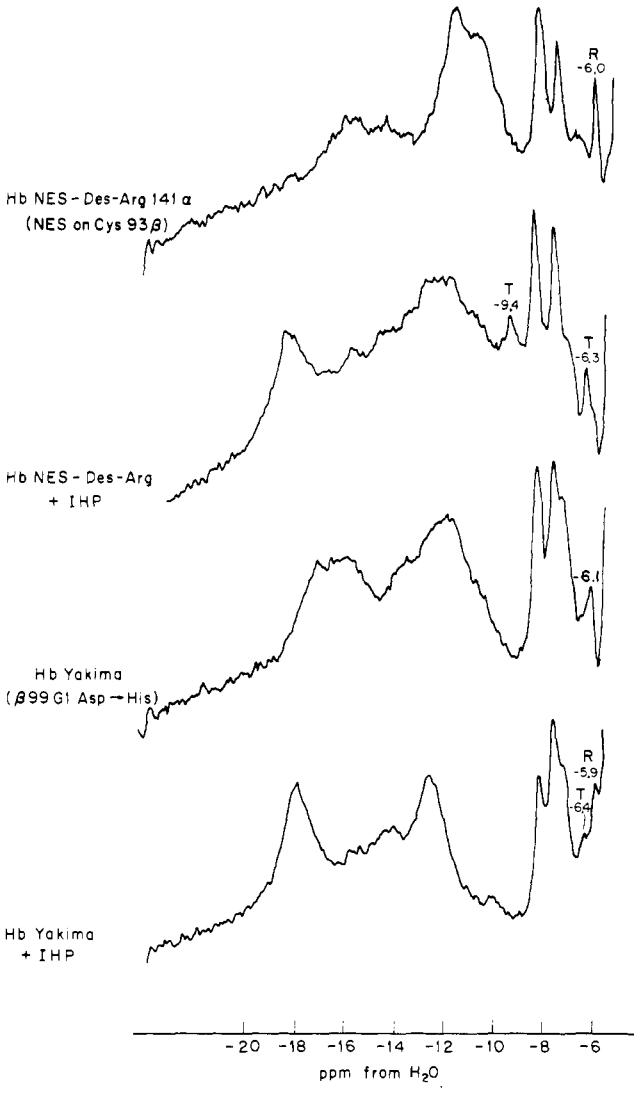

FIGURE 5: The 250-MHz ^1H NMR spectra of 12% deoxyhemoglobins NES-Des-Arg and Yakima with and without IHP in H_2O and 0.1 *M* bis-tris at pH 6.6 and 27°.

to the transition from the oxy to the deoxy quaternary structure (Perutz et al., 1972; Fung, Pisciotta, and Ho, unpublished results).

Figures 4 and 5 show the deoxy spectra of hemoglobins Des-Arg, Des-His, NES-Des-Arg, Chesapeake, Yakima, and Kempsey. There are no observable differences in the NH and/or OH proton resonances among Hb A, Hb Chesapeake, Hb New York, Hb Des-Arg, and Hb Des-His in the deoxy form (Table I). However, the exchangeable proton resonances of deoxy Hb NES-Des-Arg, Hb Yakima, and Hb Kempsey are significantly different from those of deoxy Hb A. The resonances at −9.4 and −6.4 ppm from H_2O are missing in deoxy Hb NES-Des-Arg but reappear upon the addition of IHP to the sample (Figure 5). The resonance at −9.4 ppm in the spectra of deoxy Hb Yakima and Hb Kempsey is missing, even in the presence of IHP (Figures 4 and 5; Table I) and the resonance at −6.4 ppm is shifted slightly upfield (~0.2 ppm) in these mutants. It should be noted that the resonance at −5.8 ppm from H_2O (characteristic of the oxy conformation) shows up in the deoxy spectrum of Hb Yakima plus IHP at −5.9 ppm.

In Figure 6, the spectra of Hb with the spin-label *N*-(1-oxyl-2,2,6,6-tetramethylpiperidinyl)iodoacetamide covalently attached at cysteine-β93 are essentially the same as those of unlabeled Hb A. In the presence of ascorbic acid, where the spin-label on the hemoglobin molecule is reduced to a diamagnetic species (Kornberg and McConnell, 1971), the NMR spectra remain unchanged. Due to the high oxygen affinity of the spin-labeled Hb A, IHP was added to facilitate the process of deoxygenation and thus to reduce methemoglobin formation.

Table I summarizes the exchangeable proton resonances of the various hemoglobins studied.

Discussion

The identification of proton resonances in the hemoglobin molecule in the region from −5 to −10 ppm from H_2O as the exchangeable NH and/or OH proton resonances is based on the following two types of experiments. One is the conventional method of comparing the ^1H NMR spectra of samples in H_2O with those in D_2O. Another one is by the double resonance technique, whereby the exchangeable proton resonances can be cross saturated when the water proton resonance is irradiated by a rf pulse in a double resonance experiment (Gupta and Redfield, 1970). The detection of these exchangeable proton resonances implies that the exchange between these protons and those of water is slow on the NMR time scale. The earlier studies by Ogawa

Table 1: Proton Nuclear Magnetic Resonances of Human Hemoglobins in H_2O and 0.1 *M* Bis-tris at pH 6.6 and at 27°.[a]

	Hb	Hb + IHP	Hb^+	Hb^+ + IHP	HbO_2	HbO_2 + IHP	HbCO	HbCO + IHP
			At pH 6.2	At pH 6.5				
Hb A	−9.4	−9.4		−10.0				
	−8.3	−8.3	−8.2	−8.0	−8.2	−8.2	−8.2	−8.2
	−7.6	−7.6	−7.4	−7.3	−7.3	−7.3	−7.4	−7.4
	−6.4	−6.4	−6.4					
			−5.9		−5.8	−5.8	−5.5	−5.5
			At pH 7.6	At pH 7.8				
			−8.1	−8.1				
			−7.3	−7.2				
			−5.9	−5.9				
Hb M Milwaukee	−9.5	−9.5						−9.4
(E11β67 Val → Glu)	−7.8	−7.8					−8.2	−8.1
	−7.3	−7.3					−7.5	−7.5
	−6.4	−6.4						−6.4
							−5.5	
Hb Des-Arg	−9.4	−9.4						
(α141)	−8.4	−8.4			−8.2		−8.2	
	−7.6	−7.6			−7.5		−7.4	
	−6.4	−6.4						
					−5.9		−5.5	
Hb Des-His	−9.4	−9.4						
(β146)	−8.2	−8.2			−8.1		−8.2	
	−7.6	−7.6			−7.3		−7.3	
	−6.4	−6.4						
					−5.9		−5.5	
Hb Chesapeake	−9.3	−9.3						
(FG4α92 Arg → Leu)	−8.2	−8.2			−8.1	−8.1	−8.3	
	−7.5	−7.5			−7.4	−7.4	−7.3	
	−6.5	−6.4						
					−5.8	−5.8	−5.5	
Hb NES (on β93		−9.4						
Cys)-Des-Arg	−8.4	−8.4			−8.3	−8.3		
(α141)	−7.6	−7.6			−7.6	−7.6		
		−6.3						
	−6.0				−5.9	−6.0		
Hb Kempsey	−8.2	−8.2			−8.0		−8.2	
(G1β99 Asp → Asn)	−7.6	−7.5			−7.3		−7.4	
	−6.3	(−6.5)[b]						
					−5.8		−5.5	
Hb Yakima	−8.3	−8.2			−8.2		−8.2	−8.2
(G1β99 Asp → His)	−7.6	−7.6			−7.6		−7.4	−7.4
	(−6.5)[b]	(−6.4)[b]						
	−6.1	(−5.9)[b]			−5.9		−5.5	−5.5
Hb Malmö							−8.2	
(FG4β97 His → Gln)							−7.5	
							−5.5	
Hb Titusville							−8.1	
(G1α94 Asp → Asn)							−7.4	
							−5.5	
Hb New York	−9.4							
(G14β113 Val → Glu)	−8.3						−8.2	
	−7.6						−7.3	
	−6.6							
							−5.6	
Spin-labeled		−9.3						
Hb (on SH of		−8.2					−8.2	
β93 Cys) at pH		−7.6					−7.4	
7.4		−6.5						
							−5.6	

[a] Units are in parts per million (ppm) from water proton signal with ±0.2 ppm accuracy. [b] This bracket indicates that those resonances have relatively low intensity.

and coworkers have shown that the proton resonance at −9.4 ppm from H_2O (or ~14 ppm from DSS) can be used as a quaternary structural probe for the hemoglobin molecules (Ogawa et al., 1972, 1974; Mayer et al., 1973). However, they did not make a specific assignment to this resonance. The present work has confirmed their earlier suggestion that the −9.4-ppm resonance is a quaternary structural probe. In addition, we have observed several other resonances which can also be used as spectroscopic probes to monitor the structural transitions of the hemoglobin molecule during oxygenation and have made assignments to two of these resonances.

It is clear that the −9.4- and the −6.4-ppm resonances in Figure 1 are associated with some specific exchangeable protons in the deoxyhemoglobin molecule, whereas the −5.8 ppm (or −5.5 ppm in HbCO) resonance is associated with an exchangeable proton in HbO_2 (or HbCO)..The spectra of Hb^+ A under various conditions further demonstrate the unique characteristics of the −9.4, −6.4, and −5.8-ppm resonances as quaternary structural indicators. It has been reported that aquomethemoglobin has a structure intermediate between the oxy and deoxy conformations and that Hb^+ A can be converted to the deoxy conformation upon the addition of IHP (Kilmartin, 1973; Perutz et al., 1974b,c). We have found that at pH 6.2, Hb^+ A has exchangeable resonances at −6.4 and −5.8 ppm (characteristic of the deoxy and oxy conformations, respectively) suggesting that an equilibrium between deoxy and oxy conformations can exist in Hb^+ A in solution. In the presence of IHP, the other T-type structural indicator at −9.4 ppm reappears but is shifted to −10.0 ppm (Figure 2). This IHP effect on Hb^+ A disappears at higher pH's. At pH 7.6 and above, the spectra contain only the −5.9-ppm resonance, suggesting that the molecule is in the oxy conformation even in the presence of IHP. Other studies, such as those using optical difference spectra, redox equilibrium measurements, and X-ray diffraction measurements, have suggested that aquomethemoglobin at pH 6.5 contains both deoxy and oxy quaternary structures in dynamic equilibrium (Kilmartin, 1973; Perutz et al., 1974b,c). This equilibrium can be shifted either toward the deoxy quaternary structure by the addition of IHP at this pH, or toward the oxy quaternary structure by increasing the pH above 7.0 (Kilmartin, 1973; Anderson, 1973; Perutz et al., 1974b,c). The credibility of the assignments of these resonances as quaternary structural probes is further tested on Hb M Milwaukee. NMR and other studies have shown that the addition of IHP can revert fully ligated Hb M Milwaukee to a deoxy-like quaternary structure (Perutz et al., 1972, 1974c; Fung, Pisciotta and Ho, unpublished results). As shown in Figure 3, the T-type structural indicators, −9.4- and −6.4-ppm resonances, reappear in the spectrum when IHP is added to Hb M Milwaukee saturated with carbon monoxide. (The absence of hyperfine resonances between −10 and −14 ppm indicates that the sample remains fully ligated.) In conclusion, the −9.4- and −6.4-ppm resonances are spectroscopic probes which reflect some characteristics of the deoxy quaternary structure and the −5.8-ppm resonance is a characteristic signal of the oxy conformation.

We have investigated the origin of these low-field exchangeable proton resonances. The appearance of the −8.2-, −7.3-, and −5.8-ppm resonances in HbO_2 A and the −9.4- and −6.4-ppm resonances in HbCO M Milwaukee in the presence of IHP indicates that these resonances cannot be paramagnetically shifted exchangeable proton reso-

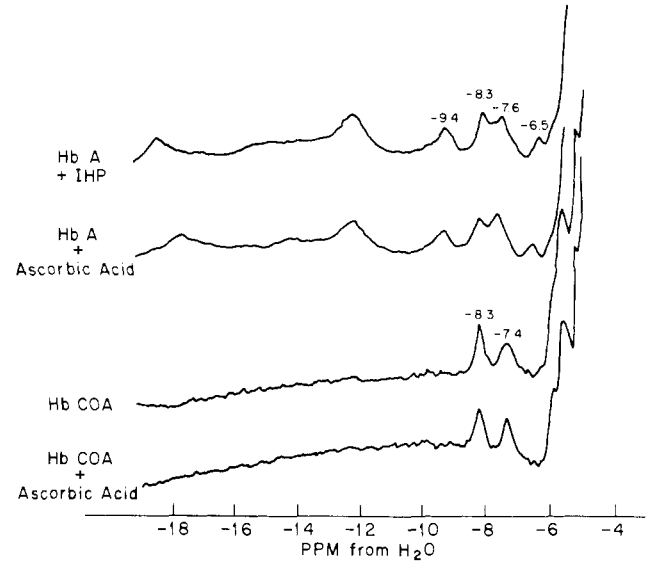

FIGURE 6: The 250-MHz ^1H NMR spectra of 10% Hb A reacted with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide at cysteine-β93 in 0.1 *M* bis-tris plus IHP at pH 7.4 and 27°.

nances. They are likely shifted downfield through the formation of hydrogen bonds. In general, when atom Y is bonded to a molecule H–X, there is a reduction of the diamagnetic circulation in the H-X bond by the electrostatic field of the donor Y, and the proton nuclear magnetic resonance is shifted downfield (Schneider et al., 1958; Pimentel and McClellan, 1959; Kollman and Allen, 1972). X-Ray diffraction studies have shown that the hydrogen bonds anchoring the $\alpha_1\beta_2$ subunit interface in the deoxy state are different from those in the oxy state. Most recent X-ray studies of human deoxy Hb at 2.5-Å resolution has found three water molecules bound in the $\alpha_1\beta_2$ contact. One of those is bound to the carboxyl of Asp-β99 (G. Fermi, personal communication, manuscript submitted to *J. Mol. Biol.*). Water molecules are also likely to be found in the $\alpha_1\beta_2$ contacts in horse Hb^+ by Dr. R. C. Ladner at 2.0-Å resolution (M. F. Perutz, personal communication). These findings further confirm that water molecules are accessible to the exchangeable protons in the subunit interfaces.

The Origin of the −9.4-ppm Resonance. Two important hydrogen bonds in the deoxy conformation, one between lysine at C5α40 and histidine at HC3β146 and another one between tyrosine at C7α42 and aspartic acid at G1β99, are broken upon oxygenation and new bonds are formed between aspartic acid at G1α94 and asparagine at G4β102 and possibly between threonine at C6α41 and histidine at FG4β97 in the oxy conformation (Perutz and Ten Eyck, 1971). Our NMR results show that in deoxyhemoglobin Yakima and Kempsey, even in the presence of IHP, the −9.4-ppm resonance is absent, clearly indicating that this resonance is related to the β99 aspartic acid, which is replaced by histidine in Hb Yakima (Jones et al., 1967) and by asparagine in Hb Kempsey (Reed et al., 1968). X-Ray diffraction studies of single crystals of Hb Yakima at 3.5-Å resolution have shown that deoxy Hb Yakima is isomorphous with deoxy Hb A, but the important $\alpha_1\beta_2$ subunit hydrogen bond between Tyr at α42 and Asp at β99 is missing (Pulsinelli, 1973). Crystals of deoxy Hb Kempsey are found to have a structure similar to that of human oxyhemoglobin A (Perutz et al., 1974a). The same hydrogen bond which is not found in deoxy Hb Yakima is also missing in deoxy Hb

Kempsey. Our NMR studies indicate that deoxyhemoglobins Yakima and Kempsey also have their heme environments altered as revealed by the hyperfine shifted proton resonances, but these resonances can be converted to those similar to Hb A upon the addition of IHP [Figure 5; for detailed discussions on the hyperfine shifted protons of these hemoglobins and the effects of organic phosphates on them in D_2O, refer to references (Davis et al., 1971; Ho et al., 1973; Lindstrom et al., 1973; Perutz et al., 1974a)]. However, the hydrogen bond between Tyr at α42 and Asp at β99 in the deoxy conformation cannot be formed even in the presence of IHP due to the amino acid substitution at β99. Hence, we have assigned the −9.4-ppm resonance to the phenolic hydroxyl proton of Tyr at C7α42, which is hydrogen bonded to the carboxyl of Asp at G1β99. A chemically modified hemoglobin, deoxy Hb NES-Des-Arg, which has a quaternary structure similar to deoxy Hb Kempsey (Perutz et al., 1974a), has also been studied. We have shown that deoxy Hb NES-Des-Arg can have either an oxy-like (R type) or a deoxy-like (T type) quaternary structure depending on the absence or presence of IHP, respectively (Figure 5; Perutz et al., 1974a). In this modified hemoglobin, the −9.4-ppm resonance is missing in the deoxy form. In the presence of IHP it reappears, although with somewhat reduced intensity. This finding indicates that it is possible to have deoxy Hb NES-Des-Arg in the T-type structure with the formation of the crucial deoxy hydrogen bond in the presence of IHP. The reduced intensity of this resonance may imply that the molecules have not been fully converted to the T-type quaternary structure. This suggestion is supported by the broadening of the hyperfine shifted resonances of this modified hemoglobin. The X-ray studies have indicated that the two deoxy hydrogen bonds made by histidine at β146 are inhibited in the NES-Des-Arg (M. F. Perutz, personal communication). Therefore, the appearance of the −9.4-ppm resonance in Hb Des-His and Hb NES-Des-Arg indicates that this resonance cannot be due to those hydrogen bonds made by β146 histidine.

The Origin of the −6.4-ppm Resonance. In the spectrum of Hb^+ A at pH 6.2 in the absence of IHP, the −6.4-ppm resonance coexists with the −5.8-ppm resonance. The −9.4-ppm resonance is missing in the absence of IHP, but reappears at −10.0 ppm in the presence of IHP (Figure 2). Though the properties of Hb^+ A indicate that it has a quaternary T structure as defined by the distances between the iron atoms and the dovetailing of the $\alpha_1\beta_2$ contacts the T type structure of Hb^+ A at pH 6.2 is not identical with that of deoxy Hb A as suggested by our NMR results. The −6.4-ppm resonance, as a deoxy-like structural probe, is more sensitive to the differences between deoxy Hb A and Hb^+ A, but possibly less crucial than the −9.4-ppm resonance. This −6.4-ppm resonance clearly comes from an amino acid residue which changes its conformation upon ligation. There are several amino acid residues that fall into this category. The penultimate tyrosines (HC2) occupy pockets between helicies F and H of both α and β chains in the deoxy form and their phenolic hydroxyls are hydrogen bonded to the carboxyls of Val at FG5. In the oxy or R form, these tyrosines are pushed out of the heme pockets and form no such hydrogen bonds (Perutz, 1970). It has been shown that the conformation of these tyrosines is not affected when the C-terminal arginines (Hb Des-Arg) in the α chains and histidines (Hb Des-His) in the β chains are deleted (Perutz and Ten Eyck, 1971). Thus, the normal spectra of deoxyhemoglobins Des-His and Des-Arg with IHP do not eliminate the penultimate tyrosine as a possible origin of this resonance. By imbedding Hb^+ A crystals in the lattice of acrylamide, Anderson (1973) has shown that Hb^+ A retains several structural features similar to those of deoxy Hb A as seen in the X-ray diffraction patterns. The hydrogen bond between Tyr at α42 and Asp at β99 is beginning to break. Meanwhile the hydrogen bond between Tyr at HC2α140 and Val at FG5α93 of the same α chain is weakened. We have observed a downfield shift of the −9.4-ppm resonance and are unable to detect the −6.4-ppm resonance in the spectrum of methemoglobin with IHP at pH 6.5. Therefore, the speculation that a possible origin of the −6.4-ppm resonance as due to the intrachain hydrogen bond between Tyr and Val is not inconsistent with Anderson's results. The NH proton of tryptophan at C3β37 could be another candidate for the −6.4-ppm resonance. In hen egg white lysozyme, some exchangeable protons from tryptophans give resonances in the region between −5 to −7 ppm from H_2O (Glickson et al., 1969). In Hb A, the tryptophan at C3β37 is in the $\alpha_1\beta_2$ subunit interface and is forming a rather long hydrogen bond with Asp-α94 in deoxy Hb. Therefore its conformation is sensitive to a change in the quaternary structure (Perutz and Ten Eyck, 1971; M. F. Perutz, personal communication). The presence of the −6.4-ppm resonance in the spectra of deoxyhemoglobins Des-Arg and Des-His with IHP eliminates the likelihood of having the salt bridges of the C-terminal histidine of the β chains (β146) and of the C-terminal arginines of the α chains (α141) as possible candidates for the origin of this resonance. Further work is needed to make a definitive assignment of the resonance at −6.4 ppm.

The Origin of the −5.8-ppm Resonance. The appearance of a resonance at −5.9 ppm in deoxy Hb Yakima plus IHP leads us to speculate that the −5.8-ppm resonance in HbO_2 A may come from the proton of the hydrogen bond between aspartic acid at G1α94 and asparagine at G4β102 in the $\alpha_1\beta_2$ subunit interface in the oxy conformation. According to the X-ray diffraction studies of Hb Yakima, the crucial hydrogen bond between Tyr at C7α42 and Asp at G1β99 in the $\alpha_1\beta_2$ subunit interface (in the deoxy conformation) is broken as a result of the amino acid substitution at β99 in this mutant, but a new hydrogen bond between Asp at G1α94 and Asn at G4β102, which is normally found in the oxy form of Hb A, is formed (Pulsinelli, 1973). The spectrum of Hb Malmö (FG4β97 His→Gln) (Lorkin et al., 1970) provides an indirect support for this speculation. The −5.5-ppm resonance is present in the spectrum of HbCO Malmö (Table I, Fung, Fairbanks, and Ho, unpublished results). Thus, this resonance is not likely to originate from the hydrogen bond between Thr at C6α41 and His at FG4β97, which is possibly another important hydrogen bond in the $\alpha_1\beta_2$ subunit interface characteristic of the oxy conformation (Perutz and Ten Eyck, 1971). As indicated in Table I, the spectrum of HbCO Titusville (G1α94 Asp →Asn) (H. Lehmann, R. G. Schneider, and M. F. Perutz, personal communication) is identical with that of HbCO A. This normal spectrum of HbCO Titusville does not invalidate our assignment that the resonance at −5.5 ppm in HbCO (or −5.8 ppm in HbO_2) is due to the proton of the hydrogen bond between the residues at G1α94 and G4β102. It is because the replacement of Asp by Asn at α94 does not prevent the formation of this particular hydrogen bond between the residues at α94 and β102 in the oxy conformation according to Perutz (personal communication). The amide NH on Asn at β102 of this mutant can form a hydrogen

bond with the side chain carbonyl group of Asn at α94 (M. F. Perutz, personal communication). The fact that the chemical shift of the −5.5-ppm resonance remains unaffected by the amino acid substitution at β102 is not fully understood. A detailed description about the structure of this region of the Hb Titusville molecule and a quantitative comparison of the bond strength of this abnormal hydrogen bond with that of Hb A may provide insight into this problem. We have observed a 0.3-ppm difference in the chemical shift of this resonance between HbO_2 A and HbCO A (Figure 1). The exact implication of this difference is not yet known. But, if they do originate from the same hydrogen bond, then this hydrogen bond in the $\alpha_1\beta_2$ subunit interface senses a slight difference in the environment between HbO_2 A and HbCO A.

The Origins of the −8.2- and −7.3-ppm Resonances. The −8.2- and −7.3-ppm resonances remain the same in the liganded and unliganded states suggesting that they may arise from the $\alpha_1\beta_1$ subunit contacts. This interface undergoes only a slight change on ligation and the number of hydrogen bonds probably remains unchanged (Perutz and Ten Eyck, 1971). There are two sets of experimental evidence to provide indirect support to this suggestion. First, the exchangeable proton resonances of the isolated α and β chains in the CO form are very different from those of HbCO A (Patel et al., 1970; Pifat, Fung, Bertoli, and Ho, unpublished results). These results suggest that these two resonances cannot come from intrachain H-bonds in the α or β chains. Second, we have obtained the exchangeable proton resonances of 3×10^{-4} *M* (2 g %) HbCO A in 0.1 *M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid plus 0.9 *M* $MgCl_2$ at pH 7.3 and have found that there is a reduction in the intensity of the resonances at −8.3, −7.3, and −5.5 ppm relative to those in the absence of $MgCl_2$ (Fung and Ho, unpublished results). Hewitt et al. (1972) reported that 3×10^{-5} *M* HbCO A in 0.1 *M* *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid plus 0.9 *M* $MgCl_2$ at pH 7.2 and at 20° dissociates readily into $\alpha_1\beta_1$ dimers. One would expect that the exchangeable proton resonances of the $\alpha_1\beta_1$ dimer would be very different from those of the intact HbCO A. The reduced intensities of the exchangeable resonances at −8.2, −7.3 and −5.5 ppm suggest that there is an incomplete dissociation of tetramers into dimers at a higher concentration of HbCO used for NMR experiments and that the $\alpha_1\beta_1$ dimer has a structure different from that of the intact tetramer. However, another possibility is that these resonances at −8.2 and −7.3 ppm may originate from tryptophans at A12 of both α and β chains and/or other residues which remain unchanged during the transition from deoxy- to oxyhemoglobin (Perutz et al., 1974a). We have also investigated the exchangeable proton resonances of Hb New York (G15β113 Val→Glu) (Ranney et al., 1967), a mutant having an amino acid substitution near the $\alpha_1\beta_1$ interface (Perutz and Lehmann, 1968). We have found that the exchangeable proton resonances of this mutant in both deoxy and CO forms are identical with those of Hb A (Table I). This result suggests that the amino acid substitution in Hb New York does not alter the conformations of those amino acid residues that give rise to the exchangeable proton resonances at −8.2 and −7.3 ppm. Further work is needed to ascertain the origin of these two resonances (see note Added in Proof).

Effects of a Spin-Label at Cysteine-β93 and of Unpaired Electrons on the Exchangeable Resonances. Since the cysteine at β93 and the α and β heme groups are close to the $\alpha_1\beta_2$ subunit interface (Perutz, 1970), it is reasonable to investigate whether a paramagnetic spin-label attached to β93 or whether the unpaired electrons of the heme iron in deoxyhemoglobin would affect the resonances in question. The observed line widths of the exchangeable protons are ranging from 70 to 125 Hz for both the oxy and deoxy structures and show no significant change upon spin-labeling at β93. Under the conditions of this study, the principal effect to be expected from a paramagnetic center would be a decrease in the transverse relaxation times (T_2) for protons located near the center. (For a recent review on the relaxation in paramagnetic systems, see Dwek (1973).) The line-width increase which would result from a decrease in T_2 is inversely proportional to the sixth power of the distance between the paramagnetic center and the nucleus in question and is also approximately proportional to the correlation time, τ_c, of this dipolar interaction. This correlation time, in turn, is determined by the sum: $1/\tau_c = 1/\tau_r + 1/\tau_s$ where τ_r is the rotational correlation time of the paramagnetic center and τ_s is the electron spin relaxation time. Thus, τ_c is effectively determined by the shorter of τ_r or τ_s.

The structure of horse carbonmonoxyhemoglobin with *N*-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide covalently attached to the sulfhydryl of β93 cysteine has been determined at 3.5-Å resolution by X-ray difference Fourier techniques (Moffat, 1971). The X-ray diffraction and electron paramagnetic resonance (EPR) studies of spin-labeled HbCO indicate that the spin-label can assume two orientations with respect to the hemoglobin molecule (Ogawa et al., 1968; McConnell et al., 1969; Moffat, 1971). These orientations correspond to the weakly immobilized and strongly immobilized components of the EPR spectra. According to McConnell et al. (1969), these two orientations of the spin-label are in equilibrium with a lifetime for interconversion $\geq 10^{-8}$ sec. The EPR spectrum of spin-labeled deoxyhemoglobin shows that in this case only the weakly immobilized component is present (Ogawa et al., 1968; Baldassare et al., 1970). Based on the spin-label studies reported by McConnell and coworkers (McConnell et al., 1969; McCalley et al., 1972), the rotational correlation time for the weakly immobilized conformation can be estimated as $\sim 9 \times 10^{-9}$ sec, while for the strongly immobilized conformation, the correlation time is essentially that of the Hb molecule, i.e., $\sim 3 \times 10^{-8}$ sec. Hence, to an accuracy of 0.2 ppm (50 Hz), no line broadening would be observed in the exchangeable proton resonances of spin-labeled Hb when the nitroxyl group is $\gtrsim$10 Å from the sites giving rise to these resonances. By placing the spin-label at β93 into the atomic models of deoxy- and methemoglobin, Dr. M. F. Perutz has kindly measured the following distances for us: the distance between the midpoint of the NO of the spin-label at β93 and the H atom of the OH group of Tyr at α42 is ~16 Å in the deoxy structure; the distance between the midpoint of the NO of the spin-label at β93 and the H atom of the amide group in the side chain of Asn at β102 is ~18 Å for the weakly immobilized orientation and is ~10 Å for the strongly immobilized orientation in the oxy structure. We have also estimated these distances by means of a graphic display computer at the National Institutes of Health using the computer program developed by Feldman et al. (1973). Our results are as follows: the distance from the N atom of the NO group of the spin-label at β93 to the O atom of the OH group of Tyr at α42 is 18.6 Å in the deoxy structure; the distance from the N atom of the NO group of the spin-label at β93 to the N atom of the amide

group in the side chain of Asn at β102 is ~16.8 Å in the weakly immobilized orientation and is ~19.6 Å in the strongly immobilized orientation in the oxy structure. The input of this study consists of the X-ray coordinates of deoxy- and methemoglobin provided to Mr. R. J. Feldman by Dr. M. F. Perutz and of the spin-label provided to us by Dr. J. K. Moffat. The orientation of the spin-label at β93 is determined by minimizing the van der Waals interactions among the atoms of the spin-label and those of the side chain residues of the hemoglobin molecule. The agreement between these two sets of distances obtained by two different techniques is quite good except that of the distance between NO of the spin-label and NH of Asn at β102 of the oxy structure in the strongly immobilized orientation.[2] Since the distances between the paramagnetic NO group and the hydrogen bonds in question are greater than 10 Å, no significant line broadening would be expected if these assignments are correct. Thus, the lack of any observable line broadening of the resonances at −9.4 and −5.5 ppm would appear to provide at least indirect support to the above assignments of these resonances. For the paramagnetic iron in deoxy Hb, τ_c is determined by τ_s which is in the range 10^{-12}–10^{-13} sec (Dwek, 1973). According to Perutz (personal communication), the distance between the iron atom in the α chain to the OH group of Tyr at α42 is ~11 Å and that between the iron atom in the β chain to the OH group of Tyr at α42 is ~16 Å. The effect of the unpaired electrons in the ferrous atoms of deoxy Hb on the nuclear resonance line widths of protons ≥10 Å from the Fe^{2+} atom would be extremely small (i.e., much less than 1 Hz).

In summary, we have observed the resonances of the exchangeable protons involved in the hydrogen bonds present in the subunit interfaces in the hemoglobin molecule. Two of these resonances are among the characteristic properties of the deoxy conformation and one of them is among those of the oxy conformation. These resonances not only can be used to identify the structural characteristics of the subunit interface of a hemoglobin molecule under certain specific experimental conditions but also allow us to monitor the conformational transition of the subunits of normal and abnormal human hemoglobins during the ligand binding process. *It should be emphasized that in understanding the molecular mechanism for the cooperative oxygenation of hemoglobin it is essential to monitor the conformational changes of different regions of the molecule. One cannot conclude with confidence that the hemoglobin molecule is in the deoxy or oxy quaternary structure or a structure in between these two by just observing the characteristic properties of one or two spectroscopic probes in the molecule. The appearance or disappearance of a specific spectroscopic probe does not necessarily imply that the entire hemoglobin molecule has been converted to one of the two quaternary structures (i.e., the formation or breaking of all those constraining "salt bridges"). This is especially true for studies of modified hemoglobins.* An important aim of our hemoglobin research is to assign as many proton resonances to specific amino acid residues and to monitor their changes during the ligand binding process. Thus, the observation and characterization of some of the exchangeable protons have extended the horizon of high-resolution proton NMR spectroscopy to investigate the structure-function relationships of hemoglobins in aqueous solution.

[2] According to Dr. Perutz (personal communication), the distance of 19.6 Å between the N atom of NO group of the spin-label and the N atom of the amide group in the side chain of Asn at β102 in the strongly immobilized conformation provided by the computer is incorrect because it would have put the spin-label into a wrong orientation in the hemoglobin molecule. If the coordinates of the strongly immobilized orientation of the spin-label attached to β93 were determined at a higher resolution by X-ray diffraction, this discrepancy may be resolved.

Added in Proof

Our most recent ^1H NMR results on Hb Philly (C1β35 Tyr→Phe) and on pH titration studies of Hb A suggest that the −8.3-ppm resonance is associated with the β35 tyrosine in the $\alpha_1\beta_1$ subunit interface (Pifat, Fung, and Ho, unpublished results).

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