

# A PROTON NUCLEAR MAGNETIC RESONANCE INVESTIGATION OF PROXIMAL HISTIDYL RESIDUES IN HUMAN NORMAL AND ABNORMAL HEMOGLOBINS

## A Probe for the Heme Pocket

SEIZO TAKAHASHI, ALLISON K.-L. C. LIN, AND CHIEN HO

*Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213*

**ABSTRACT** Proton nuclear magnetic resonance spectroscopy at 250 MHz has been used to investigate the conformations of proximal histidyl residues of human normal adult hemoglobin, hemoglobin Kempsey [ $\beta 99(\text{G1}) \text{ Asp} \rightarrow \text{Asn}$ ], hemoglobin Osler [ $\beta 145(\text{HC2}) \text{ Tyr} \rightarrow \text{Asp}$ ], and hemoglobin McKees Rocks [ $\beta 145(\text{HC2}) \text{ Tyr} \rightarrow \text{Term}$ ] around neutral pH in  $\text{H}_2\text{O}$  at  $27^\circ\text{C}$ , all in the deoxy form. Two resonances that occur between 58 and 76 ppm downfield from the water proton signal have been assigned to the hyperfine shifted proximal histidyl NH-exchangeable protons of the  $\alpha$ - and  $\beta$ -chains of deoxyhemoglobin. These two resonances are sensitive to the quaternary state of hemoglobin, amino acid substitutions in the  $\alpha_1\beta_2$ -subunit interface and in the carboxy-terminal region of the  $\beta$ -chain, and the addition of organic phosphates. The experimental results show that there are differences in the heme pockets among these four hemoglobins studied. The structural and dynamic information derived from the hyperfine shifted proximal histidyl NH-exchangeable proton resonances complement that obtained from the ferrous hyperfine shifted and exchangeable proton resonances of deoxyhemoglobin over the spectral region from 5 to 20 ppm downfield from  $\text{H}_2\text{O}$ . The relationship between these findings and Perutz's stereochemical mechanism for the cooperative oxygenation of hemoglobin is discussed.

### INTRODUCTION

In spite of the considerable efforts that have been devoted to the hemoglobin (Hb)<sup>1</sup> problem during the past two decades, the detailed molecular basis for the cooperative oxygenation of this protein molecule is not fully understood. Perutz, in his stereochemical mechanism, has proposed that there is a movement of the proximal histidyl residue (F8) (which is linked to the heme iron atom) toward the porphyrin plane by  $\sim 0.6 \text{ \AA}$  on ligation and that this movement is responsible for initiating the cooperative oxygenation process of Hb (Perutz, 1970; 1972; 1979). Hence, a direct spectroscopic probe that can monitor the conformation as well as the ligand-induced conformational changes of the proximal histidyl residues in the  $\alpha$ - and

$\beta$ -chains can provide valuable insight into the cooperative oxygenation of Hb.

High-resolution proton nuclear magnetic resonance (NMR) spectroscopy has been used quite successfully to correlate the structure-function relationship in human normal and abnormal hemoglobins in solution. For a recent review, refer to Ho and Russu (1981). Of special interest to the present study are two  $^1\text{H}$  NMR spectral regions: one, from 6 to 20 ppm downfield from the proton resonance of the residual  $\text{H}_2\text{O}$  in  $\text{D}_2\text{O}$ , arises from the hyperfine interactions between the unpaired electrons of the heme iron atom and the protons of the heme group and/or of the neighboring amino acid residues of deoxy Hb (the so-called ferrous hyperfine shifted proton resonances); the other region from 58 to 76 ppm downfield from  $\text{H}_2\text{O}$  is due to the hyperfine shifted NH-exchangeable protons of the proximal histidyl residues of the  $\alpha$ - and  $\beta$ -chains of deoxy Hb (the so-called hyperfine shifted proximal histidyl NH-exchangeable proton resonances). The ferrous hyperfine shifted proton resonances of deoxyhemoglobins have been extensively studied by a number of investigators (see Ho and Russu [1981] and the references cited). La Mar et al. (1977) reported two very low-field exchangeable proton resonances and attributed them to the hyperfine shifted proximal histidyl NH-exchangeable proton resonances. The

Dr. Takahashi's present address is the Department of Chemistry, Japan Women's University, 2-8-1, Mejirodai, Bunkyo-ku, Tokyo 112, Japan. Address all correspondence and reprint requests to Dr. Chien Ho, Department of Biological Sciences, Carnegie-Mellon University, 4400 Fifth Avenue, Pittsburgh, PA 15213.

<sup>1</sup>*Abbreviations used in this paper:* Hb, hemoglobin; Hb A, normal human adult hemoglobin; NMR, nuclear magnetic resonance; IHP, inositol hexaphosphate; 2,3-DPG, 2,3-diphosphoglycerate; bis-Tris, Bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane; DSS, 2,2-dimethyl-2-silpentane-5-sulfate; and EXAFS, extended X-ray absorption fine structure.

specific assignment of the proton resonances at  $\sim 59$  and  $\sim 71$  ppm to the proximal histidyl NH-exchangeable proton resonances of the  $\alpha$ - and  $\beta$ -chains of human normal adult deoxyhemoglobin (Hb A) was made by this laboratory (Takahashi et al., 1980). This problem was also investigated by La Mar et al. (1980) and they confirmed our assignment. These two proton resonances could, in principle, provide information about the conformations of the heme pockets and the ligand-induced conformational changes in the heme pockets. In addition, the  $^1\text{H}$  NMR spectra of Hb solutions in  $\text{H}_2\text{O}$  show several proton resonances that are between 5 and 10 ppm downfield from  $\text{H}_2\text{O}$ , and arise from the exchangeable hydrogen atoms in the protein molecule (Patel et al., 1970; Ho et al., 1973; Breen et al., 1974; Fung and Ho, 1975). These resonances vanish when the Hb samples are in  $\text{D}_2\text{O}$  or when the proton resonance of  $\text{H}_2\text{O}$  is irradiated by a second radiofrequency pulse in a double resonance experiment. The exchangeable proton resonances that are relevant to the present work are at 9.4 and 6.4 ppm downfield from  $\text{H}_2\text{O}$  in the spectrum of deoxy Hb A and at 5.8 ppm in the spectrum of oxy Hb A. The resonances at 9.4 and 6.4 ppm have been shown to be specific probes for the deoxy structure (Ogawa et al., 1972; Mayer et al., 1973; Ho et al., 1973; Breen et al., 1974; Fung and Ho, 1975). The resonance at 9.4 ppm has been assigned to the intersubunit hydrogen bond between  $\alpha 42(\text{C7})$  Tyr and  $\beta 99(\text{G1})$  Asp and is thus an excellent probe for the deoxy quaternary structure of Hb A (Fung and Ho, 1975). The resonance at 6.4 ppm has tentatively been assigned to the intrasubunit hydrogen bond between  $\beta 145(\text{HC2})$  Tyr and  $\beta 98(\text{FG5})$  Val and is a deoxy tertiary structural probe (Viggiano et al., 1978). The resonance at 5.8 ppm has been assigned to the intersubunit hydrogen bond between  $\alpha 94(\text{G1})$  Asp and  $\beta 102(\text{G4})$  Asn and is a good probe for the oxy quaternary structure of Hb A. The main difference between the exchangeable proton resonances over the spectral region from 5 to 10 ppm and the low-field proximal histidyl proton resonances is that the former are not subjected to a paramagnetic shifted mechanism because the NH protons involved are too far from the heme iron atoms (Fung and Ho, 1975) while the latter are subjected to the influence of the paramagnetic iron atoms in the heme groups of deoxyhemoglobin.

In the present work, we have selected three mutant hemoglobins, i.e., Hb Kempsey [ $\beta 99(\text{G1})$  Asp  $\rightarrow$  Asn], Hb Osler [ $\beta 145(\text{HC2})$  Tyr  $\rightarrow$  Asp], and Hb McKees Rocks [ $\beta 145(\text{HC2})$  Tyr  $\rightarrow$  Term], in addition to Hb A for our investigation. Hb Kempsey, Hb Osler, and Hb McKees Rocks all have a very high affinity and low cooperativity in  $\text{O}_2$  binding (Reed et al., 1968; Bunn et al., 1974; Charache et al., 1975; Arnone et al., 1976; Winslow et al., 1976). Hb Kempsey has a mutation located in the  $\alpha_1\beta_2$ -subunit interface (Reed et al., 1968). Present experimental evidence strongly suggests that deoxy Hb Kempsey has an oxylike quaternary structure and that the addition of inositol hexaphosphate (IHP) to deoxy Hb Kempsey can

convert this Hb to a deoxylike quaternary structure (Perutz et al., 1974). It should be mentioned that we have observed an exchangeable proton resonance at  $\sim 5.6$  ppm from  $\text{H}_2\text{O}$  (an indicator of oxylike quaternary structure) in deoxy Hb Kempsey in the absence of inositol hexaphosphate (IHP; unpublished results). This mutant Hb can, thus, provide an excellent test of the sensitivity of the hyperfine shifted proximal histidyl NH-exchangeable proton resonances as probes for (a) the conformations and the ligand-induced conformational changes of the heme pockets; (b) the effect of allosteric effectors, such as organic phosphates, on the heme pockets; and (c) the effect of the amino acid substitution in the  $\alpha_1\beta_2$ -subunit interface on the heme pockets of the  $\alpha$ - and  $\beta$ -chains. According to the atomic model of hemoglobin (Perutz, 1970; Fermi, 1975; Baldwin and Chothia, 1979), the penultimate tyrosine residues (HC2) of both the  $\alpha$ - and  $\beta$ -chains of deoxy Hb A fit nicely in the space created by the F and H helices and form hydrogen bonds with the valine residues (FG5). On oxygenation, the penultimate tyrosyl residues are expelled from the heme pockets (Perutz, 1970). Hence, Hb Osler and Hb McKees Rocks provide good models to investigate the effect of the amino acid substitution at the carboxy-terminal region of the  $\beta$ -chain on the heme pocket.

It should be mentioned that both the ferrous hyperfine shifted and exchangeable proton resonances of Hb A, Hb Kempsey, Hb Osler, and Hb McKees Rocks have been investigated by this laboratory (Davis et al., 1971; Ho et al., 1973; Lindstrom et al., 1973; Perutz et al., 1974; Fung and Ho, 1975; Viggiano et al., 1978; and Viggiano and Ho, 1979). These published results can provide a direct comparison with the information provided by the proximal histidyl NH-exchangeable proton resonances of deoxyhemoglobins.

## MATERIALS AND METHODS

### Materials

Hb A was prepared by the standard procedure used in this laboratory from erythrocytes obtained from the local Blood Bank (Lindstrom and Ho, 1972). The purification procedure for Hb Kempsey has been described by Davis et al. (1971) and the purification procedures for Hb Osler and Hb McKees Rocks have been described previously (Viggiano et al. [1978] and the references cited). Organic phosphates were removed from Hb according to the procedure of Berman et al. (1971). Bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane (bis-Tris) was purchased from Aldrich (Milwaukee, WI). 2,3-Diphosphoglycerate (2,3-DPG) was purchased from Calbiochem-Behring Corp. (San Diego, CA) as the pentacyclohexyl-ammonium salt, and converted to the acid form by exchange on Bio-Rad AG50W-X8 resin (Bio-Rad Laboratories, Richmond, CA) and then neutralized to pH 7 with NaOH. IHP was obtained from Sigma Chemical Co. (St. Louis, MO). Hb samples were adjusted to the appropriate pH values by adding 1 M bis-Tris buffer and the final concentration of bis-Tris buffer was 0.1 M. The deoxygenation of the Hb samples was carried out by the procedure used previously in our laboratory (Lindstrom and Ho, 1972). Sodium dithionite which was purchased from Vine Chemicals, Ltd. (Cheshire, England) was added to some of the sealed Hb NMR tubes by syringe to convert the residual ferrihemoglobin to the ferrous state.

## Methods

The  $^1\text{H}$  NMR spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer, interfaced to a Sigma-5 computer, using the NMR correlation technique of Dadok and Sprecher (1974). Proton chemical shifts were referenced to the solvent  $\text{H}_2\text{O}$  signal, which was 4.83 ppm downfield from the proton signal of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, Stohler Isotope Chemicals, Rutherford, NJ) at  $27^\circ\text{C}$ , the ambient probe temperature. The chemical shift scale is presently defined as positive in the low-field direction with respect to the reference signal, i.e., water proton.<sup>2</sup> The spectrometer settings used to obtain the hyperfine shifted proximal histidyl NH-exchangeable proton resonances have been described in a previous report (Takahashi et al., 1980). The accuracy of the chemical-shift measurement depends on the line width of the resonances, but it is better than  $\pm 0.3$  ppm. The accuracy of the line-width measurement is about  $\pm 75$  Hz.

## RESULTS AND DISCUSSION

### Effect of Organic Phosphates on Hb A

Within the experimental accuracy, both the chemical shift and the line width of the hyperfine shifted proximal histidyl NH-exchangeable proton resonance of the  $\alpha$ -chain of deoxy Hb A at 58.6 ppm are unaffected by either 2,3-DPG or IHP (Fig. 1 and Table I). On the other hand, the chemical shift of the proximal histidyl NH-exchangeable proton resonance of the  $\beta$ -chain of deoxy Hb A at 71.0 ppm is shifted upfield by  $\sim 0.5$  ppm upon the addition of IHP (no observable shift by 2,3-DPG is detected as shown in Fig. 1 and Table I). The line width of the resonance at  $\sim 59$  ppm is  $\sim 635$  Hz and that of the resonance at  $\sim 71$  ppm is about 685 Hz. The effect of IHP on the resonance at  $\sim 71$  ppm is analogous to that observed on the ferrous hyperfine shifted proton resonance of the  $\beta$ -chain of deoxy Hb A at  $\sim 17.5$  ppm downfield from HDO. In the latter case, the addition of either 2,3-DPG or IHP shifts the hyperfine shifted proton resonance at  $\sim 17.5$  to  $\sim 18$  ppm (Ho et al., 1973; Wiechelman et al., 1976). The exchangeable proton resonances at 9.4 and 6.4 ppm of deoxy Hb A are not affected by the addition of organic phosphates (Fung and Ho, 1975).

### Hb Kempsey

Fig. 2 shows the proximal histidyl NH-exchangeable proton resonances of deoxy Hb Kempsey with and without IHP. In the absence of IHP, they occur at 64.3 and 72.0 ppm for the  $\alpha$ - and  $\beta$ -chains, respectively, and the line width of the  $\alpha$ -chain resonance is about twice that of the  $\beta$ -chain (1,248 vs. 593 Hz as shown in Table I). These two resonances are very different from those of deoxy Hb A. Upon binding of IHP, both the  $\alpha$ - and  $\beta$ -proximal histidyl

<sup>2</sup>In conforming with the recommendation for the presentation of NMR data for publication in chemical journals proposed by IUPAC (1974), we have adopted the IUPAC convention; namely, the chemical-shift scale is defined as positive in the low-field (or high-frequency) direction rather than as negative in the low-field direction. This convention is different from that used by this laboratory prior to 1980. Hence, this change in the sign of the chemical-shift scale should be noted when referring to earlier publications reported by this laboratory.

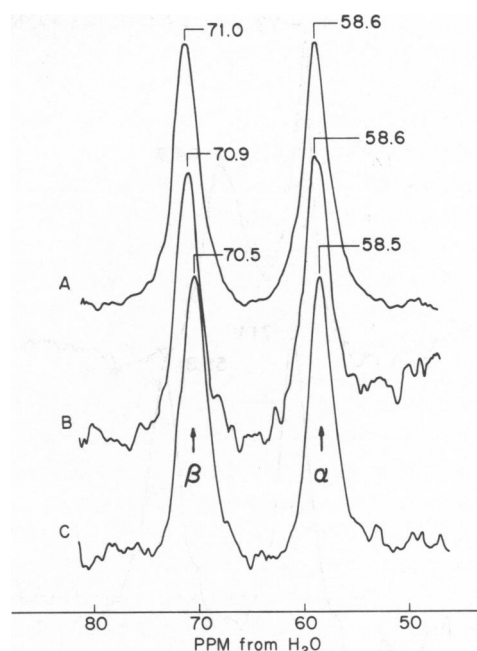


FIGURE 1 Effects of 2,3-DPG and IHP on hyperfine shifted proximal histidyl NH-exchangeable proton resonances of  $\sim 12\%$  deoxy Hb A in  $\text{H}_2\text{O}$  at pH 6.4 and  $27^\circ\text{C}$ : A, deoxy Hb A in 0.1 M bis-Tris; B, deoxy Hb A in 0.1 M bis-Tris plus 0.06 M 2,3-DPG; and C, deoxy Hb A in 0.1 M bis-Tris plus 0.06 M IHP.

NH-exchangeable resonances approach those of deoxy Hb A (see Fig. 1 and Table I). However, the chemical shifts of these two resonances found in deoxy Hb Kempsey in the presence of IHP (59.8 and 71.1 ppm) are not exactly the same as those of deoxy Hb A as shown in Figs. 1 and 2. The

TABLE I  
PROTON CHEMICAL SHIFTS AND LINE WIDTHS OF HYPERFINE SHIFTED PROXIMAL HISTIDYL NH-EXCHANGEABLE PROTON RESONANCES OF DEOXY-HEMOGLOBINS IN 0.1-M BIS-TRIS BUFFER AT  $27^\circ\text{C}$

Hemoglobin	Chemical shift		Line width	
	$\alpha$ -chain	$\beta$ -chain	$\alpha$ -chain	$\beta$ -chain
	ppm	ppm	Hz	Hz
Hb A at pH 6.4	58.6	71.0	641	676
Hb A + 0.06 M 2,3-DPG at pH 6.4	58.6	70.9	626	681
Hb A + 0.06 M IHP at pH 6.4	58.5	70.5	639	697
Hb Kempsey at pH 6.4	64.3	72.0	1,248	593
Hb Kempsey + 0.06 M IHP at pH 6.4	59.8	71.1	786	759
Hb Osler at pH 6.8	59.3	75.6	664	1,085
Hb Osler + 0.06 M IHP at pH 6.8	59.0	73.7	590	723
Hb McKees Rocks at pH 6.8	59.1	73.5	546	666
Hb McKees Rocks + 0.06 M IHP at pH 6.8	58.8	71.0	700	819
		73.6		505

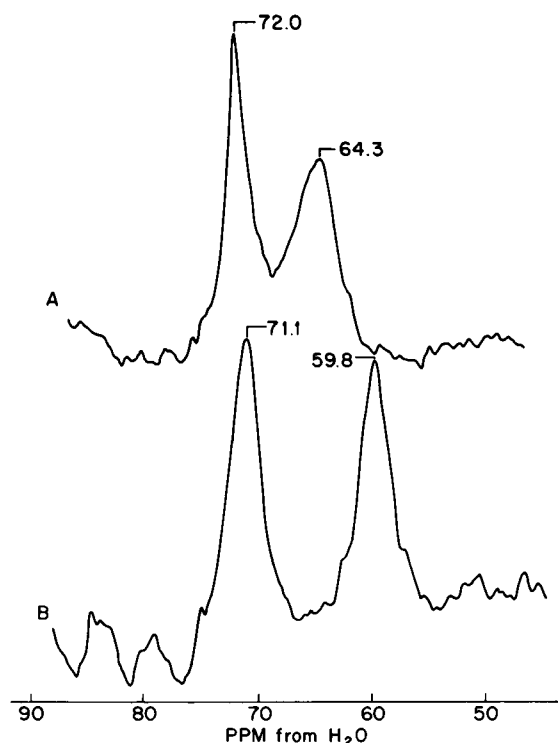


FIGURE 2 Effect of IHP on the 250-MHz hyperfine shifted proximal histidyl NH-exchangeable proton resonances of ~10% Hb Kempsey [ $\beta 99(\text{G1}) \text{ Asp} \rightarrow \text{Asn}$ ] in  $\text{H}_2\text{O}$  at pH 6.4 and  $27^\circ \text{C}$ : *A*, deoxy Hb Kempsey in 0.1 M bis-Tris; and *B*, deoxy Hb Kempsey in 0.1 M bis-Tris plus 0.06 M IHP.

$\alpha$ -chain proximal histidyl NH-exchangeable resonance of deoxy Hb Kempsey shifts from 64.3 to 59.8 ppm and sharpens from 1,248 to 786 Hz, while the  $\beta$ -chain resonance shifts only 1 ppm from 72.0 to 71.1 ppm and broadens from 593 to 759 Hz. These resonances of deoxy Hb Kempsey in the presence of IHP are broader than those of deoxy Hb A under the same conditions (Table I). Again,

the ferrous hyperfine shifted proton resonances over the spectral region from 6 to 20 ppm downfield from HDO of deoxy Hb Kempsey are very different from those of deoxy Hb A in the absence of organic phosphate (Lindstrom et al., 1973; Perutz et al., 1974). The addition of IHP converts the ferrous hyperfine shifted proton resonance pattern of deoxy Hb Kempsey to a pattern similar, but not identical to that of deoxy Hb A. It should also be pointed out that the spectral change is more drastic in the resonance which is due to the proximal histidyl residue of the  $\alpha$ -chain even though the amino acid substitution in Hb Kempsey is at  $\beta 99$ . This confirms one of our earlier conclusions that the amino acid substitution in the  $\alpha_1\beta_2$ -subunit contact region can affect the heme pockets of both the  $\alpha$ - and  $\beta$ -chains (Davis et al., 1971). The exchangeable proton resonance at 9.4 ppm is missing in the spectrum of deoxy Hb Kempsey (Fung and Ho, 1975). This is to be expected because the amino acid substitution of this mutant Hb is at the  $\beta 99(\text{G1})$  aspartic acid and the normal intersubunit hydrogen bond between  $\alpha 42(\text{C7}) \text{ Tyr}$  and  $\beta 99(\text{G1}) \text{ Asp}$  cannot be formed.

### Hb Osler and Hb McKees Rocks

Fig. 3 *A* shows the spectra of deoxy Hb Osler and deoxy Hb McKees Rocks in the absence of IHP. The proximal histidyl NH-exchangeable resonances of the  $\alpha$ - and  $\beta$ -chains of deoxy Hb Osler are observed at 59.3 and 75.6 ppm downfield from  $\text{H}_2\text{O}$ , respectively, while those of deoxy Hb McKees Rocks are observed at 59.1 and 73.5 ppm. As shown in Figure 3 *A* and Table I, the line width of the  $\beta$ -chain resonance of deoxy Hb Osler is considerably broader (~1,085 Hz for the  $\beta$ -chain and ~664 Hz for the  $\alpha$ -chain) and that of the  $\alpha$ -chain of deoxy Hb McKees Rocks is narrower (~546 Hz for the  $\alpha$ -chain and ~666 Hz for the  $\beta$ -chain) than the corresponding resonances of deoxy Hb A. Hence, the resonances of these two mutant

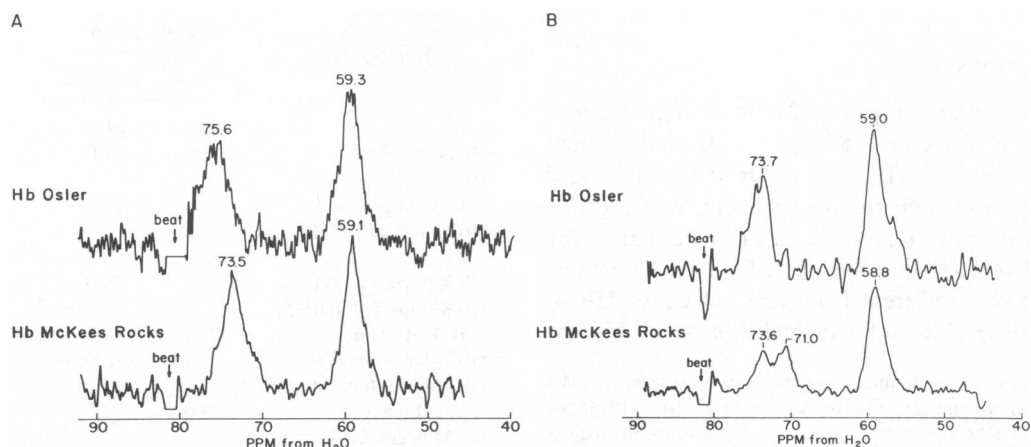


FIGURE 3 Effect of IHP on the 250-MHz hyperfine shifted proximal histidyl NH-exchangeable proton resonances of ~10% deoxy Hb Osler [ $\beta 145(\text{HC2}) \text{ Tyr} \rightarrow \text{Asp}$ ] and ~10% deoxy Hb McKees Rocks [ $\beta 145(\text{HC2}) \text{ Tyr} \rightarrow \text{Term}$ ]: *A*, deoxy Hb Osler and deoxy Hb McKees Rocks in 0.1 M bis-Tris; and *B*, deoxy Hb Osler and deoxy Hb McKees Rocks in 0.1 M bis-Tris plus 0.06 M IHP. The beat appearing at ~80 ppm in the spectra is generated by the 20-kHz time-sharing operation of the spectrometer and is not a signal.

hemoglobins are very different from those of Hb A under the same conditions.

In the presence of IHP, the  $\alpha$ - and  $\beta$ -chain resonances of the proximal histidyl NH-exchangeable protons are found at 58.9 and 71.3 ppm for deoxy Hb Des-His (results not shown) and 59.0 and 73.7 ppm for deoxy Hb Osler (Fig. 3 B). The line width of the  $\beta$ -chain resonance of deoxy Hb Osler ( $\sim 723$  Hz) is appreciably broader than that of the  $\beta$ -chain resonance for deoxy Hb Des-His ( $\sim 594$  Hz). The line width of the  $\alpha$ -chains for deoxy Hb Des-His and deoxy Hb Osler are  $\sim 523$  and  $\sim 590$ , respectively. The spectral change of deoxy Hb McKees Rocks upon binding of IHP is indeed very remarkable as shown in Fig. 3 B. The  $\beta$ -chain proximal histidyl NH-exchangeable proton resonance clearly splits into two peaks at 71.0 and 73.6 ppm, and their intensity ratio is nearly 1:1. These results suggest that there are two conformations of the proximal histidyl residues in the  $\beta$  chains of deoxy Hb McKees Rocks in the presence of IHP. The chemical shift at 71.0 ppm is almost the same as that of deoxy Hb A and the chemical shift at 73.6 ppm is close to that of deoxy Hb McKees Rocks in the presence of IHP. The chemical shift of the  $\alpha$ -chain proximal histidyl NH-exchangeable proton resonance for deoxy Hb McKees Rocks is not much affected by the addition of IHP as shown in Fig. 3, and is essentially the same as the corresponding resonance in deoxy Hb A. The  $\alpha$ -chain proximal histidyl NH resonance of deoxy Hb McKees Rocks in the presence of IHP is somewhat broadened compared with the line width of the same molecule without IHP or the line width of deoxy Hb A (700 vs. 546 or 635 Hz for Hb A). Due to the overlapping of the two resonances of the  $\beta$ -chain, it is difficult to estimate their individual line width. However, it appears that the line width at 71.0 ppm ( $\sim 819$  Hz) is broader than that at 73.6 ppm ( $\sim 505$  Hz).

Again, there are similarities in the spectral features between the ferrous hyperfine shifted proton resonances and the hyperfine shifted proximal histidyl NH-exchangeable proton resonances of deoxy Hb Osler and deoxy Hb McKees Rocks. In the absence of IHP, the ferrous hyperfine shifted proton resonances of these two hemoglobins are very different from those of deoxy Hb A (Viggiano et al., 1978). Their respective resonance patterns are similar to that of deoxy Hb Kempsey in the absence of IHP (Lindstrom et al., 1973; Perutz et al., 1974). Upon the addition of an excess amount of IHP, the ferrous hyperfine shifted proton resonance pattern of these two hemoglobins is converted to one similar to (but not identical to) that of deoxy Hb A. The IHP-induced spectral changes in the proximal histidyl NH-exchangeable proton resonances of deoxy Hb Osler are analogous to those observed in the ferrous hyperfine shifted proton resonances. The spectral changes induced by IHP in deoxy Hb McKees Rocks are very different from those in the rest of the hemoglobins investigated.

The normally occurring exchangeable proton resonances

at 9.4 and 6.4 ppm downfield from  $\text{H}_2\text{O}$  are missing in the spectra of deoxy Hb Osler and deoxy Hb McKees Rocks in 0.1 M bis-Tris at pH 7 (Viggiano et al., 1978). Since the mutation sites in these two mutant hemoglobins are at the penultimate tyrosyl residues, it is to be expected that the usual intrasubunit hydrogen bond between  $\beta 145(\text{HC2})$  Tyr and  $\beta 98(\text{FG5})$  Val cannot be formed. Hence, the disappearance of the resonance at 6.4 ppm is to be expected. Several pieces of experimental evidence suggest that both deoxy Hb Osler and deoxy Hb McKees Rocks in 0.1 M bis-Tris at neutral pH exist in an oxylike quaternary structure and that upon addition of excess IHP, these two hemoglobins are converted to a deoxylike quaternary structure. These predictions are supported by the exchangeable proton resonance at 9.4 ppm. In the absence of IHP, this resonance does not appear in these two deoxyhemoglobins. On the other hand, an exchangeable proton resonance at  $\sim 9.3$  ppm is observed upon the addition of IHP to deoxy Hb Osler and deoxy Hb McKees Rocks (Viggiano et al., 1978).

The  $^1\text{H}$  chemical shifts and the line widths of the proximal histidyl NH-exchangeable proton resonances as well as their variations with IHP show clearly that there are detailed differences in the heme pockets among the four hemoglobins examined in the deoxy state. It appears that for the Hb mutants with high  $\text{O}_2$  affinity and low cooperativity, such as Hb Kempsey, Hb Osler, and Hb McKees Rocks, the proximal histidyl NH-exchangeable proton resonances of both the  $\alpha$ - and  $\beta$ -chains are shifted downfield compared with the corresponding ones in Hb A. The addition of IHP to these mutant hemoglobins produces upfield shifts, to a pattern similar to that of Hb A, with the exception of Hb McKees Rocks. Nagai et al. (1982) have carried out a similar  $^1\text{H}$  NMR investigation on Hb Kempsey, Hb NES-Des-Arg( $\alpha 141$ ), Hb Des-His( $\beta 146$ ), Hb Des-His( $\beta 146$ )-Des-Arg( $\alpha 141$ ), and Hb Des-Arg( $\alpha 141$ ) in the deoxy state. They have attempted to correlate the chemical shifts of the hyperfine shifted proximal histidyl exchangeable proton resonances to the strain of the iron-proximal histidine bond in Perutz's stereochemical model of hemoglobin and to interpret the change in the chemical shifts of these resonances to a change in the covalency of this bond. The following discussion will show that such a simple relationship does not exist.

First, it should also be noted that the results obtained from the extended x-ray absorption fine structure (EXAFS) determination of the iron-nitrogen bond distances in deoxy- and oxy-hemoglobins show that any differences of iron to porphyrinato-nitrogen bond lengths in the oxy and deoxy states are  $< 0.01$  Å (Eisenberger et al., 1976; 1978). These EXAFS experiments were performed on deoxy Hb A, oxy Hb A, and deoxy Hb Kempsey. Eisenberger et al. (1976; 1978) reported that the iron-nitrogen bond distances in these two hemoglobins are equal, within an accuracy of  $\pm 0.02$  Å. Based on the EXAFS results, they concluded that the strain energy of

the iron-nitrogen bond in Hb A introduced by the transition from the oxy to the deoxy quaternary structure is less than the thermal energy ( $\sim 0.6$  kcal). They further suggested that the doming and buckling of the porphyrin ring may play a more important role in the mechanism for the cooperative oxygenation than the movement of the proximal histidyl residue toward the porphyrin plane. Second, Nagai and Kitagawa (1980) have analyzed the Fe(II)—N<sub>i</sub>(HisF8) stretching frequency in the resonance Raman spectra in order to estimate the strain energy imposed on the iron-proximal histidyl bond. According to this approach, the square root of the stretching frequency of the iron-proximal histidyl bond in the resonance Raman spectra should be a direct measure of the strain in this bond (Nagai and Kitagawa, 1980; Nagai et al., 1980). This forms the basis for using the chemical shifts of the proximal histidyl NH-exchangeable proton resonances as indicators of the iron-proximal histidyl bond in hemoglobin by Nagai et al.<sup>3</sup> If this relationship holds, one would expect that the resonance Raman stretching frequency of the iron-proximal histidyl bond should vary with the chemical shift of the proximal histidyl NH-exchangeable proton resonance in some consistent manner. On plotting the published data (Kincaid et al., 1979; La Mar et al., 1980; Nagai and Kitagawa, 1980; Nagai et al., 1980 and 1982; the present results) in the form of observed stretching frequencies of Raman scattering vs. chemical shifts of proximal histidyl NH proton resonances, we have found that there is a poor correlation between these two sets of data (results not shown). This is not surprising because Kincaid et al. (1979) reported that the low-frequency Raman spectrum in deoxy Hb Kempsey is not affected by the addition of IHP and is the same as that in deoxy Hb A within the experimental error ( $\pm 2$  cm<sup>-1</sup>). On the other hand, the proximal histidyl NH-exchangeable proton resonances of deoxy Hb Kempsey are very sensitive to the presence of IHP (Fig. 2). It should also be noted that Stein et al. (1980) proposed an alternative interpretation of the frequency shifts of the Fe(II)—N<sub>i</sub>(HisF8) stretching mode that may involve the hydrogen bonding of the proximal histidyl NH proton to a peptide backbone. Using the appropriate hemoglobins with mutations in the  $\alpha_1\beta_2$ -subunit interface, the heme pocket, and the carboxyl-terminal region of the  $\beta$ -chain, we have shown that there are complex variations in the details of the heme pockets in these mutant hemoglobins. Hence, no simple conclusion on the strain of the iron-proximal histidine bond and the covalency of this bond can be drawn from the proximal histidyl NH-exchangeable proton resonances alone. For example, according to the recent x-ray crystallographic results (Perutz, 1979), the displacement of N<sub>i</sub> of the proximal histidine (F8) from the porphyrin is 2.6 Å in the  $\alpha$ - and 2.8 Å in the  $\beta$ -chains of deoxy Hb A, so that one would expect that there is a greater strain in the heme iron-proximal histidyl bond in the  $\beta$ -chain than in the  $\alpha$ -chain of deoxy Hb A according to Perutz's mechanism.

On the other hand, the resonance Raman results show that there is a greater strain in the  $\alpha$ -chain of Hb A relative to that in the  $\beta$ -chain (Nagai and Kitagawa, 1980). Yet, the observed proximal histidyl NH-exchangeable proton resonance for the  $\beta$ -chain is shifted  $\sim 12$  ppm further downfield than that for the  $\alpha$ -chain (Fig. 1). This is contrary to the relation between the NH hyperfine shift and the strain in the iron-histidine bond proposed by Nagai et al. (1982). Hence, further research is clearly needed to gain insight into this important region of the Hb molecule.

The present results show that there are large differences in the line widths of the proximal histidyl NH-exchangeable proton resonances among the four hemoglobins studied and that there is a complex variation of the line widths of these two resonances upon the addition of IHP to these hemoglobin solutions (Table I). The line width of these resonances depends upon a number of factors, such as the exchange rates between the NH groups of these proximal histidyl residues and the solvent H<sub>2</sub>O, the detailed conformations of the heme pockets, the detailed electronic properties of the heme iron atoms, etc. Even though the proximal histidyl residue of deoxy Hb A is buried deeply inside the heme pocket and is protected by hydrophobic amino acid residues (Fermi, 1975; Baldwin and Chothia, 1979), one would expect that the exchange between the NH of proximal histidine and the solvent H<sub>2</sub>O proton should be slow on the NMR time scale (the separation between the H<sub>2</sub>O proton signal and the NH resonances is  $> 58$  ppm). However, in the mutant hemoglobins with amino acid substitutions in the  $\alpha_1\beta_2$ -subunit interface, the heme pocket, and/or the carboxyterminal region, the exchange rate between the NH of the proximal histidyl residue and the solvent H<sub>2</sub>O could be altered. The ferrous hyperfine shifted proton resonances of Hb Kempsey, Hb Osler, and Hb McKees Rocks differ significantly from those of Hb A. This clearly suggests that the electronic structure of the heme groups in these three hemoglobins is different from that of Hb A. Again, more work is clearly needed to understand the factors affecting the line width of the hyperfine shifted proximal histidyl NH-exchangeable proton resonances of deoxy Hb.

In conclusion, the present results show that the hyperfine shifted proximal histidyl NH-exchangeable proton resonances of deoxy Hb are sensitive to the quaternary structure and can also be used to investigate the details of the conformation and dynamics of the heme pocket in hemoglobin and that they complement nicely the ferrous hyperfine shifted and exchangeable proton resonances over the spectral region from 6 to 20 ppm downfield from H<sub>2</sub>O. Upon oxygenation, these hyperfine shifted proton resonances shift back to their usual diamagnetic positions. We have used the ferrous hyperfine shifted proton resonances to monitor the binding of O<sub>2</sub>, CO, or *n*-butyl isocyanide to the  $\alpha$ - and  $\beta$ -chains of Hb A (Lindstrom et al., 1971; Lindstrom and Ho, 1972; Johnson and Ho, 1974; Viggiano et al., 1979) and the exchangeable proton resonances to

monitor tertiary and quaternary structural changes associated with the cooperative oxygenation of Hb A (Viggiano and Ho, 1979). Similarly, the hyperfine shifted proximal histidyl NH-exchangeable proton resonances can also be used to measure the binding of ligands to the  $\alpha$ - and  $\beta$ -chains of Hb A (Takahashi, Viggiano, and Ho, unpublished results). Preliminary results suggest that there are advantages in using the two proximal histidyl NH-exchangeable proton resonances to measure the amount of O<sub>2</sub> bound to the  $\alpha$ - and  $\beta$ -chains of Hb A over using the ferrous hyperfine shifted proton resonances. These results will be published elsewhere.

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