High-Resolution Proton Nuclear Magnetic Resonance Studies of Sickle Cell Hemoglobin†

Leslie W.-M. Fung, Kuo-Ling Chao Lin, and Chien Ho*

ABSTRACT: High-resolution proton nuclear magnetic resonance spectroscopy at 250 MHz has been used to investigate sickle cell hemoglobin. The hyperfine shifted, the ring-current shifted, and the exchangeable proton resonances suggest that the heme environment and the subunit interfaces of the sickle cell hemoglobin molecule are normal. These results suggest that the low oxygen affinity in sickle cell blood is not due to conformational alterations in the heme environment or the subunit interfaces. The C-2 proton resonances of certain histidyl residues can serve as structural probes for the surface conformation of the hemoglobin molecule. Several sharp resonances in sickle cell hemoglobin are shifted upfield from their positions in normal adult hemoglobin. These upfield shifts, which are observed in both oxy and deoxy forms of the molecule under various experimental conditions, suggest that some of the surface residues of sickle cell hemoglobin are altered and they may be in a more hydrophobic environment as compared with that of normal human adult hemoglobin. These differences in surface conformation are pH and ionic strength specific. In particular, upon the addition of organic phosphates to normal and sickle cell hemoglobin samples, the differences in their aromatic proton resonances diminish. These changes in the surface conformation may, in part, be responsible for the abnormal properties of sickle cell hemoglobin.

Due to a mutation in the globin gene, the glutamic acid residue at $\beta 6$ of normal human adult hemoglobin (Hb A)[1] is replaced by a valine residue in sickle cell hemoglobin (Hb S) (Ingram, 1957). Under certain experimental conditions, the sickle cell hemoglobin molecules polymerize either within the sickle cell erythrocytes (Bessis et al., 1958; Stetson, 1966; Döbler and Bertles, 1968) or in concentrated deoxy Hb S solution (Harris, 1950; Bertles et al., 1970). Blood samples from sickle cell anemia patients have been found to exhibit an abnormally low oxygen affinity (Becklake et al., 1955; Fraimow et al., 1958; Bromberg and Jensen, 1967), which is believed to be closely associated with the concentration of Hb S but not with the 2,3-diphosphoglycerate (DPG) level (Seakins et al., 1973). Therefore, it is essential to investigate whether this decrease in oxygen affinity is due to an alteration in the mechanism of cooperative oxygen binding or whether it is due to increased intermolecular interactions leading to the polymerization of deoxyhemoglobin molecules at high Hb S concentration. The early oxygen equilibrium studies of Allen and Wyman (1954) have shown identical properties of Hb S and Hb A. However, a more fundamental and direct approach is to examine the structure of Hb S molecule in solution with emphasis on the functionally active regions, such as the heme environment and the subunit interfaces, and on the surface of the molecule.

High-resolution proton nuclear magnetic resonance (NMR) spectroscopy has been recognized as a unique technique to investigate the structural and functional properties of human normal and abnormal hemoglobins in solution (Davis et al., 1971; Ogawa and Shulman, 1972; Lindstrom and Ho, 1972; Ho et al., 1973; Johnson and Ho, 1974; Wiechelman et al., 1974; Fung and Ho, 1975). The hyperfine shifted proton resonances of deoxyhemoglobins are very sensitive to tertiary and quaternary structural changes in the region of the heme pockets of the α and β chains and can also be used to investigate the ligand binding properties of the α and β chains in intact Hb tetramers (Davis et al., 1969, 1971; Lindstrom et al., 1971, 1972a; Lindstrom and Ho, 1972; Ho et al., 1973; Perutz et al., 1974; Johnson and Ho, 1974). In an oxy- or carbonmonoxyhemoglobin (HbO_2 or HbCO) molecule, the ring-current shifted proton resonances are very sensitive to the tertiary structure around the heme pockets (Lindstrom et al., 1972b; Ho et al., 1973; Lindstrom and Ho, 1973). The lowfield exchangeable proton resonances of the hemoglobin molecule in H_2O have been shown to provide detailed information about the conformations of the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces. These resonances describe several important hydrogen bonds which anchor the subunits and are characteristic of the quaternary structures of Hb A (Fung and Ho, 1975).

In a normal human adult hemoglobin molecule, there are 38 histidyl residues (Dayhoff, 1972). According to Perutz's atomic models of hemoglobin and the studies of the titratable histidines in the hemoglobin molecule (Tanford and Nozaki, 1966; DeBruin and Van Os, 1968; Janssen et al., 1972), at least 20 of these histidines are located on the surface of the molecule. Hence, these histidine residues can serve as structural probes for the surface conformation of the hemoglobin molecule. In addition, the C-2 proton resonances of these freely rotating surface histidines which usually occur in the aromatic spectral region between −3 and

† From the Department of Biophysics and Microbiology and the Department of Biochemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received March 17, 1975.* Supported by research grants from the National Institutes of Health (HL-10383, HL-15165, and RR-00292). The material was presented in part at the 1st National Symposium on Sickle Cell Disease, June 27–29, 1974, Washington, D.C., at the 3rd International Conference on Red Cell Metabolism and Function, Oct. 16–19, 1974, Ann Arbor, Mich., and at the 19th National Meeting of the Biophysical Society, Feb. 18–21, 1975, Philadelphia, Pa.

[1] Abbreviations used are: Hb A, normal human adult hemoglobin; Hb S, sickle cell hemoglobin; Hb F, fetal homoglobin; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; bis-tris, 2,3-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol.

−4 ppm from H_2O are distinctive from the majority of aromatic proton resonances of the molecule (Greenfield and Williams, 1972; Kilmartin et al., 1973). Thus, these C-2 proton resonances of histidines in the hemoglobin molecule can be used to monitor its surface conformation.

We have applied high-resolution proton NMR techniques to investigate the structure of different regions of the Hb S molecule in solution. In this communication, we wish to present the results obtained from our studies of the hyperfine shifted, ring-current shifted, exchangeable, and aromatic proton resonances and to discuss the implications of these results in respect to the structure–function relationship in sickle cell hemoglobin.

Experimental Procedure

Materials. Sickle cell and normal adult human blood samples were washed and lysed by standard procedures (Drabkin, 1946) to obtain hemolysates of Hb S and Hb A, respectively. In case of heterozygous sickle cell blood samples the hemolysates were passed through a demountable DEAE-Sephadex (A-50, Pharmacia) column with 0.05 *M* Tris buffer at pH 8.1 and at room temperature. After the elution of Hb A_2 (a fast moving component) and when Hb S was in the lower half of the column, the column was separated and Hb S was eluted with 0.1 *M* NaCl in 0.01 *M* Tris buffer at pH 7.4 (H. F. Bunn, personal communication). Homozygous hemolysates were purified by a Bio-Rex 70 column with a 0.05 *M* sodium phosphate buffer at pH 7.2 and 4°. After the elution of fetal hemoglobin (Hb F), Hb S was eluted with 0.1 *M* sodium phosphate buffer at pH 7.2 (J. V. Kilmartin, personal communication). The purity of Hb S, obtained from homozygous or heterozygous hemolysate samples, was checked by cellulose acetate strip electrophoresis. All hemoglobin samples were freed from phosphates by passing through a column of Sephadex G-25 (Pharmacia) equilibrated with 0.01 *M* Tris-HCl buffer containing 0.1 *M* NaCl at pH 7.5 (Berman et al., 1971). The Hb samples were then exchanged repeatedly with either deuterium oxide (Merck, Sharp, and Dohme of Canada, Ltd) or deionized water through an ultrafiltration membrane (UM-20E, Amicon) to remove the remaining inorganic ions and the samples were concentrated to about 15 g %. Either 2,3-bis(hydroxyethyl)-2,2′,2″-nitrilotrimethanol (bis-tris) (Aldrich) or potassium phosphate buffer at a specific pH was added to the samples. A stock solution of sodium chloride, sodium inositol hexaphosphate (IHP) (Sigma), or 2,3-diphosphoglycerate (DPG) (Calbiochem) was added when required. Carbonmonoxy, oxy, and deoxy (non-gelled) samples for NMR studies were prepared by the usual methods used in this laboratory (Lindstrom and Ho, 1972). Gelled deoxyhemoglobin was transferred to ultracentrifuge tubes in a nitrogen atomsphere and insulated from air by layering paraffin oil onto the surface of the gelled sample (Bertles et al., 1970). After ultracentrifugation for 1 hr at 35,000 rpm in a Beckman Model L2-65 ultracentrifuge using a Beckman Model SW36 swing bucket rotor at 25°, the supernatant was then transferred to a standard 5-mm NMR sample tube under a nitrogen atmosphere. All pH values reported were taken on the HbCO samples and were direct readings of a Radiometer Model 4 pH meter equipped with a Beckman 39030 frit junction combination electrode.

Methods. High-resolution ^1H NMR spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer (Dadok et al., 1970), interfaced with a Sigma 5 computer and the signal-to-noise ratio was enhanced by the NMR correlation technique (Dadok and Sprecher, 1974). The frequency sweep field was set for 6000 Hz with 0.8-sec sweep time for the region of hyperfine shifted and exchangeable proton resonances and 1500 Hz with 3-sec sweep time for the region of aromatic and ring-current shifted proton resonances. The sensitivity and resolution of the ^1H NMR spectra were improved by the accumulation of multiple scans: 50–100 scans were needed for the aromatic proton resonances, 200 scans for the ring-current shifted resonances, and several hundred to thousand scans for the hyperfine shifted and the exchangeable proton resonances. 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 at 27°, the ambient temperature of the probe. Chemical shifts downfield from water were assigned negative values with ±0.2 ppm accuracy for the spectra with 6000-Hz sweep field and ±0.05 ppm for the 1500-Hz sweep field.

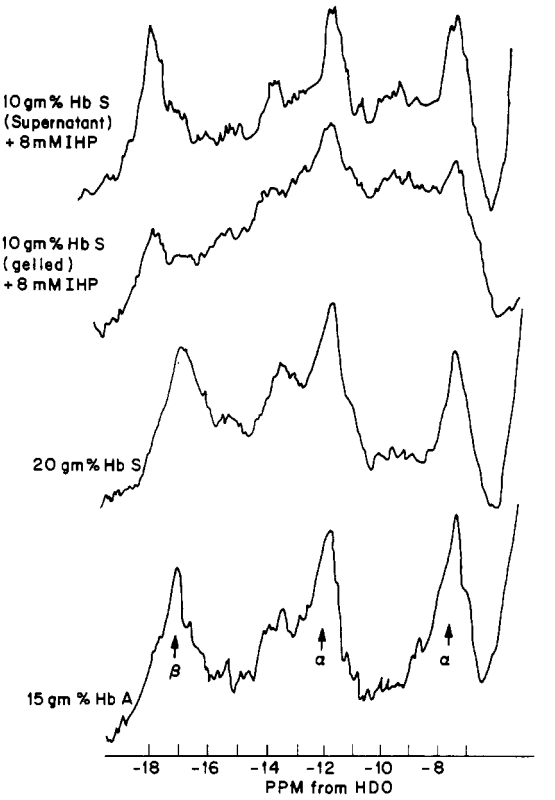

FIGURE 1: The 250-MHz hyperfine shifted proton resonances of deoxyhemoglobins A and S in 0.1 *M* bis-tris in D_2O at pH 6.6 and 27°. The assignments for the α- and β-heme resonances are taken from Davis et al. (1971) and Lindstrom et al. (1972a).

Results

Hyperfine and Ring-Current Shifted Proton Resonances. Figure 1 shows the hyperfine shifted proton resonances of the α and β hemes of 20 g % ungelled deoxy Hb S in 0.1 *M* bis-tris buffer in D_2O at pH 6.6 and 27°. This spectrum as well as those of 13 and 15 g % deoxy Hb S appear identical with the spectrum of deoxy Hb A. Three prominent resonances at −17.6, −12.0, and −8.0 ppm from HDO are found. In the presence of DPG or IHP, the β-heme resonance of Hb S at −17.6 ppm is shifted downfield to ∼−18 ppm. This downfield shift is also observed in Hb A (Lindstrom and Ho, 1972; Ho et al., 1973). Under certain experi-

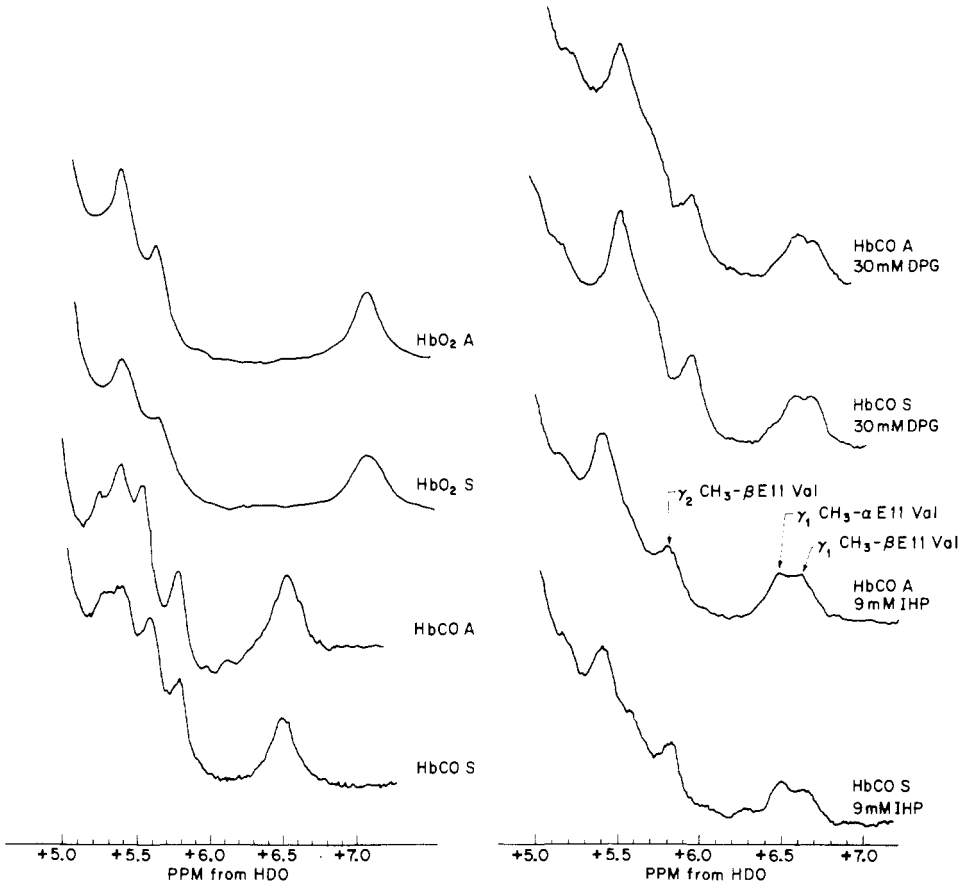

FIGURE 2: The 250-MHz ring-current shifted proton resonances of 10 g % oxy- and carbonmonoxyhemoglobins A and S in 0.1 *M* bis-tris in D_2O at pH 6.6 and 27° with and without organic phosphates. The assignments of γ_1 and γ_2 methyl groups of E11 valine residues in HbCO A in 9 m*M* IHP are taken from Lindstrom et al. (1972b).

mental conditions, such as at high Hb concentration and in the presence of organic phosphate, deoxy Hb S molecules polymerize to form a gel. The tumbling rate of these polymers in solution becomes slow, i.e., the rotational correlation time is long on the NMR time scale. Consequently, incomplete averaging of the dipole-dipole interactions results and the proton resonances of the molecule become too broad to give distinct resonances. The observed resonances in the gelled samples presumably come from the residual nonpolymerized molecules. We have also obtained the NMR spectrum of the "supernatant" of the gelled deoxy Hb S, which is the same as that of the ungelled sample (Figure 1). In Figure 2, the ring-current shifted resonances of HbCO S and HbO_2 S in 0.1 *M* bis-tris buffer in D_2O at pH 6.6 and 27° are found to be identical with those of HbCO A and HbO_2 A, respectively. The addition of DPG or IHP causes the extreme upfield ring-current shifted resonance of HbCO S to split into two peaks at +6.5 and +6.6 ppm. This spectral change induced by organic phosphate is similarly observed in the HbCO A molecule (Figure 2).

Exchangeable Proton Resonances. The spectra of 12 g % deoxy Hb S in water given in Figure 3 show that the −9.4, −8.3, −7.6, and −6.4 ppm resonances of a nongelled sample in 0.1 *M* bis-tris buffer at pH 6.6 and 27° are the same as those of deoxy Hb A under the same experimental conditions. Similarly, the resonances at −8.2, −7.3, and −5.8 ppm of HbO_2 S in water are identical with those of HbO_2 A.

Aromatic Proton Resonances. The proton resonances of a protein molecule in D_2O in the region between −2 to −4 ppm from HDO are generally associated with nonexchangeable protons on the aromatic rings of amino acid residues. In the spectra of hemoglobin samples, many of the sharp low-field aromatic resonances (−3 to −4 ppm from HDO) are from the C-2 protons of surface histidine residues (Kilmartin et al., 1973). These aromatic resonances of Hb S in deuterated solvent in either liganded or unliganded states exhibit rather unique features. Several resonances with full width at half-height of about 10 Hz appear to have different chemical shifts from those of Hb A but can be conditionally returned to "normal" by altering the pH and/or ionic environment of the sample. These resonances are very pH and ionic strength dependent. As shown in Figure 4a, in stripped HbCO S at pH 7.7 in D_2O, various resonances between −2.6 and −3.6 ppm are shifted upfield with respect to those of HbCO A. Upon addition of an excess amount of IHP (threefold molar excess), the spectrum of HbCO S changes to one similar to that of HbCO A (Figure 4b). In 0.1 *M* bis-tris buffer in D_2O at pH 6.5, the resonances between −3 and −4 ppm in HbCO S are different from those of HbCO A (Figure 4c), but again become the same when IHP is added (Figure 4d). In 0.2 *M* potassium phosphate buffer plus 0.2 *M* NaCl in D_2O at pH 6.3, the spectra of HbCO S and HbCO A are essentially identical except for a very slight upfield shift for the resonances between −3.5 and −3.7 ppm (Figure 5a). A particular resonance at about −3.6 ppm is shifted upfield by a small amount. In the same potassium phosphate buffer with NaCl but at pH 6.9, there

seem to be more resonances shifted upfield to a greater extent than those at pH 6.3 (Figure 5b). However, the binding of IHP to the samples again changes the spectrum of HbCO S to one similar to that of HbCO A (Figure 5c). In the deoxy samples of Hb S and Hb A in 0.1 *M* bis-tris buffer at pH 6.5, the resonances between −3 and −3.5 ppm exhibit distinct differences. A resonance at about −3.2 ppm has moved upfield, resulting in a doublet in the spectrum of Hb S (Figure 6a). Yet the spectrum of deoxy Hb S in 0.1 *M* potassium phosphate buffer containing 0.2 *M* NaCl at pH 6.5 is very similar to that of deoxy Hb A (Figure 6b). The addition of 6 m*M* IHP to the sample in 0.1 *M* bis-tris buffer in D_2O at pH 6.5 causes the sample to gel (even at a very low Hb concentration for NMR studies, ~5 g %), but the envelope of the broad resonance seems to suggest that the resonances have features similar to those of Hb A (Figure 6c).

Discussion

The identical hyperfine shifted proton resonances of deoxy Hb A and Hb S and ring-current shifted proton resonances of HbCO A or HbO_2 A and HbCO S or HbO_2 S (Figures 1 and 2) strongly suggest that there are no alterations in heme environment in sickle cell hemoglobin. This normal heme structure of Hb S persists over a wide range of hemoblobin concentrations and of experimental conditions. Our finding of indistinguishable heme environments in Hb A and Hb S agrees with the recent proton relaxation data on methemoglobins A and S reported by Gupta and Mildvan (1975). Deoxy- and oxyhemoglobin S (or HbCO S) respond to organic phosphates in a manner identical with those observed for Hb A (Lindstrom and Ho, 1972, 1973; Ho et al., 1973). The conformations of the E11 valines of α and β chains in HbCO S (or HbO_2 S) are identical with those of HbCO A (or HbO_2 A). Therefore, these NMR results allow us to conclude that the observed decrease in oxygen affinity of sickle cell blood is not due to an alteration in the heme environment of the Hb S molecule. The mutation at β6 has not induced any significant change in the heme pocket to account for the abnormal functional properties of Hb S. It should be emphasized that the ring-current shifted and hyperfine shifted proton resonances are very sensitive to small structural alterations in the vicinity of the heme groups and in the $\alpha_1\beta_2$ subunit interface (Davis et al., 1971; Lindstrom et al., 1972a,b, 1973; Ho et al., 1973).

It has been shown that hemoglobins with mutations at the $\alpha_1\beta_2$ subunit interface exhibit decreased heme–heme interactions (Perutz and Lehmann, 1968). Some of these mutants have low cooperativity as measured by the Hill coefficient, *n*, and low oxygen affinity; for example, Hb Kansas (G4β102 Asn→Thr) (Bonaventura and Riggs, 1968) has low oxygen affinity with n = 1.3 (Reismann et al., 1961). Therefore, it is essential to examine the subunit interfaces of the Hb S molecule to see if they have been altered. The NMR spectra of Hb S samples in water indicate that all of the observed exchangeable proton resonances are normal in both deoxy and oxy forms. In Hb Kansas, the −9.4-ppm resonance is observed in the oxy form, suggesting an abnormal quaternary structure (Ogawa et al., 1972). The resonance at −9.4 ppm of the deoxy sample is due to the crucial hydrogen bond between the tyrosine at C7α42 and the aspartic acid at G1β99, which anchors the $\alpha_1\beta_2$ subunits of deoxy Hb A (Fung and Ho, 1975). The −5.8 ppm resonance of the oxy sample is believed to come from the hydrogen bond between aspartic acid at G1α94 and asparagine at

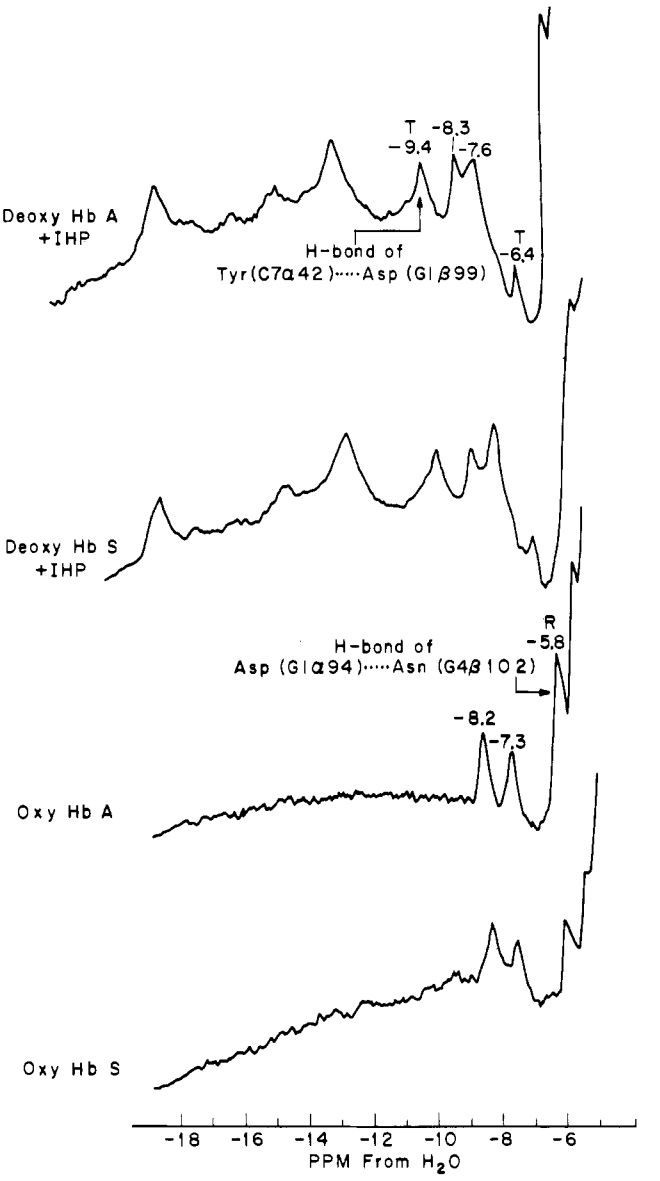

FIGURE 3: The 250-MHz hyperfine shifted and exchangeable proton resonances of Hb A and Hb S in 0.1 *M* bis-tris in H_2O at pH 6.6 and 27°. The assignments for the resonance at −9.4 and at −5.8 ppm are taken from Fung and Ho (1975). The symbols T and R represent spectroscopic probes for deoxy and oxy quaternary structures.

G4β102 which is also in the $\alpha_1\beta_2$ subunit interface (Fung and Ho, 1975). Thus, the normal exchangeable proton resonances of Hb S indicate that the subunit interfaces of the molecule are again normal. Hence, the β6 mutation has not induced any observable change in this functionally critical region of the subunit interface which may be responsible for the low oxygen affinity of the sickle cell blood.

Bookchin et al. (1970) have investigated the interactions of various mutant hemoglobins with deoxy Hb S and suggested that the β6, β73, and β121 residues may be crucial in Hb S–Hb S interactions. According to X-ray crystallographic data, these residues all lie at the surface of the molecule (Perutz, 1970). Our NMR results indicate that the proton spectra of surface histidines of Hb A and Hb S are different. Under certain conditions, when the HbCO sample is in 0.2 *M* potassium phosphate buffer containing 0.2 *M* NaCl at pH 6.3, the resonances of HbCO S and HbCO A in the entire spectral region are essentially identical except

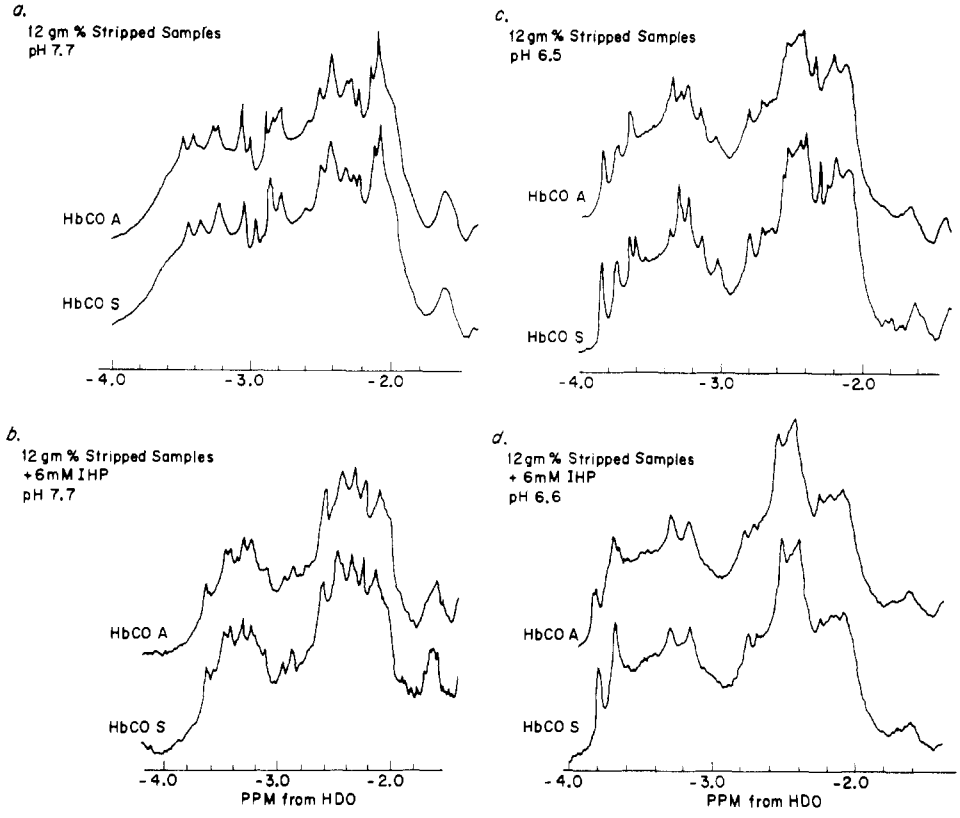

FIGURE 4: The 250-MHz aromatic proton resonances of HbCO A and HbCO S in 0.1 *M* bis-tris in D_2O at 27° with and without IHP.

for the two resonances between −3.5 and −3.7 ppm. In particular, the resonance at −3.6 ppm, which has been identified as the β2 histidine resonance (Fung et al., 1975), is shifted upfield very slightly. It is not surprising to have the β2 histidine in Hb S in a slightly altered electronic environment since this residue is close to the mutation site. Under other experimental conditions, the spectral differences become more dramatic, in both the CO and deoxy forms. Therefore, it is desirable first to relate these aromatic resonances with the histidine residues on the surface of the molecule. In addition to the β2 histidine assignment stated earlier, Kilmartin et al. (1973) identified β146 histidine resonances in both HbCO A and deoxy Hb A samples. With approximately 20 surface histidine residues in the molecule, as discussed in the introduction, the remaining task of assigning all of the resonances is not simple. However, the differences observed in the resonances have already suggested certain unique surface properties of the Hb S molecule. Under the conditions where the spectra of Hb A and Hb S are different, the resonances of Hb S remain sharp but are generally shifted upfield. The upfield shift of resonances in NMR spectra usually indicates that the corresponding protons are in a somewhat more electronically shielded (i.e., more hydrophobic) environment (Roberts and Jardetzky, 1970). In the NMR titration studies of a protein molecule, it has been found that the histidine resonances shift upfield upon deprotonation of histidine residues (Markley, 1975). The new magnetic environment in Hb S, as indicated by the upfield shift of the aromatic resonances, may suggest a different spatial orientation either of the side chains of the surface histidines at which the resonating protons reside or of the neighboring groups near these histidine residues. The line widths of these resonances, which remain narrow, suggest that the side chains of the residues of the corresponding resonances still rotate rather freely. The slight upfield shift of the β2 histidine in HbCO S in 0.2 *M* potassium phosphate buffer and 0.2 *M* NaCl at pH 6.3 may be either a reflection of the substitution of its neighboring residue at β6 from glutamic acid to valine or a slight movement of the β2 residue due to the amino acid substitution at β6.

Recently, Asakura et al. (1974) found that the oxy form of Hb S is mechanically unstable, i.e., HbO_2 S precipitates much more readily than HbO_2 A upon shaking. They suggested that this difference between the properties of Hb S and Hb A is due to the difference in the conformation of these two proteins. Our finding that there are differences in the histidine proton resonances between HbCO S and HbCO A indicates that there are differences between the surface conformations and/or net charge of surface residues of these two hemoglobins. More recently, Elbaum et al. (1975) have measured the surface tensions of Hb A and Hb S and have found that there are differences in the surface properties between sickle cell and adult hemoglobins. Upon addition of phosphate ions, the NMR spectral differences between Hb S and Hb A begin to diminish. In the presence of IHP, the spectra of both Hb S and Hb A in the deoxy or CO form under various experimental conditions are essentially identical. This may very well be related to the protective effect of organic phosphates on HbO_2 S observed by Adachi and Asakura (1974). The binding of a highly charged IHP molecule to the Hb molecule may either cause a change in the net charge of the Hb molecule and/or a change in the surface conformation of the protein molecule, thus making the Hb A and Hb S molecules more alike. Our NMR results further suggest that the conformational alterations in the Hb S molecule are pH and ionic strength specific, which may, in part, explain the pH and ionic strength effects on the gelation of deoxy Hb S re-

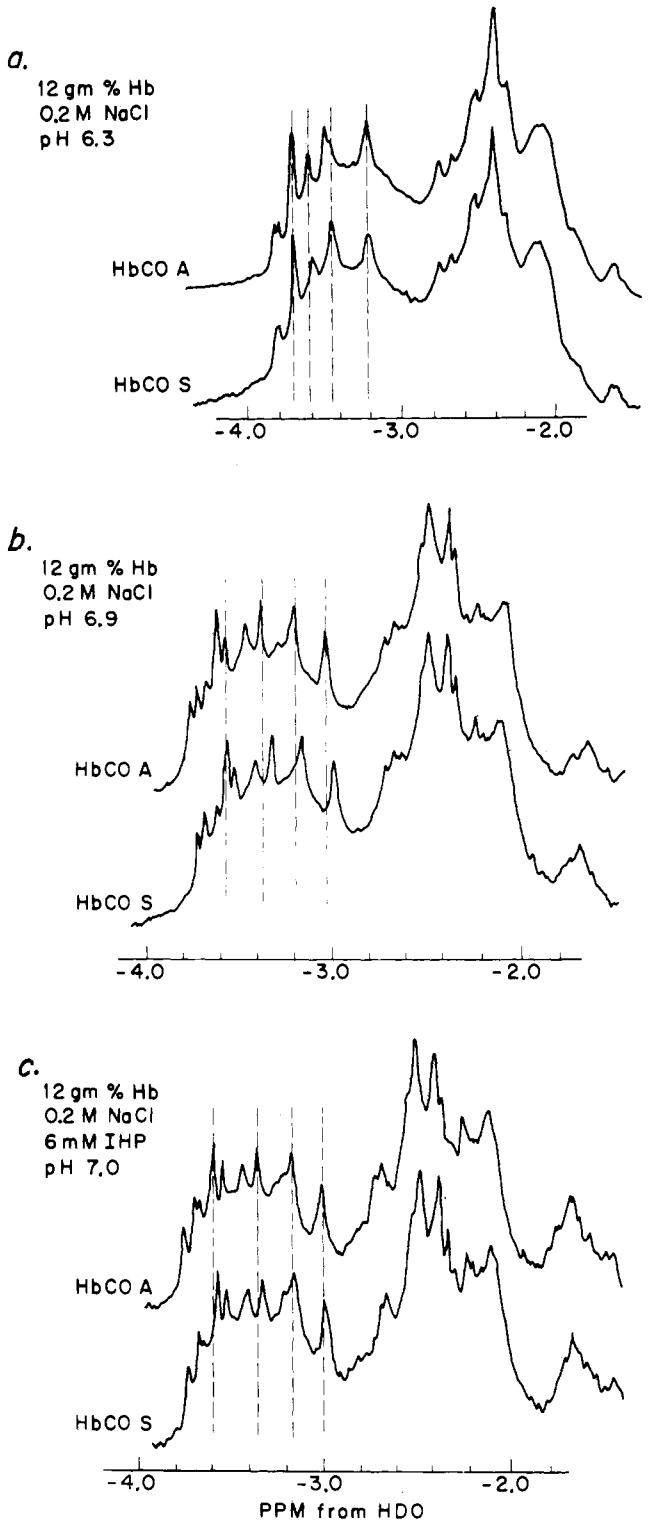

FIGURE 5: The 250-MHz aromatic proton resonances of HbCO A and HbCO S in 0.2 *M* potassium phosphate plus 0.2 *M* NaCl in D_2O at 27°.

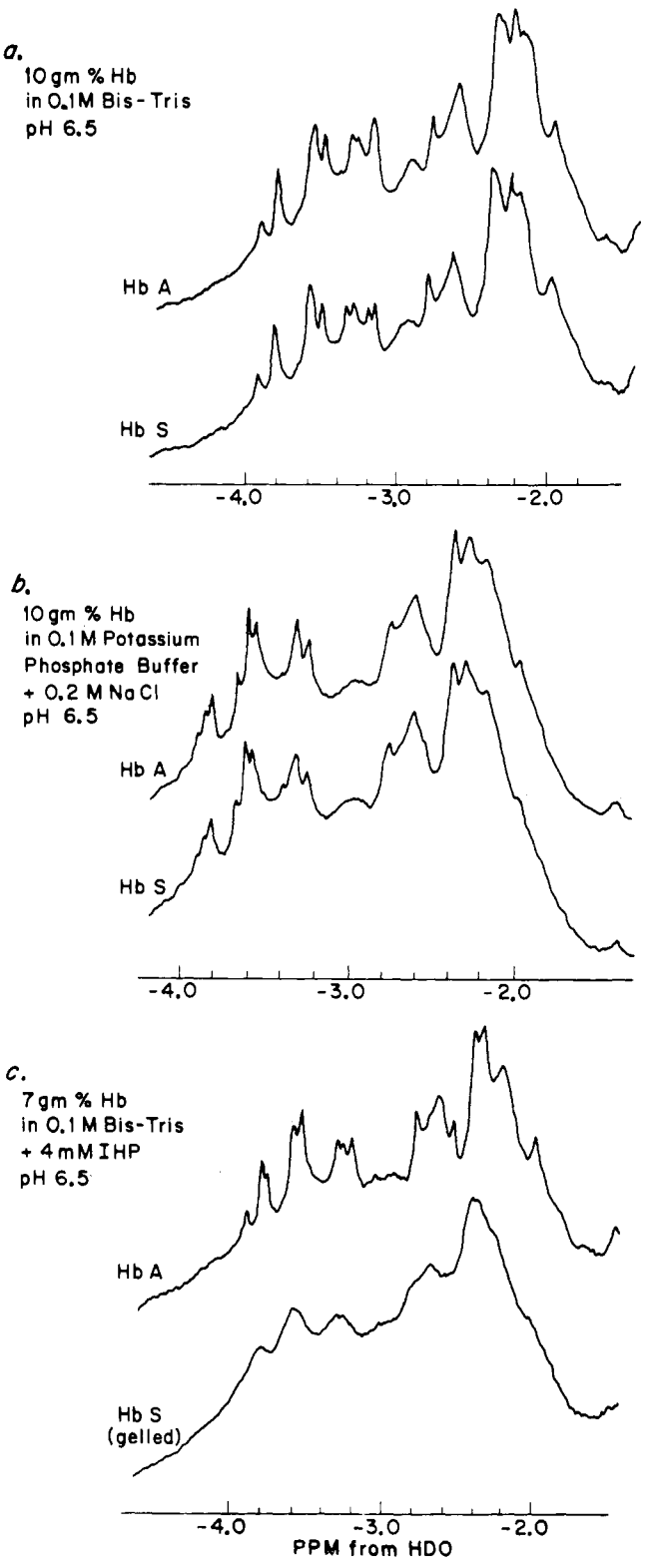

FIGURE 6: The 250-MHz aromatic proton resonances of deoxyhemoglobins A and S in D_2O at 27°.

ported by several investigators (Briehl and Ewert, 1973; Cottom et al., 1973).

In conclusion, the high-resolution ^1H NMR spectra of Hb S and Hb A molecules indicate that, between these two molecules, there are no observable differences in the heme environment and in the functionally crucial regions of the subunit interfaces. However, the NMR results suggest that the abnormality of Hb S due to the single amino acid substitution at $\beta 6$ is propagated over the surface of the molecule. Some of the surface residues of the sickle cell hemoglobin are in a more hydrophobic environment and/or are more shielded (as measured by the chemical shifts of the histidine residues), but the rotational freedom of most of these residues remains unchanged. Studies extended from this work on the proton NMR studies of aromatic residues in sickle cell hemoglobin as a function of various experimental parameters, such as pH and concentration, will be

published elsewhere.

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