A Proton Nuclear Magnetic Resonance Investigation of Histidyl Residues in Sickle Hemoglobin†

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ABSTRACT: Using high-resolution proton nuclear magnetic resonance spectroscopy at 250 MHz, we have determined the individual pK values of 22 surface histidyl residues (11 per $\alpha\beta$ dimer) of sickle hemoglobin in both deoxy and carbon monoxy forms. Seven histidyl residues in the deoxy form and three in the carbon monoxy form are found to have pK values and chemical shifts different from the corresponding ones in human normal adult hemoglobin. Two of these histidyl residues are the β2 histidine and the β146 histidine, indicating that the conformations of the amino- and carboxyl-terminal regions of the β chain in sickle hemoglobin are altered compared to those in human normal adult hemoglobin. The differences in the pK values of the additional surface histidyl residues between sickle and normal hemoglobins suggest that the effect of the amino acid substitution at the sixth position of the β chain in sickle hemoglobin, namely, glutamic acid replaced by valine, is not restricted to the region around the mutation site but can extend to other regions in the protein molecule. In the deoxy form, the histidyl residues of sickle hemoglobin that have altered pK values and chemical shifts compared to the corresponding ones in human normal adult hemoglobin have been found to be sensitive to the early stages of the polymerization process [Russu, I. M., & Ho, C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6577–6581].

Sickle hemoglobin (Hb S)[1] is a genetic variant of the human normal adult hemoglobin (Hb A) in which the valyl residue at the sixth position of the β chain replaces the normally occuring glutamyl residue (Ingram, 1956). In the deoxygenated state and under certain experimental conditions, Hb S molecules can polymerize in solution as well as inside the red blood cells. The polymerization of deoxy-Hb S is believed to be the main pathogenic process in patients with sickle cell anemia. Extensive research carried out during the last decade has provided a large amount of information on the molecular and cellular properties of Hb S and of its polymerization process [for a review, see Dean & Schechter (1978a–c)]. However, the detailed molecular mechanism of the polymerization process is not yet fully understood. At the molecular level, one current problem of great significance is the existence or nonexistence of conformational differences between the Hb S and Hb A. X-ray diffraction studies of crystals of Hb S have revealed the structures of the Hb S and Hb A molecules to be similar in both the oxy and deoxy forms (Perutz et al., 1951; Wishner et al., 1975, 1976; Love et al., 1978). Furthermore, the proton nuclear magnetic resonance (NMR) studies of Hb S carried out previously in our laboratory have demonstrated that in solution the environments of the hemes as well as the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in the Hb S molecule are the same as those in Hb A (Fung et al., 1975). The absence of a large change in the conformations of these key regions in the Hb S molecule as compared to those of Hb A provides a structural basis for the intrinsic similarities between these two molecules in the oxygen affinities (Allen & Wyman, 1954; Charache et al., 1970), the ligand binding kinetics (Pennelly & Noble, 1978), and the subunit dissociation kinetics (Ip et al., 1976).

In spite of the absence of gross alterations in these functionally crucial regions of Hb S, several other experimental studies have revealed that differences in the surface conformations between the Hb S and Hb A molecules do exist under certain experimental conditions. Fronticelli (1978) has found that the circular dichroism in the Soret region of Hb S solution differs from that of Hb A, in the carbon monoxy form, whereas in the oxy and deoxy forms, no differences are present as reported by Li & Johnson (1969). On the basis of these results, Fronticelli (1978) has suggested that there is a change in the local conformation of β15 Trp in Hb S compared to that in Hb A. Schechter and co-workers (Young et al., 1975; Curd et al., 1976) have purified several antibodies that bind to Hb S but not to Hb A. This specificity appears to be localized at the valine substitution at the β6 position, suggesting the existence of a conformational difference between Hb S and Hb A in the amino-terminal region of the β chain. Further support for the existence of alterations in the surface conformation of the Hb S molecule has been provided by the abnormal rates of precipitation during mechanical shaking for Hb S solutions in the ligated forms [reported by Asakura et al. (1974)].

Previous research carried out in our laboratory has demonstrated that high-resolution ^{1}H NMR spectroscopy represents a unique experimental technique to monitor the differences in the surface conformations between Hb S and Hb A. Of special interest to this work are the histidyl residues situated on the surface of the Hb molecule. The protons of these His residues give rise to resonances that are well resolved and separated from other resonances in the ^{1}H NMR spectrum of Hb. As previously shown in our laboratory, one can monitor and compare the environment and the conformation of individual His residues in Hb S and Hb A over a wide range of experimental conditions by ^{1}H NMR spectroscopy. Fung et al. (1975) have found that the ^{1}H resonances of several of these His residues are shifted upfield in the ^{1}H NMR spectra of Hb S compared to those of Hb A, in both the deoxy and ligated forms, under certain conditions of pH and ionic strength. Ho et al. (1976) have suggested that these spectral changes originate from small differences between Hb S and Hb A in the

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[1] Abbreviations: Hb S, sickle hemoglobin; Hb A, human normal adult hemoglobin; HbCO, (carbon monoxy)hemoglobin; NMR, nuclear magnetic resonance; ppm, parts per million; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate.

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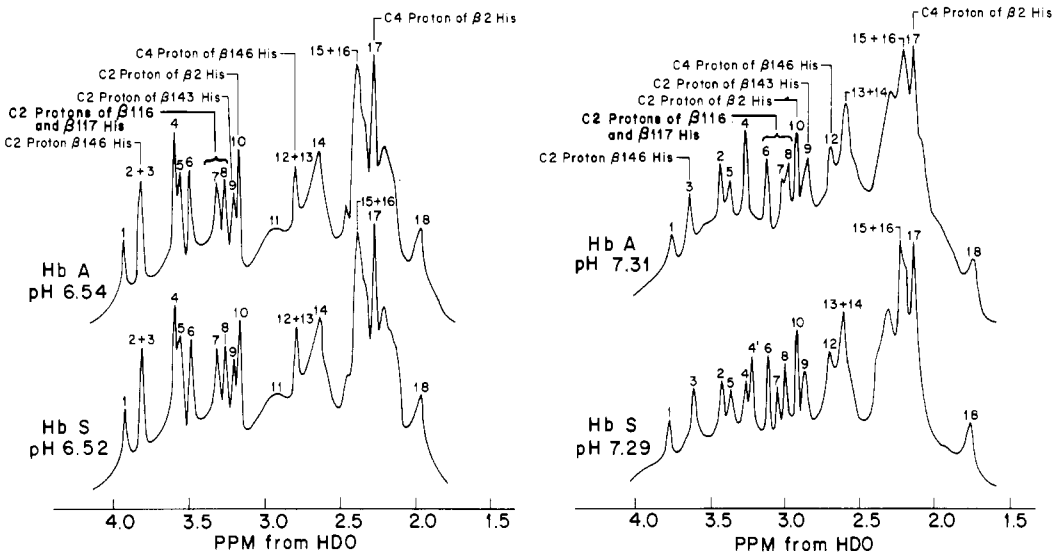

FIGURE 1: 250-MHz ^{1}H NMR spectra of 10% deoxy-Hb S and deoxy-Hb A in 0.1 M Bis-Tris buffer at 27 °C.

pK values of at least three His residues in the deoxy form and at least two in the carbon monoxy form. Furthermore, they have found that these differences, as well as the spectral pattern of the ^{1}H resonances of the histidyl residues, are dependent specifically upon the factors known to affect the gelation properties of Hb S, such as the concentrations of 2,3-diphosphoglycerate (2,3-DPG) and of Hb. On the basis of these results, Ho et al. (1976) have concluded that Hb S has a unique localized surface conformation.

In view of these previous findings, we have carried out a detailed ^{1}H NMR titration of the surface His residues in Hb S, in both the deoxy and carbon monoxy forms, under a given set of experimental conditions: 10% Hb solutions in the presence of 0.1 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) or 0.1 M tris(hydroxymethyl)-aminomethane (Tris) with chloride ion concentrations ranging from 5 to 60 mM at 27 °C. The rationale for choosing these experimental conditions is that, under these experimental conditions, the largest number of distinct proton resonances of the histidyl residues can be observed in the ^{1}H NMR spectra of Hb A and Hb S, in both the deoxy and carbon monoxy forms. A preliminary account of this work has been published elsewhere (Ho & Russu, 1978).

Experimental Procedures

Materials. Hb A was prepared by the standard procedure from fresh blood samples obtained from the local blood bank (Lindstrom & Ho, 1972). Hb S was isolated and purified from blood samples of homozygous (SS) or heterozygous (AS) donors provided by the Sickle Cell Society, Inc. The red blood cells were washed and lysed by the same procedure as that used for the normal blood (Drabkin, 1946). Hb S was purified from the hemolysate by cation-exchange chromatography on a Bio-Rex 70 column (200–400 mesh, sodium form, Bio-Rad) equilibrated with 0.05 M sodium phosphate buffer at pH 7.2. The purity of the Hb S preparation was checked by cellulose–acetate strip electrophoresis. The organic phosphates were removed by passing the Hb solutions through a Sephadex G-25 column equilibrated with 0.01 M Tris-HCl in the presence of 0.1 M NaCl buffer at pH 7.6 (Berman et al., 1971). All the Hb solutions used in the present work were exchanged 4 times with D_2O (99.8% deuterium content, Bio-Rad) in order to suppress the intense water proton resonance in the ^{1}H NMR spectra. Hb samples in 0.1 M Bis-Tris or 0.1 M Tris were prepared as described in the preceding paper (Russu et al., 1982). The deoxygenation of the Hb samples was carried out as described previously (Lindstrom & Ho, 1972).

Methods. High-resolution ^{1}H NMR spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer, interfaced with a Sigma 5 computer, by the technique of NMR correlation spectroscopy (Dadok & Sprecher, 1974). The spectrometer settings were the same as those described in the preceding paper (Russu et al., 1982). The proton chemical shifts are expressed as parts per million (ppm) relative to the residual water proton resonance. The chemical shift scale is presently defined as positive in the low-field direction.[2] The accuracy of the chemical shift measurement is ±0.02 ppm.

The proton chemical shifts, δ, of the histidine resonances were fitted individually as a function of pH to the following equation for a simple proton dissociation equilibrium of a single ionizable group (Markley, 1975):

$$\delta = (\delta^+[H^+] + \delta^0 K)/([H^+] + K) \quad (1)$$

where $[H^+]$ is the concentration of hydrogen ions, K is the proton dissociation equilibrium of the His residue, and δ^+ and δ^0 are the proton chemical shifts in the protonated and unprotonated forms of the His residue, respectively. The chemical shifts of the His C2 proton resonances were also fitted as a function of pH to the following equation (Markley, 1975):

$$\delta = (\delta^+[H^+]^n + \delta^0 K^n)/([H^+]^n + K^n) \quad (2)$$

where n is the titration coefficient for the ^{1}H NMR titration of the His residue and the rest of the symbols have the same meaning as those in eq 1. The nonlinear least-squares program NLIN in the computer center at the University of Pittsburgh has been used for both these analyses of the ^{1}H NMR data. For details, refer to the preceding paper (Russu et al., 1982).

Results

We have monitored the aromatic proton resonance region (1.5–5.0 ppm downfield from HDO) of 10% Hb S solutions

[2] 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. This convention is different from that used by this laboratory prior to 1980. Previously, we had used the negative sign to indicate that the chemical shift of a given resonance is downfield from the proton resonance of the residual water (HDO). Hence, this change in the sign of the chemical shift scale should be noted when referring to earlier publications reported by this laboratory.

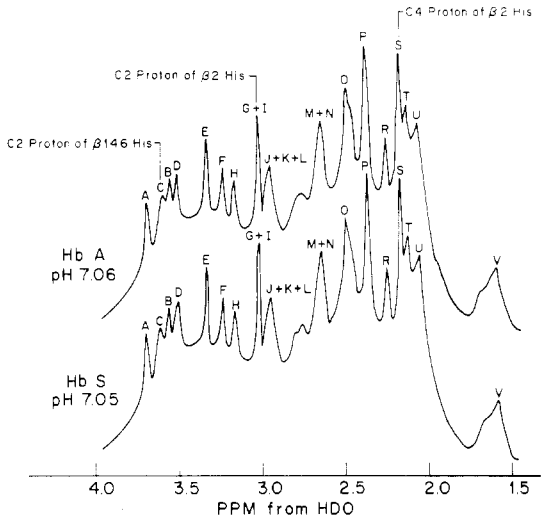

FIGURE 2: 250-MHz ^{1}H NMR spectra of 10% HbCO S and HbCO A in 0.1 M Bis-Tris buffer at 27 °C.

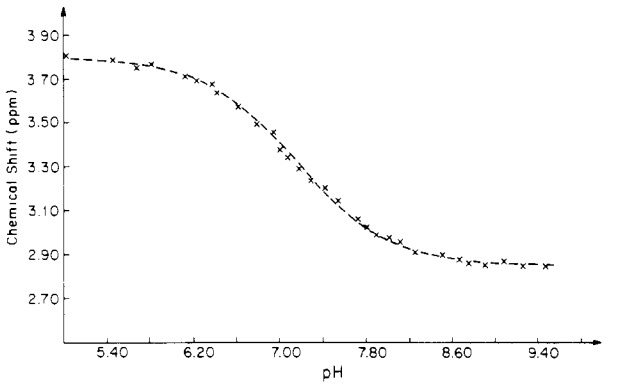

FIGURE 3: ^{1}H NMR titration of resonance labeled E in 10% HbCO in 0.1 M Bis-Tris buffer at 27 °C: (×) experimental points in HbCO S; (dashed line) fitted titration curve in HbCO A [taken from the preceding paper (Russu et al., 1982)].

in 0.1 M Bis-Tris or 0.1 M Tris buffer, in both deoxy and carbon monoxy forms, as a function of pH over the pH range from 5.0 to 9.5. Several representative ^{1}H NMR spectra of Hb S in the deoxy and the carbon monoxy forms are shown in Figures 1 and 2, respectively, in comparison with the corresponding ones of Hb A. In the pH range examined, the same aromatic proton resonances are observed in the ^{1}H NMR spectra of Hb S as in those of Hb A. They are labeled from 1 to 18 in the ^{1}H NMR spectra of deoxy-Hb A and deoxy-Hb S and from A to V for HbCO A and HbCO S. In the deoxy form, the resonances labeled 3 and 12 are assigned to the C2 and C4 protons of β146 His, respectively; the resonances labeled 10 and 17 are assigned to the C2 and C4 protons of β2 His, respectively; the resonance labeled 9 is tentatively assigned to the C2 proton of β143 His. In the carbon monoxy form, the resonances labeled G and S are assigned to the C2 and C4 protons of β2 His, respectively, and the resonance labeled C is assigned to the C2 proton of β146 His. These spectral assignments are described in detail in the preceding paper (Russu et al., 1982).

By following the chemical shifts of each of these aromatic proton resonances of deoxy-Hb S and HbCO S as a function of pH, we have found that most of them have ^{1}H NMR titration curves that are identical to those found in Hb A. For illustration of this finding, Figure 3 shows the experimental data (×) for the titration of the resonance labeled E in HbCO S in comparison with titration curve previously found for resonance E in HbCO A (--). One can see that, in Hb S, the ^{1}H NMR titration of this resonance follows exactly that found in Hb A. Nevertheless, there are several aromatic proton resonances of Hb S in both deoxy and CO forms that show small but consistent titration differences from the corresponding ones in Hb A as shown below.

Figure 4 summarizes the results for the histidine resonances that show differences in the ^{1}H NMR titration between deoxy-Hb S and deoxy-Hb A. These resonances are labeled as follows: 2 (Figure 4A); 3, assigned to β146 His C2 proton (Figure 4B); 4 (Figure 4C); 5 (Figure 4D); 6 (Figure 4E); 10, assigned to β2 His C2 proton (Figure 4F); 12, assigned to β146 His C4 proton (Figure 4G); 13 (Figure 4H); 18 (Figure 4I). In addition to the differences in the ^{1}H NMR titration of these aromatic proton resonances, we have also found that, in deoxy-Hb S in the pH range between 7.1 and 7.6, the resonance labeled 4 resolved into two resonances, which we labeled 4 and 4′ (Figure 1). Both these resonances are shifted upfield in the ^{1}H NMR spectrum of deoxy-Hb S as compared to their positions in the spectrum of deoxy-Hb A. This result is presented in Figure 4C where the experimental points for the ^{1}H NMR titration of resonance 4′ are shown with a different symbol, when different from those of resonance 4 [(○) for 4′ and (×) for 4].

In the carbon monoxy form, there are three aromatic proton resonances in Hb S that are consistently shifted upfield relative to their positions in the spectrum of Hb A. These resonances are resonance C, assigned to the β146 His C2 proton, resonance F, and resonance G, assigned to the β2 His C2 proton. The ^{1}H NMR titration of these three resonances in HbCO S is presented in Figure 5A–C in comparison with the titration curves obtained for the same resonances in HbCO A.

The results of the fitting of the chemical shifts vs. pH to eq 1 for the aromatic proton resonances in the ^{1}H NMR spectra of deoxy-Hb S and HbCO S are given in Tables I and II, respectively. For comparison, these tables also include the corresponding results reported in the preceding paper for the aromatic proton resonances of deoxy-Hb A and HbCO A, respectively.

Discussion

We have previously shown that from the 18 aromatic proton resonances that can be resolved in the ^{1}H NMR spectrum of deoxy-Hb, the resonances labeled 1–10 originate from the C2 protons of His residues, whereas those labeled 12, 13, 17, and 18 originate from the C4 protons of His residues (Russu et al., 1982). Similarly, in the ^{1}H NMR spectrum of HbCO, the aromatic proton resonances labeled A–K originate from His C2 protons and those labeled L–V from His C4 protons. Furthermore, it is most likely that the His residues responsible for these ^{1}H NMR resonances are situated, in both deoxy and ligated states, on the surface of the Hb molecule (Russu et al., 1982).

In the present work, we have found that seven surface His C2 proton resonances and three surface His C4 proton resonances in deoxy-Hb S and three surface His C2 proton resonances in HbCO S have pK values and intrinsic chemical shifts (δ^+ and δ^0) that are different from the corresponding ones in Hb A. These results confirm the previous findings of our laboratory that at least three His residues in deoxy-Hb S and at least two His residues in HbCO S have pK values that are different from the corresponding ones in Hb A (Ho et al., 1976). Among the resonances showing differences between Hb S and Hb A, resonances 10 and G in the ^{1}H NMR spectra of deoxy and HbCO, respectively, originate from the C2 protons of the β2 His residues. The β2 His residues are sit-

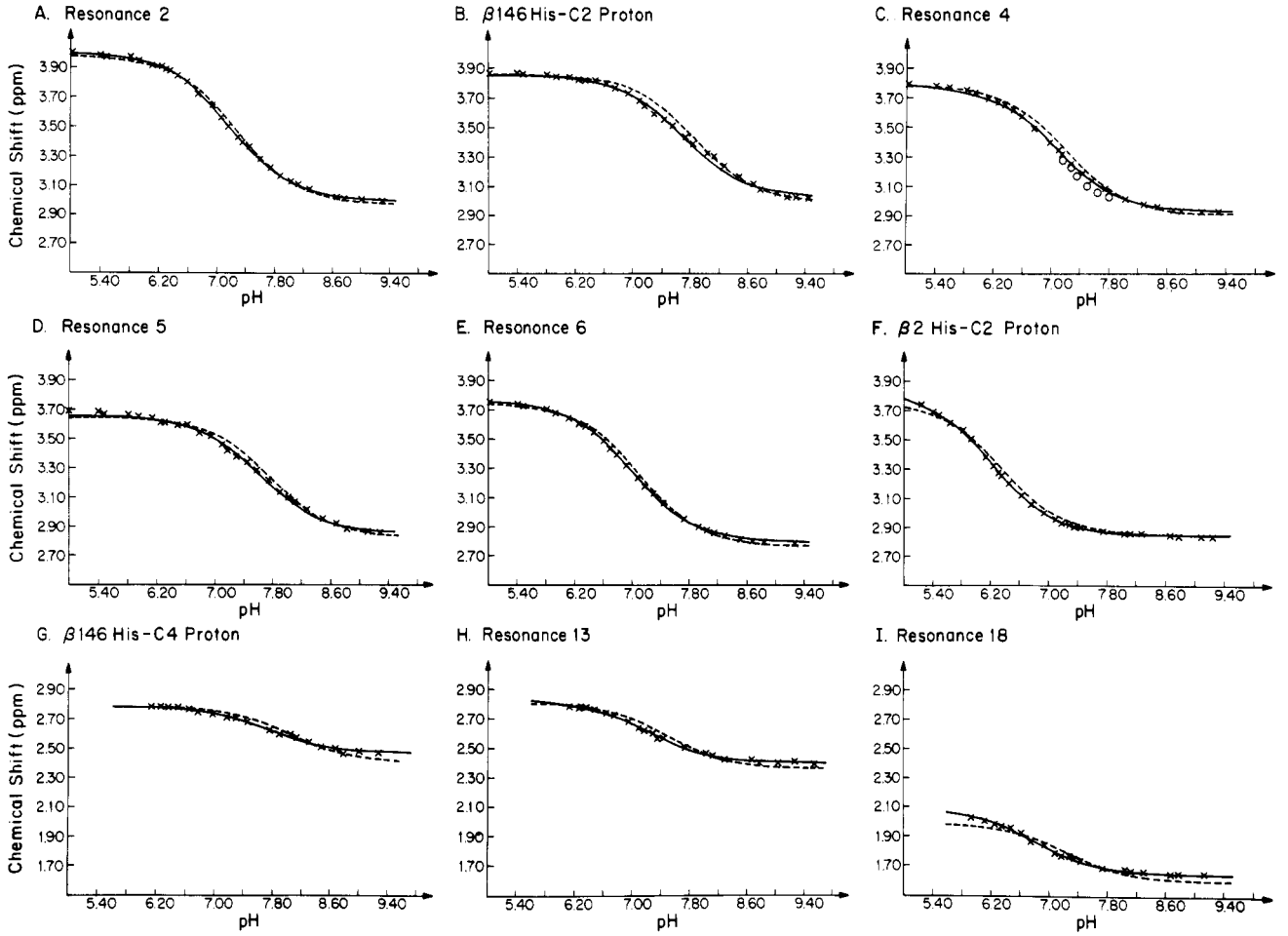

FIGURE 4: ^{1}H NMR titration of histidine resonances in 10% deoxy-Hb S in 0.1 M Bis-Tris buffer at 27 °C: (A) resonance labeled 2; (B) resonance labeled 3 (β146 His C2 proton); (C) resonances labeled 4 and 4′; (D) resonance labeled 5; (E) resonance labeled 6; (F) resonance labeled 10 (β2 His C2 proton); (G) resonance labeled 12 (β146 His C4 proton); (H) resonance labeled 13; (I) resonance labeled 18. Symbols used were the following: (×) experimental points in deoxy-Hb S and (o) in C, experimental points for resonance labeled 4′. Solid lines are experimental titration curves in deoxy-Hb S and dashed lines are experimental titration curves in deoxy-Hb A [taken from the preceding paper (Russu et al., 1982)]. The titration curves were obtained by fitting the corresponding experimental data to eq 1 for the resonances labeled 2–4, 6, 10, 12, 13, and 18 and to eq 2 for the resonance labeled 5.

Table I: ^{1}H NMR Titration of Histidyl Residues in Deoxy-Hb S and Deoxy-Hb A, 10% Solutions in 0.1 M Bis-Tris and/or 0.1 M Tris Buffer at 27 °C[a]

	deoxy-Hb S			deoxy-Hb A		
resonance no.	pK	δ^+	δ^0	pK	δ^+	δ^0
1	8.00 ± 0.04	3.95 ± 0.01	3.01 ± 0.02	8.07 ± 0.04	3.95 ± 0.01	2.97 ± 0.02
2	7.19 ± 0.02	4.00 ± 0.01	3.00 ± 0.01	7.28 ± 0.02	3.98 ± 0.01	2.97 ± 0.01
3 (β146 C2)	7.70 ± 0.04	3.86 ± 0.01	3.03 ± 0.02	7.98 ± 0.02	3.86 ± 0.01	2.90 ± 0.02
4	7.07 ± 0.02	3.79 ± 0.01	2.92 ± 0.01	7.20 ± 0.03	3.78 ± 0.01	2.90 ± 0.01
4′	7.04 ± 0.03	3.80 ± 0.01	2.92 ± 0.01			
5	7.62 ± 0.04	3.65 ± 0.02	2.84 ± 0.02	7.76 ± 0.04	3.64 ± 0.01	2.81 ± 0.02
6	7.01 ± 0.03	3.76 ± 0.01	2.79 ± 0.01	7.10 ± 0.03	3.74 ± 0.03	2.76 ± 0.01
7	7.05 ± 0.05	3.52 ± 0.02	2.74 ± 0.02	7.07 ± 0.06	3.55 ± 0.02	2.72 ± 0.02
8	6.62 ± 0.05	3.63 ± 0.02	2.83 ± 0.01	6.71 ± 0.06	3.63 ± 0.03	2.82 ± 0.02
9 (β143 C2)	6.71 ± 0.06	3.56 ± 0.03	2.63 ± 0.02	6.82 ± 0.07	3.56 ± 0.03	2.60 ± 0.02
10 (β2 C2)	6.22 ± 0.04	3.83 ± 0.03	2.84 ± 0.01	6.35 ± 0.04	3.76 ± 0.02	2.84 ± 0.01
12 (β146 C4)	7.73 ± 0.08	2.78 ± 0.01	2.46 ± 0.01	8.03 ± 0.06	2.78 ± 0.01	2.40 ± 0.01
13	7.23 ± 0.06	2.83 ± 0.01	2.40 ± 0.01	7.49 ± 0.05	2.81 ± 0.01	2.36 ± 0.01
17 (β2 C4)	6.21 ± 0.05	2.51 ± 0.02	2.11 ± 0.01	6.31 ± 0.05	2.50 ± 0.01	2.10 ± 0.01
18	6.81 ± 0.07	2.09 ± 0.02	1.65 ± 0.01	7.24 ± 0.05	1.99 ± 0.01	1.60 ± 0.01

[a]Nonlinear least-squares fitting of the experimental data to eq 1. The results for deoxy-Hb A are reproduced from the preceding paper (Russu et al., 1982).

uated in close proximity to the β6 mutation sites in the Hb S molecule. Therefore, the difference in their pK values between Hb S and Hb A indicates the existence of a change in the conformation of the region around the mutation site in the Hb S molecule in both deoxy and CO states. This conclusion is supported by our longitudinal relaxation rate (T_1^{-1}) measurements of the His residues in deoxy-Hb S and deoxy-Hb A (Russu & Ho, 1980). We have found that, under certain experimental conditions where the extent of pregelation aggregation in the Hb S solutions is negligible, the C2 protons

Table II: ^{1}H NMR Titration of Histidyl Residues in HbCO S and HbCO A, 10% Solutions in 0.1 M Bis-Tris and/or 0.1 M Tris Buffer at 27 °C[a]

	Hb S			Hb A		
resonance	pK	δ^+	δ^0	pK	δ^+	δ^0
A	7.83 ± 0.03	3.85 ± 0.01	2.90 ± 0.02	7.87 ± 0.02	3.86 ± 0.01	2.88 ± 0.01
B	7.36 ± 0.03	3.92 ± 0.01	2.92 ± 0.01	7.42 ± 0.03	3.92 ± 0.01	2.88 ± 0.01
C (β146 C2)	7.76 ± 0.04	3.76 ± 0.01	2.91 ± 0.01	7.85 ± 0.03	3.77 ± 0.01	2.90 ± 0.02
D	7.60 ± 0.04	3.77 ± 0.02	2.82 ± 0.02	7.65 ± 0.02	3.78 ± 0.01	2.80 ± 0.01
E	7.12 ± 0.03	3.80 ± 0.01	2.85 ± 0.01	7.15 ± 0.02	3.80 ± 0.01	2.85 ± 0.01
F	6.91 ± 0.03	3.84 ± 0.01	2.85 ± 0.01	7.00 ± 0.03	3.84 ± 0.01	2.85 ± 0.01
G (β2 C2)	6.39 ± 0.02	3.75 ± 0.02	2.84 ± 0.01	6.53 ± 0.02	3.77 ± 0.01	2.85 ± 0.01
I	6.71 ± 0.04	3.65 ± 0.03	2.72 ± 0.01	6.65 ± 0.03	3.70 ± 0.02	2.72 ± 0.01
J	6.84 ± 0.04	3.42 ± 0.02	2.68 ± 0.01	6.81 ± 0.03	3.45 ± 0.01	2.68 ± 0.01
H	6.26 ± 0.07	3.83 ± 0.06	3.05 ± 0.01	6.20 ± 0.05	3.88 ± 0.05	3.06 ± 0.01
K	6.78 ± 0.07	3.39 ± 0.03	2.67 ± 0.01	6.76 ± 0.06	3.42 ± 0.03	2.67 ± 0.01
M	7.32 ± 0.05	2.81 ± 0.01	2.39 ± 0.01	7.38 ± 0.05	2.81 ± 0.01	2.36 ± 0.01
P	7.13 ± 0.05	2.60 ± 0.01	2.12 ± 0.01	7.13 ± 0.06	2.61 ± 0.01	2.11 ± 0.01
R	6.90 ± 0.08	2.46 ± 0.01	2.10 ± 0.01	6.99 ± 0.05	2.47 ± 0.01	2.10 ± 0.01
S (β2 C4)	6.56 ± 0.07	2.42 ± 0.02	2.08 ± 0.01	6.68 ± 0.05	2.43 ± 0.01	2.09 ± 0.01
T	6.47 ± 0.09	2.39 ± 0.02	2.07 ± 0.01	6.50 ± 0.06	2.41 ± 0.01	2.04 ± 0.01

[a] Nonlinear least-squares fitting of the experimental data to eq 1. The results for HbCO A are reproduced from the preceding paper (Russu et al., 1982).

of the β2 His residues in deoxy-Hb S have a T_1^{-1} value about 3 times larger than that in deoxy-Hb A. We have previously suggested that this difference between Hb S and Hb A in the T_1^{-1} value of β2 His C2 protons could reflect an intrinsic change in the distances between β2 His and β1 Val and β3 Leu in the deoxy-Hb S molecule as compared to deoxy-Hb A. The existence of a conformational change of the amino-terminal region of the β chain in Hb S has also been inferred from the functional studies carried out by Ueda et al. (1978) and by Elbaum & Nagel (1981). Ueda et al. (1978) have found that 2,3-DPG and inositol hexaphosphate (IHP) lower the oxygen affinity of Hb S less than that of Hb A and that 2,3-DPG is displaced by CO_2 from the β chain of Hb S more readily than from the normal β chain. Since 2,3-DPG binds in the central cavity of the Hb molecule (Arnone, 1972), these findings have been interpreted as reflecting a conformational change in the amino-terminal region of the β chain in deoxy-Hb S compared to that in deoxy-Hb A. Elbaum & Nagel (1981) have found that the rate of hydrolysis of *p*-nitrophenyl acetate in the presence of oxy-Hb S is about 2 times slower than that in the presence of oxy-Hb A. They have shown that this effect originates, very likely, from subtle conformational differences between oxy-Hb S and oxy-Hb A involving the β2 His residues and/or the amino acid residues situated in their vicinity. More recently, X-ray crystallography results on Hb S also suggest that the amino-terminal region of the β chain in deoxy-Hb S (in particular the A helix) is different from that in deoxy-Hb A (W. E. Love, personal communication). Our present ^{1}H NMR results are consistent with these findings, suggesting that the structural basis for these effects consists of a difference in the electrostatic microenvironments of the β2 His residues between Hb A and Hb S in both deoxy and CO forms.

In the present work, we have also found that the pK values of the β146 His residues in Hb S, in both deoxy and ligated forms, differ from those in Hb A (Tables I and II). In the deoxy form, both C2 and C4 protons of β146 His (resonances labeled 3 and 12) give a pK value for β146 His in Hb S that is ~0.28 pH unit lower than that in Hb A. Furthermore, in deoxy-Hb S, both C2 and C4 proton resonances of β146 His in the unprotonated form are shifted downfield relative to their positions in deoxy-Hb A (δ^0 in Table I). According to the X-ray structure of deoxy-Hb A (Fermi, 1975), the β146 His residue from one β chain is situated within ~12 Å of the β6 site on the adjacent β chain. Given this spatial proximity, the differences between Hb S and Hb A in the ^{1}H NMR titration parameters of β146 His could reflect a direct effect of the amino acid substitution at the β6 position on the electrostatic microenvironment of β146 His in the Hb S molecule. It should be pointed out that the difference in the pK values of β146 His between Hb S and Hb A, in both deoxy and CO forms, is not expected to produce a significant difference in the Bohr effect between these two hemoglobins. We have previously shown that, under the experimental conditions used in the present study, the β146 His residues do not contribute significantly to the alkaline Bohr effect of Hb A (Russu et al., 1980).

In addition to the conformational change around the mutation site, the present ^{1}H NMR results indicate that, in the deoxy-Hb S molecule, there are several other regions where the conformations and/or the environments are altered compared to the corresponding one in Hb A. This conclusion is based on the differences between deoxy-Hb S and deoxy-Hb A found in the present work for the resonances labeled 2, 4, 4′, 5, 6, 13, and 18 (Figure 4 and Table I). The origin of these histidine resonances is not yet known. However, the X-ray structure of deoxy-Hb A (Fermi, 1975) indicates that 13 out of the 19 His residues per $\alpha\beta$ dimer of Hb are situated on the surface of the molecule. For these 13 surface His residues all but β2 His, β143 His, and β146 His are located away from the β6 position. Therefore, the additional His resonances for which differences in the ^{1}H NMR titration are detected in deoxy-Hb S compared with deoxy-Hb A should all originate from regions of the Hb S molecule other than that around the mutation site. Five of these resonances (namely, resonances labeled 2, 4, 4′, 5, and 6) are surface His C2 proton resonances. Thus, we could suggest that, in addition to the region around the mutation site, there are at least five other areas on the surface of the deoxy-Hb S molecule where the conformations and/or the electrostatic environments differ from the corresponding ones in deoxy-Hb A. The difference in the ^{1}H NMR titration of the His C4 proton resonance labeled 13 between Hb A and Hb S is more difficult to interpret since, as explained in the preceding paper (Russu et al., 1982), the NMR titration of the resonance labeled 13 probably reflects the average titration of several surface His C4 protons. Thus, the observed difference in deoxy-Hb S may not reflect a conformational change in a specific region on the surface of the Hb S molecule.

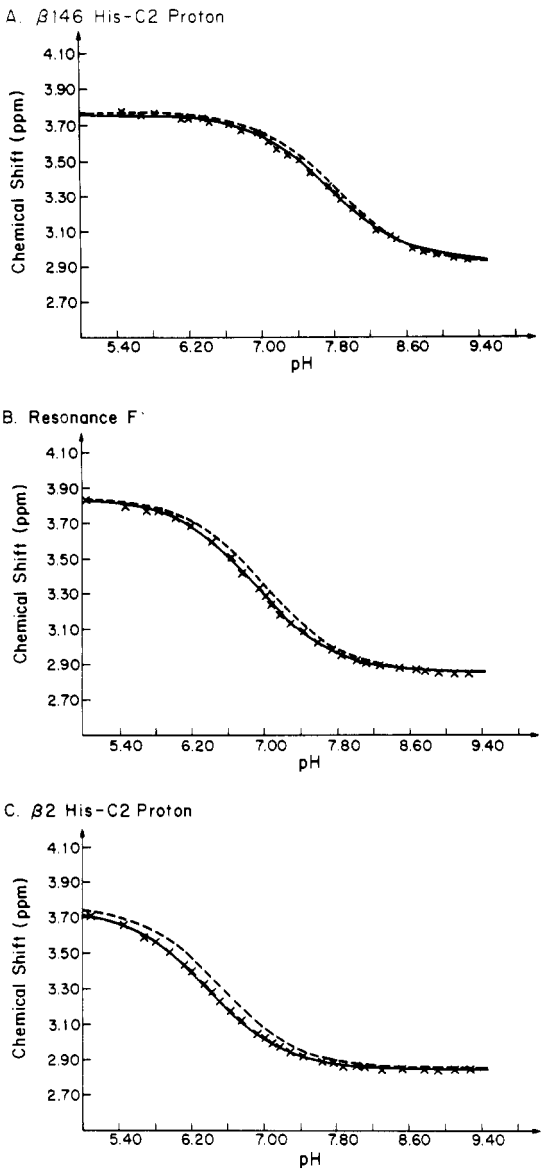

FIGURE 5: ^{1}H NMR titration of histidine resonances in 10% HbCO S in 0.1 M Bis-Tris buffer at 27 °C: (A) resonance labeled C (β146 His C2 proton); (B) resonance labeled F; (C) resonance labeled G (β2 His C2 proton). (×) denotes the experimental points in HbCO S; solid lines are experimental titration curves in HbCO S; dashed lines are experimental titration curves in HbCO A [taken from the preceding paper (Russu et al., 1982)]. The titrated curves were obtained by fitting the corresponding experimental data to eq 1.

On the other hand, it is very likely that the His C4 proton resonance labeled 18 could originate from a surface His residue whose C2 proton is not observed in the present experiments (Russu et al., 1982). If this is the case, the resonance 18 should define an additional area on the surface of the Hb S molecule where a conformational change occurs.

In the carbon monoxy form, we have found that the number of His residues having different pK values in Hb S and Hb A and the magnitude of these differences are greatly diminished. In addition to the β2 and β146 His residues, only one other His C2 proton resonance (namely, the resonance labeled F) has a different pK value in HbCO S compared to that in HbCO A (Figure 5 and Table II). These findings are consistent with the proton binding data of Scholberg et al. (1980), which show that the differential titration of HbCO S and HbCO A deviates only slightly from that expected for the β6 Val → Glu substitution. Our present ^{1}H NMR results suggest that the extent of the conformational changes induced by the amino acid substitution in HbCO S is reduced relative to those in the deoxy form. This suggestion is also consistent with the absence of gelation in ligated Hb S solutions.

The exact nature of the conformational differences observed in this work between Hb S and Hb A is not yet fully understood. At present, it is clear that these changes modify the electrostatic microenvironment as well as the magnetic shielding of specific His C2 protons on the surface of the Hb S molecule. For most of the His residues observed in the present work, these alterations in the electrostatic and magnetic environments are present over the entire pH range investigated (Figures 4 and 5). However, for several His C2 proton resonances in deoxy-Hb S, the deviations of the ^{1}H NMR titration curves from those in deoxy-Hb A appear to occur over specific pH ranges. For instance, the experimental data presented in Figure 4B show that, for pH values lower than 7 and higher than 8, the ^{1}H NMR titration of the β146 His C2 proton in deoxy-Hb S follows quite closely that in deoxy-Hb A whereas, in the pH range from 7 to 8, the β146 His C2 proton in deoxy-Hb S is consistently shifted upfield from its position in deoxy-Hb A. This finding suggests that the difference in the pK value of β146 His between deoxy-Hb S and deoxy-Hb A originates from a specific change in the microenvironment of β146 His over the pH range from 7 to 8. Another example of this kind of pH-dependent change is provided by the ^{1}H NMR titration of the resonance labeled 4′. As shown in Figure 4C, in deoxy-Hb S, resonance 4′ titrates separately from resonance 4 over the pH range from 7.1 to 7.6. However, the pK values and the intrinsic chemical shifts of resonances 4 and 4′ in deoxy-Hb S are, within experimental error, the same (Table I). Therefore, the separate ^{1}H NMR titration of resonance 4′ in deoxy-Hb S very likely reflects an additional specific change in the microenvironment of the corresponding His residue over the pH range from 7.1 to 7.6. These two findings suggest that in deoxy-Hb S, there are surface His residues whose local conformations and/or environments are specifically altered from the corresponding ones in deoxy-Hb A over pH ranges close to that inside the red blood cells. Our present suggestion is consistent with the findings of Bookchin et al. (1978), which show that, over the specific pH range from 7.0 to 7.2, the oxygen affinity of sickle cell blood is significantly different from that in normal blood.

Deviations of ^{1}H NMR Titration of Histidyl Residues of Hb S from Simple Proton Dissociation Equilibrium. As in the case of Hb A, we have found that several histidyl residues of Hb S have ^{1}H NMR titration curves that deviate from that predicted by a simple proton dissociation equilibrium of a single ionizable group (eq 1). To quantitate these deviations, we have also analyzed the experimental data for the eleven His C2 proton resonances in deoxy and HbCO S in terms of eq 2 for asymmetrical ^{1}H NMR titration curves. The results of this analysis are presented in Tables III and IV for deoxy-Hb S and HbCO S, respectively. The results of the same analysis for the His C2 proton resonances of deoxy-Hb A and HbCO A, reported in the preceding paper, are also included for comparison. We have not carried out a similar analysis for the His C4 proton resonances because, for these resonances, the characterization of an asymmetrical titration curve is less accurate due to their lower resolution and their smaller titration shift ($\delta^+ - \delta^0$).

As presented in Table IV, the titration coefficients for the ^{1}H NMR titration of the His C2 proton resonances of HbCO S are, within the experimental error, the same as the corresponding ones in HbCO A. This finding provides an additional support for our suggestion that the β6 Glu → Val substitution

Table III: ^{1}H NMR Titrations of Histidyl Residues in Deoxy-Hb S and Deoxy-Hb A, 10% Solutions in 0.1 M Bis-Tris or 0.1 M Tris Buffer at 27 °C[a]

	deoxy-Hb S				deoxy-Hb A			
resonance no.	pK	δ^+	δ^0	n	pK	δ^+	δ^0	n
1	8.10 ± 0.05	3.97 ± 0.01	2.91 ± 0.04	0.79 ± 0.05	8.13 ± 0.06	3.97 ± 0.04	2.92 ± 0.04	0.87 ± 0.07
2	7.19 ± 0.02	4.00 ± 0.01	2.99 ± 0.01	0.99 ± 0.02	7.29 ± 0.02	4.00 ± 0.01	2.95 ± 0.01	0.91 ± 0.04
3 (β146 C2)	7.76 ± 0.05	3.90 ± 0.01	2.94 ± 0.03	0.74 ± 0.05	7.96 ± 0.05	3.88 ± 0.01	2.91 ± 0.03	0.80 ± 0.05
4	7.07 ± 0.02	3.80 ± 0.02	2.91 ± 0.02	0.95 ± 0.06	7.21 ± 0.03	3.81 ± 0.01	2.86 ± 0.01	0.85 ± 0.04
4′	7.04 ± 0.04	3.79 ± 0.02	2.93 ± 0.02	1.03 ± 0.08				
5	7.75 ± 0.09	3.70 ± 0.02	2.74 ± 0.07	0.67 ± 0.07	7.92 ± 0.05	3.70 ± 0.01	2.65 ± 0.04	0.63 ± 0.04
6	7.01 ± 0.03	3.75 ± 0.02	2.79 ± 0.02	1.00 ± 0.07	7.10 ± 0.03	3.79 ± 0.01	2.72 ± 0.01	0.81 ± 0.04
7	7.03 ± 0.08	3.68 ± 0.07	2.59 ± 0.06	0.57 ± 0.09	7.02 ± 0.08	3.76 ± 0.06	2.54 ± 0.05	0.48 ± 0.06
8	6.59 ± 0.07	3.68 ± 0.05	2.81 ± 0.02	0.75 ± 0.10	6.63 ± 0.09	3.77 ± 0.07	2.73 ± 0.03	0.59 ± 0.08
9 (β143 C2)	6.53 ± 0.07	3.77 ± 0.06	2.56 ± 0.02	0.64 ± 0.06	6.61 ± 0.07	3.84 ± 0.06	2.47 ± 0.03	0.52 ± 0.04
10 (β2 C2)	6.26 ± 0.04	3.78 ± 0.04	2.85 ± 0.01	1.12 ± 0.10	6.32 ± 0.05	3.80 ± 0.03	2.83 ± 0.01	0.88 ± 0.07

[a]Nonlinear least-squares fitting of the experimental data for the His C2 proton resonances to eq 2. The results for HbCO A are reproduced from the preceding paper (Russu et al., 1982).

Table IV: ^{1}H NMR Titration of Histidyl Residues in HbCO S and HbCO A, 10% Solutions in 0.1 M Bis-Tris or 0.1 M Tris Buffer at 27 °C[a]

	HbCO S				HbCO A			
resonance	pK	δ^+	δ^0	n	pK	δ^+	δ^0	n
A	7.89 ± 0.04	3.88 ± 0.01	2.83 ± 0.04	0.81 ± 0.06	7.90 ± 0.03	3.87 ± 0.01	2.84 ± 0.02	0.87 ± 0.05
B	7.83 ± 0.03	3.94 ± 0.02	2.88 ± 0.03	0.87 ± 0.07	7.42 ± 0.02	3.96 ± 0.01	2.84 ± 0.02	0.83 ± 0.04
C (β146 C2)	7.80 ± 0.05	3.79 ± 0.01	2.86 ± 0.03	0.83 ± 0.07	7.86 ± 0.04	3.79 ± 0.01	2.84 ± 0.03	0.82 ± 0.06
D	7.65 ± 0.05	3.82 ± 0.02	2.76 ± 0.04	0.74 ± 0.07	7.68 ± 0.03	3.82 ± 0.01	2.74 ± 0.02	0.82 ± 0.05
E	7.11 ± 0.03	3.84 ± 0.02	2.82 ± 0.02	0.85 ± 0.06	7.14 ± 0.03	3.86 ± 0.02	2.81 ± 0.01	0.81 ± 0.04
F	6.92 ± 0.03	3.82 ± 0.02	2.86 ± 0.01	1.01 ± 0.07	6.99 ± 0.03	3.86 ± 0.02	2.84 ± 0.01	0.91 ± 0.05
G (β2 C2)	6.45 ± 0.04	3.76 ± 0.04	2.84 ± 0.01	0.97 ± 0.07	6.49 ± 0.03	3.80 ± 0.03	2.84 ± 0.01	0.91 ± 0.05
I	6.60 ± 0.08	3.72 ± 0.07	2.69 ± 0.02	0.76 ± 0.08	6.59 ± 0.05	3.74 ± 0.04	2.70 ± 0.01	0.85 ± 0.06
J	6.79 ± 0.07	3.47 ± 0.04	2.66 ± 0.02	0.83 ± 0.10	6.78 ± 0.03	3.48 ± 0.02	2.66 ± 0.01	0.86 ± 0.05
K	5.95 ± 0.15	3.92 ± 0.04	2.53 ± 0.05	0.39 ± 0.09	6.22 ± 0.18	3.87 ± 0.02	2.55 ± 0.03	0.45 ± 0.06

[a]Nonlinear least-squares fitting of the experimental data for the His C2 proton resonances to eq 2. The results for HbCO A are reproduced from the preceding paper (Russu et al., 1982).

affects the conformation of the HbCO S molecule to a lesser extent than that in the deoxy form. On the other hand, in deoxy-Hb S, there are three His C2 proton resonances whose titration coefficients differ from the corresponding ones in deoxy-Hb A (Table III). These resonances are the β2 His C2 proton resonance and the resonances labeled 4′ and 6. In all three cases, the corresponding titration coefficient is equal or closer to unity than that in deoxy-Hb A. A titration coefficient equal or close to unity corresponds to a simple proton dissociation equilibrium of a single ionizable group (eq 1). A deviation from unity of the titration coefficient can be interpreted as indicating the presence of one or more titratable groups in the vicinity of the His residue being observed (Markley, 1975). These neighboring titratable groups can influence the ^{1}H NMR titration of the His residue by changing the pK value of the His residue and/or the intrinsic chemical shifts of the His C2 proton resonance (Shrager et al., 1972). On the basis of these general trends, one could suggest that, in deoxy-Hb S, the His residues whose resonances are labeled 4′, 6, and 10 (β2 His) change their positions relative to their neighboring titratable groups such that the corresponding mutual electrostatic interactions are attenuated or cancelled out. Such changes in the positions of the neighboring titratable groups could also be responsible for the lower pK values of these His residues in deoxy-Hb S compared to those in deoxy-Hb A.

The differences in the titration of the several surface His residues observed here between Hb S and Hb A should be interpreted with caution when attempting to correlate them with the Bohr effect of Hb S. As we have discussed here and in the preceding paper, the ^{1}H NMR titration of a His residue in Hb could reflect the influence of the neighboring titratable groups on both the pK value of the His residue and the intrinsic chemical shift of the His C2 proton resonance. Furthermore, as shown in the present study, in the case of Hb S, the ^{1}H NMR titration of several His C2 proton resonances appears to be influenced by additional changes in the microenvironment of the corresponding His residues and these changes occur over specific pH ranges. As a result, the relationship between the ^{1}H NMR titration curves observed in the present work and the corresponding fractions of charged or uncharged His residues is, at present, more difficult to ascertain in Hb S than in the case of Hb A.

Conclusions

The present ^{1}H NMR results clearly indicate that there are specific differences in the surface conformation of the Hb S molecule compared to that of the Hb A molecule. These alterations in conformation, although very small, appear to be localized in specific areas on the surface of the Hb S molecule, and they are enhanced in the deoxy form relative to the CO form. The exact molecular origin of these changes is not yet fully understood. However, our present results clearly show that ^{1}H NMR spectroscopy can be used successfully *to detect* the areas on the surface of the Hb S molecule where the conformation and/or environment are altered relative to those in Hb A in the solution state. According to the X-ray structure of the deoxy-Hb S molecule (Love et al., 1978), several surface His residues (such as α20, α45, α72, α112, β2, β77, β97, β116, β117, and β146) are situated close to the residues previously found to affect the polymerization of Hb S. This fact suggests that at least some of the His residues of Hb S for which differences in the pK values are observed in the present work could be situated at

or close to the surface areas involved in the polymerization process. This suggestion is strongly supported by our T_1^{-1} measurements of the surface His C2 protons in deoxy-Hb S and deoxy-Hb A (Russu & Ho, 1980). We have found that the seven His residues whose pK values are different between deoxy-Hb S and deoxy-Hb A (e.g., β2 His, β146 His, and the His residues whose ^{1}H NMR resonances are labeled here 2, 4, 4′, 5, and 6) also have larger T_1^{-1} values in deoxy-Hb S compared to those in deoxy-Hb A. Furthermore, the differences in the T_1^{-1} values of these His residues between deoxy-Hb S and deoxy-Hb A are enhanced when the deoxy-Hb S solutions approach gelation by increasing either the temperature or the concentration of Hb S. On the basis of these results, we have proposed that these His residues of Hb S are located at or near the intermolecular contact sites between Hb S tetramers during the early stages of the polymerization process. Thus, the differences in the ^{1}H NMR titration observed in the present work for several His residues in the Hb S and the Hb A molecules appear to be significant for the polymerization process of Hb S.

References

Allen, D. W., & Wyman, J. (1954) *Rev. Hematol. 9*, 155–157.

Arnone, A. V. (1972) *Nature (London) 237*, 146–148.

Asakura, T., Ohnishi, T., Friedman, S., & Schwartz, E. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1594–1598.

Berman, M., Benesch, R., & Benesch, R. E. (1971) *Arch. Biochem. Biophys. 145*, 236–239.

Bookchin, R. M., Ueda, Y., Nagel, R. L., & Landau, L. C. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 57–66, Academic Press, New York.

Charache, S., Grisolia, S., Fiedler, A. J., & Hellegers, A. E. (1970) *J. Clin. Invest. 49*, 806–812.

Curd, J. G., Young, N. S., & Schechter, A. N. (1976) *J. Biol. Chem. 251*, 1290–1295.

Dadok, J., & Sprecher, R. F. (1974) *J. Magn. Reson. 13*, 243–248.

Dean, J., & Schechter, A. N. (1978a) *N. Engl. J. Med. 299*, 752–763.

Dean, J., & Schechter, A. N. (1978b) *N. Engl. J. Med. 299*, 804–811.

Dean, J., & Schechter, A. N. (1978c) *N. Engl. J. Med. 299*, 863–870.

Drabkin, D. L. (1946) *J. Biol. Chem. 164*, 703–723.

Elbaum, D., & Nagel, R. L. (1981) *J. Biol. Chem. 256*, 2280–2283.

Fermi, G. (1975) *J. Mol. Biol. 97*, 237–256.

Fronticelli, C. (1978) *J. Biol. Chem. 253*, 2288–2291.

Fung, L. W.-M., Lin, A. K.-L. C., & Ho, C. (1975) *Biochemistry 14*, 3424–3430.

Ho, C., & Russu, I. M. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 179–193, Academic Press, New York.

Ho, C., Fung, L. W.-M., Lin, A. K.-L. C., Supinski, G. S., & Wiechelman, K. J. (1976) *DHEW Publ. (NIH) (U.S.) NIH 76-1007*, 65–85.

Ingram, V. A. (1956) *Nature (London) 178*, 792–794.

Ip, S. H. C., Johnson, M. L., & Ackers, G. K. (1976) *Biochemistry 15*, 654–660.

IUPAC (1974) *Inf. Bull.: Append. Provis. Nomencl., Symb., Units, Stand. (I.U.P.A.C.) No. 38.*

Li, T.-K., & Johnson, B. P. (1969) *Biochemistry 8*, 2083–2088.

Lindstrom, T. R., & Ho, C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1707–1710.

Love, W. E., Fitzgerald, P. M. D., Hanson, J. C., Royer, W. E., Jr., & Ringle, W. M. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 165–177, Academic Press, New York.

Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–89.

Pennelly, R. R., & Noble, R. W. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 401–411, Academic Press, New York.

Perutz, M. F., Liquori, A. M., & Eirich, F. (1951) *Nature (London) 167*, 929–931.

Russu, I. M., & Ho, C. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6577–6581.

Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043–1052.

Russu, I. M., Ho, N. T., & Ho, C. (1982) *Biochemistry* (preceding paper in this issue).

Scholberg, H. P. F., Fronticelli, C., & Bucci, E. (1980) *J. Biol. Chem. 255*, 8592–8598.

Shrager, R. I., Cohen, J. S., Heller, S. R., Sachs, D. H., & Schechter, A. N. (1972) *Biochemistry 11*, 541–547.

Ueda, Y., Bookchin, R. M., & Nagel, R. L. (1978) *Biochem. Biophys. Res. Commun. 85*, 526–531.

Wishner, B. C., Ward, K. B., Lattman, E. E., & Love, W. E. (1975) *J. Mol. Biol. 98*, 179–194.

Wishner, B. C., Hanson, J. C., Ringle, W. M., & Love, W. E. (1976) *DHEW Publ. (NIH) (U.S.) NIH 76-1007*, 1–29.

Young, N. S., Curd, J. G., Eastlake, A., Furie, B., & Schechter, A. N. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4759–4763.