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A Proton Nuclear Magnetic Resonance Investigation of Histidyl Residues in Human Normal Adult Hemoglobin†

Irina M. Russu, Nancy Tseng Ho, and Chien Ho*

ABSTRACT: High-resolution proton nuclear magnetic resonance (NMR) spectroscopy at 250 MHz has been used to titrate 22 individual surface histidyl residues (11 per $\alpha\beta$ dimer) of human normal adult hemoglobin in both the deoxy and the carbon monoxy forms. The proton resonances of β2, β143, and β146 histidyl residues are assigned by a parallel ^{1}H NMR titration of appropriate mutant and chemically modified hemoglobins. The pK values of the 22 histidyl residues investigated are found to range from 6.35 to 8.07 in the deoxy form and from 6.20 to 7.87 in the carbon monoxy form, in the presence of 0.1 M Bis-Tris or 0.1 M Tris buffer in D_2O with chloride ion concentrations varying from 5 to 60 mM at 27 °C. Four histidyl residues in the deoxy form and one histidyl residue in the carbon monoxy form are found to have proton nuclear magnetic resonance titration curves that deviate greatly from that predicted by the simple proton dissociation equilibrium of a single ionizable group. The proton nuclear magnetic resonance data are used to ascertain the role of several surface histidyl residues in the Bohr effect of hemoglobin under the above-mentioned experimental conditions. Under these experimental conditions, we have found that (i) the β146 histidyl residues do not change their electrostatic environments significantly upon binding of ligand to deoxyhemoglobin and, thus, their contribution to the Bohr effect is negligible, (ii) the β2 histidyl residues have a negative contribution to the Bohr effect, and (iii) the total contribution of the 22 histidyl residues investigated here to the Bohr effect is, in magnitude, comparable to the Bohr effect observed experimentally. These results suggest that the molecular mechanism of the Bohr effect proposed by Perutz [Perutz, M. F. (1970) *Nature (London) 228*, 726–739] is not unique and that the detailed mechanism depends on experimental conditions, such as the solvent composition.

High-resolution proton nuclear magnetic resonance (NMR)[1] spectroscopy is the only experimental technique currently available capable of monitoring the environments and the conformations of individual amino acid residues of a protein molecule in solution. Extensive research carried out in the last decade has shown that this technique is most suitable for the study of the structure–function relationship of hemoglobin (Hb) in solution [for a recent review, see, for example, Ho & Russu (1981)]. A large variety of spectroscopic probes for the Hb molecule are available when using ^{1}H NMR. They extend over the entire Hb molecule from protons situated close to the iron atoms and in the heme pockets (Ho et al., 1973, 1978; Lindstrom & Ho, 1973; Shulman et al., 1975; Takahashi et al., 1980) to those at the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces (Fung & Ho, 1975; Viggiano & Ho, 1979) and further to those situated on the surface of the molecule (Kilmartin et al., 1973; Fung et al., 1975; Ho et al., 1976; Russu et al., 1980). Most of these spectroscopic probes belong to those amino acid residues that have been proposed, on the basis of X-ray diffraction results, to play key roles in the function of Hb (Baldwin, 1975). Among them, the interest of the present work is concentrated on the surface histidyl (His) residues of Hb.

Several His residues of human normal adult hemoglobin (Hb A) have been proposed, on the basis of X-ray diffraction data and the results on mutant and chemically modified hemoglobins, to play an important role in the variation of the oxygen affinity with pH, known as the Bohr effect (Perutz, 1970; Perutz et al., 1980). The involvement of these surface His residues in the Bohr effect originates from the changes in their electrostatic environments accompanying the change in the conformation of the Hb molecule upon ligation. For example, Perutz (1970) has proposed that the carboxyl-terminal β146 His is one of the residues responsible for the

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[1] Abbreviations: NMR, nuclear magnetic resonance; Hb, hemoglobin; HbCO, (carbon monoxy)hemoglobin; Hb A, human normal adult hemoglobin; des-His-Hb, des-β146-histidine-hemoglobin; HbO_2, oxyhemoglobin; 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; Ac-His-MA, *N*-acetyl-L-histidine methylamide; DEAE, diethylaminoethyl.

0006-2960/82/0421-5031$01.25/0

alkaline Bohr effect. The X-ray diffraction data indicate that in the deoxy form the imidazole group of β146 His makes a salt bridge with the carboxyl group of β94 Asp. The formation of this "salt bridge" can raise the pK values of β146 His. In the ligated form, the β146 His–β94 Asp salt bridge is broken, and thus, the pK value of the β146 His should be normal (Perutz, 1970). A more exact quantification of the role of β146 His of Hb A in the alkaline Bohr effect was provided by the direct measurement of its pK values in both deoxy and carbon monoxy forms, carried out in our laboratory by means of ^{1}H NMR (Kilmartin et al., 1973; Russu et al., 1980). We have found that, in the presence of 0.2 M phosphate and 0.2 M NaCl in D_2O, the pK value of β146 His decreases, upon ligation of the Hb A molecule, by ~0.9 pH unit (Kilmartin et al., 1973). This large change in the pK value of β146 His confirmed the prediction made by Perutz (1970) that at physiological pH, this amino acid residue releases ~0.8 proton per Hb tetramer upon ligation. More recently, Russu et al. (1980) have found that, in the absence of phosphate ions and at low concentrations of chloride ions, the pK value of β146 His of Hb A decreases by only 0.13 pH unit upon the transition from the deoxy to the CO state. This result indicates that, under these experimental conditions, β146 His residues do not contribute significantly to the alkaline Bohr effect of Hb A. Nevertheless, we have also found that under the experimental conditions used in this study (i.e., no phosphate ions and chloride ion concentrations ranging from 0.005 to 0.060 M) the total Bohr effect of Hb A has the same magnitude as that in the presence of 0.2 M phosphate and 0.2 M chloride ions (Russu et al., 1980). This finding suggests that, in the absence of phosphate ions and at low concentrations of chloride ions, additional amino acid residues of Hb A must contribute to the alkaline Bohr effect.

The absence of a significant contribution of β146 His to the alkaline Bohr effect under experimental conditions of low ionic strength has been questioned recently by Kilmartin et al. (1980) and by Perutz et al. (1980). They have found that, in the chemically modified des-β146-histidine-hemoglobin (des-His-Hb) and in N-ethylsuccinimide-modified Hb, in the presence of 0.1 M chloride ions, the total Bohr effect is reduced by ~60% compared to that in Hb A. Perutz and co-workers concluded that, since the β146 His residues of these two hemoglobins are deleted or prevented from forming salt bridges with the β94 Asp residues, the corresponding reduction in the alkaline Bohr effect should mean that in Hb A the β146 His residues should be responsible for ~60% of the Bohr effect, even under low ionic strength conditions.

In view of these controversies, we have carried out a detailed ^{1}H NMR titration for 22 surface His residues of Hb (11 per $\alpha\beta$ dimer) in the absence of phosphate and in the presence of low chloride ion concentrations. Since under these experimental conditions the change in the apparent pK values of β146 His upon ligation can account for only ~5% of the Bohr effect, the remaining His residues of Hb A appear to be likely candidates for significant contributions to the alkaline Bohr effect. We have found that the total contribution of these 22 His residues to the alkaline Bohr effect of Hb A under low ionic strength experimental conditions is comparable to the Bohr effect measured experimentally. A preliminary report of our findings was published elsewhere (Russu et al., 1982).

Experimental Procedures

Materials. Hb A was prepared by standard procedures from fresh blood samples obtained from the local blood bank (Lindstrom & Ho, 1972). Hb Deer Lodge (β2 His → Arg) was purified, from a blood sample kindly provided to us by Dr. R. L. Nagel, by anion-exchange chromatography on a DEAE-Sephadex A-50 demountable column at 4 °C. Des-His-Hb was prepared by carboxypeptidase B digestion (Kilmartin et al., 1975) and was kindly supplied to us by J. Fogg. Hb Little Rock (β143 His → Glu) was kindly made available to us by Dr. James O. Alben. The organic phosphates were removed by passing the Hb solutions through a Sephadex G-25 column equilibrated with 0.01 M Tris-HCl buffer containing 0.1 M NaCl at pH 7.6 (Berman et al., 1971). The Hb solutions were exchanged 4 times with D_2O (99.8% deuterium content, Bio-Rad) in order to suppress the intense water proton resonance from the ^{1}H NMR spectra. Hb samples of various pH values were obtained by adding to the Hb solutions 1 M Bis-Tris buffer (for the pH range from 6 to 8) or 1 M Tris buffer (for the pH range from 8 to 9.5) in D_2O. The buffers were titrated with 2 N DCl or 2 N NaOD to appropriate pH values. The final buffer concentration in the Hb samples was 0.1 M. For the pH range below 6, the Hb samples were dialyzed against 0.1 M Bis-Tris buffer in D_2O at the desired pH value. Due to the pH adjustment of the buffers, the concentration of chloride ions in the Hb samples varied from 0.005 to 0.060 M in the pH range studied. The pH values of the Hb samples in the carbon monoxy form were measured on a Beckman pH meter (Model 3500) with a standard combination glass electrode. The pH values of the Hb samples in the deoxy form were measured anaerobically on the same pH meter with a Radiometer microelectrode unit (E5201a). They are given here as direct pH meter readings, without correcting for the deuterium isotope effect on the glass electrode, namely, pD = pH + 0.4 (Glasoe & Long, 1960). The accuracy of the pH measurements was estimated to be ±0.02 pH unit. The deoxygenation of the Hb samples was carried out as described previously (Lindstrom & Ho, 1972).

Methods. High-resolution ^{1}H NMR spectra were obtained by the technique of NMR correlation spectroscopy (Dadok & Sprecher, 1974) on the MPC-HF 250-MHz superconducting spectrometer interfaced with a Sigma 5 computer. The temperature inside the probe was 27 °C. The spectrometer was locked on the residual water signal in each sample. The sweep width was 1460 Hz with a sweep time of 3 s, and each spectrum is the result of 50–100 scans. The proton chemical shifts are expressed as parts per million (ppm) with respect to the residual water proton signal (HDO). The chemical shift scale is presently defined as positive in the low-field direction.[2] The resolution was improved by an exponential multiplication in the time domain of the following form: $[(1 + 2^Q)e^{-2LW_1t}]/[1 + 2^Qe^{-2LW_2t}]$. The accuracy of the chemical shift measurements was ±0.02 ppm.

The titration parameters of the histidine resonances are obtained by fitting the chemical shift, δ, of a given resonance, as a function of pH, to the ^{1}H NMR titration equation describing a simple proton dissociation equilibrium of a single ionizable group (Markley, 1975):

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

where $[H^+]$ is the concentration of hydrogen ions, K is the

[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 used the negative sign to indicate that the chemical shift of a given resonance is downfield from the proton resonance of the residual water (HDP). Hence, this change in sign of the chemical shift scale should be noted when referring to earlier publications from this laboratory.

Table I: ^{1}H NMR Titration of Histidyl Residues in 10% Deoxy-Hb A in 0.1 M Bis-Tris and/or 0.1 M Tris Buffer in D_2O at 27 °C[a]

resonance	pK	δ^+	δ^0	$\delta^+ - \delta^0$	$V \times 10^4$
1	8.07 ± 0.04	3.95 ± 0.01	2.97 ± 0.02	0.98 ± 0.03	7.6
2	7.28 ± 0.02	3.98 ± 0.01	2.97 ± 0.01	1.01 ± 0.02	4.4
3 (β146 C2)	7.98 ± 0.03	3.86 ± 0.01	2.99 ± 0.02	0.87 ± 0.03	7.3
4	7.20 ± 0.03	3.78 ± 0.01	2.90 ± 0.01	0.88 ± 0.02	5.8
5	7.76 ± 0.04	3.64 ± 0.01	2.81 ± 0.02	0.83 ± 0.03	11.7
6	7.10 ± 0.03	3.74 ± 0.01	2.76 ± 0.01	0.98 ± 0.02	7.4
7	7.07 ± 0.06	3.55 ± 0.02	2.72 ± 0.02	0.83 ± 0.04	36.1
8	6.71 ± 0.06	3.63 ± 0.03	2.82 ± 0.02	0.81 ± 0.05	25.1
9 (β143 C2)	6.82 ± 0.07	3.56 ± 0.03	2.60 ± 0.02	0.96 ± 0.05	38.3
10 (β2 C2)	6.35 ± 0.03	3.76 ± 0.02	2.84 ± 0.01	0.92 ± 0.03	7.7
12 (β146 C4)	8.03 ± 0.06	2.78 ± 0.01	2.40 ± 0.01	0.38 ± 0.02	3.3
13	7.49 ± 0.05	2.81 ± 0.01	2.36 ± 0.01	0.45 ± 0.02	3.6
17 (β2 C4)	6.31 ± 0.05	2.50 ± 0.01	2.10 ± 0.01	0.40 ± 0.02	3.7
18	7.24 ± 0.05	1.99 ± 0.01	1.60 ± 0.01	0.39 ± 0.02	3.4

[a] Nonlinear least-squares fitting of experimental data to the ^{1}H NMR equation for the proton dissociation equilibrium of a single ionizable group (eq 1). V is the variance of the nonlinear least-squares fit.

proton dissociation equilibrium constant of the His residue, and δ^+ and δ^0 are the proton chemical shifts in the respective protonated and unprotonated forms of the His residue. The variable parameters of eq 1 are K, δ^+, and δ^0. The chemical shifts of the His C2 proton resonances are also fitted as a function of pH to the following equation (Markley, 1975):

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

where n is the titration coefficient[3] for the ^{1}H NMR titration of this His residue and the rest of the symbols have the same meaning as those in eq 1. The variable parameters in the present case are K, n, δ^+, and δ^0. Both of these analyses of the ^{1}H NMR data have been carried out by using the nonlinear least-squares program NLIN in the Computer Center at the University of Pittsburgh. The Bohr effect of Hb A has been determined under the same experimental conditions as those used in the ^{1}H NMR measurements from the variation of the oxygen affinity as a function of pH, as described previously (Russu et al., 1980).

Results

We have monitored the aromatic proton resonances (1.5–5.0 ppm downfield from HDO) of 10% Hb A solutions in 0.1 M Bis-Tris or 0.1 M Tris buffer in D_2O as a function of pH at 27 °C, in both deoxy and carbon monoxy forms. The pH was varied in 0.05–0.2 pH unit increments over the range from 5.0 to 9.5, and a separate sample was used for each pH value. A parallel ^{1}H NMR titration was also carried out, in both deoxy and carbon monoxy forms, for the following histidine-mutant and histidine-modified hemoglobins: Hb Deer Lodge (β2 His → Arg), Hb Little Rock (β143 His → Gln), and des-His-Hb (β146 His deleted). These modified hemoglobins are used for spectral assignments of β2 His, β143 His, and β146 His.

Figure 1 shows the variation in the aromatic proton resonances of deoxy-Hb A over the pH range from 6.0 to 7.0; included in this figure are only those spectra corresponding to the pH values for which large changes in the spectral pattern are observed. Several representative ^{1}H NMR spectra of Hb Deer Lodge, Hb Little Rock, and des-His-Hb, in the deoxy form, are compared with the corresponding ones of deoxy-Hb A under the same experimental conditions in Figures 2 and 3. Eighteen proton resonances (labeled 1–18 in the spectra shown) can be observed individually as a function of pH in the aromatic proton resonance region of deoxy-Hb A. Among them, resonances 11 and 14 have a constant chemical shift over the entire pH range examined; resonances 15 and 16 change their chemical shift slightly as a function of pH, but due to their strong overlap, their individual ^{1}H NMR titration curves cannot be determined. The experimental results for the ^{1}H NMR titration of the rest of the aromatic proton resonances

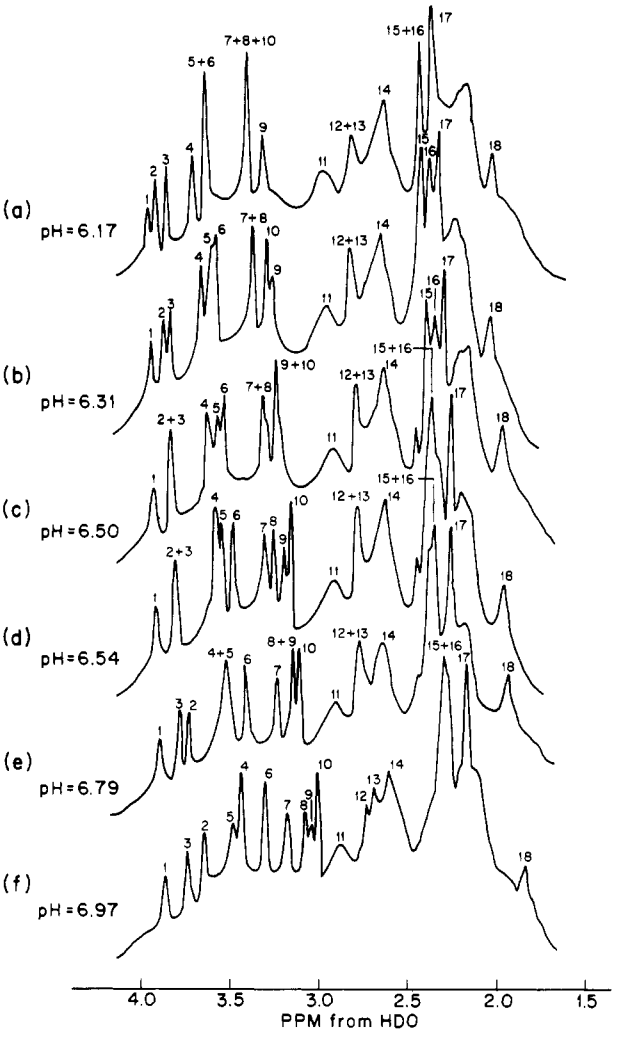

FIGURE 1: 250-MHz aromatic proton resonances of 10% deoxy-Hb A in 0.1 M Bis-Tris buffer in D_2O as a function of pH at 27 °C.

[3] The coefficient n in eq 2 is normally called the Hill coefficient for the ^{1}H NMR titration of the His residue. In the case of hemoglobin, this nomenclature could be confusing due to the well-established use of the term Hill coefficient to describe the cooperativity in oxygen binding. Thus, in the present and the following papers, we have chosen to call the coefficient n in eq 2 the titration coefficient for the ^{1}H NMR titration of the His residue.

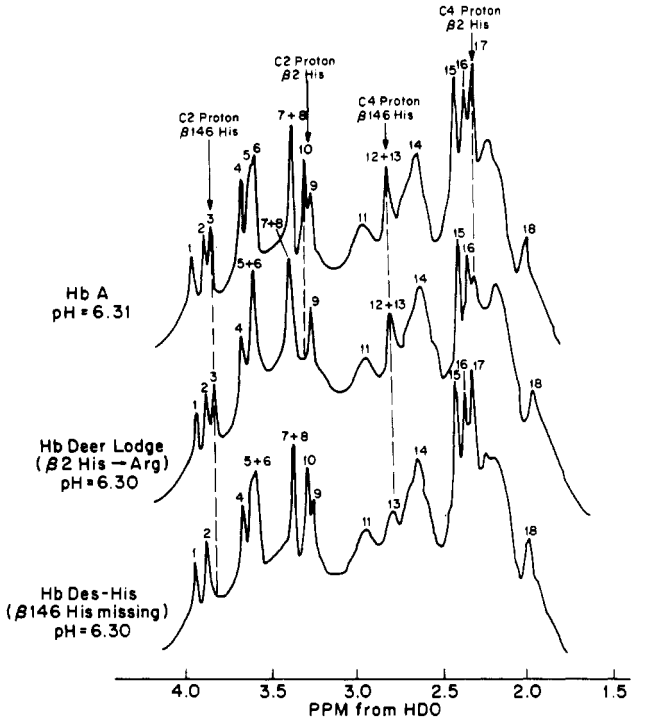

FIGURE 2: 250-MHz aromatic proton resonances of 10% deoxy-Hb A, deoxy-Hb Deer Lodge, and deoxy-des-His-Hb in 0.1 M Bis-Tris buffer in D_2O at 27 °C. Assignment of the C2 and C4 proton resonances of β2 His and β146 His.

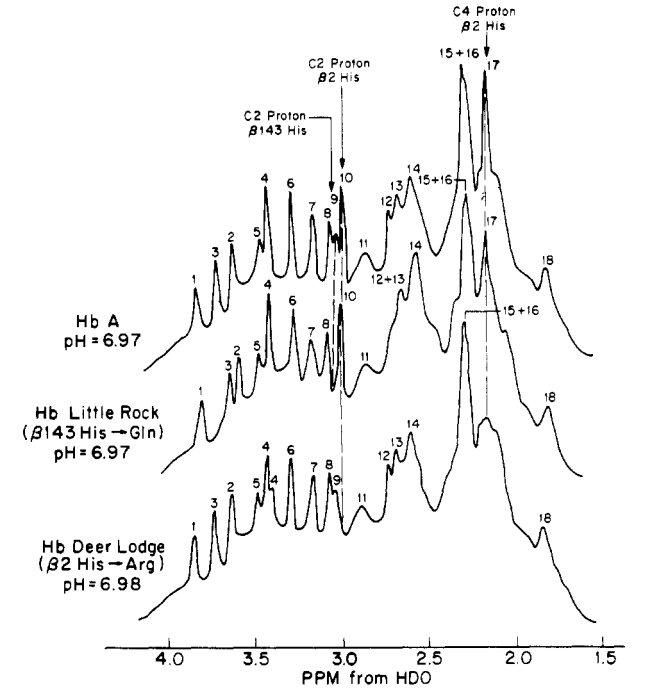

FIGURE 3: 250-MHz aromatic proton resonances of 10% deoxy-Hb A, deoxy-Hb Little Rock, and deoxy-Hb Deer Lodge in 0.1 M Bis-Tris buffer in D_2O at 27 °C. Assignment of the C2 and C4 proton resonances of β2 His and of the C2 proton resonances of β143 His.

of deoxy-Hb A are presented in Figure 4 for resonances 1–10 and in Figure 5 for resonances 12, 13, 17, and 18. The corresponding NMR titration parameters obtained by the nonlinear least-squares fit to eq 1 are presented in Table I.

The aromatic proton resonances of Hb A, in the carbon monoxy form, at pH 6.63 and at 27 °C are shown in Figure 6. A representative ^1H NMR spectrum of HbCO Deer Lodge is compared to the corresponding one of HbCO A under the

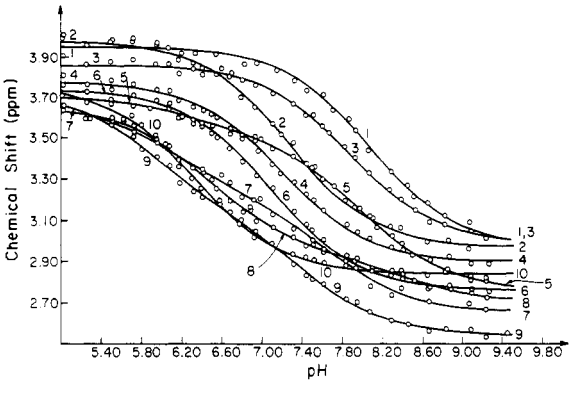

FIGURE 4: ^1H NMR titration of His C2 proton resonances in 10% deoxy-Hb A in 0.1 M Bis-Tris and/or 0.1 M Tris buffer in D_2O at 27 °C. The titration curves for the resonances labeled 1–4, 6, and 10 were obtained by fitting the experimental data to eq 1; those for the resonance labeled 5 and 7–9 were obtained by fitting the experimental data to eq 2.

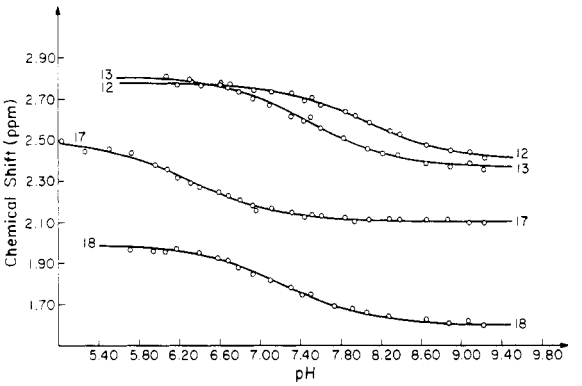

FIGURE 5: ^1H NMR titration of His C4 proton resonances in 10% deoxy-Hb A in 0.1 M Bis-Tris and/or 0.1 M Tris buffer in D_2O at 27 °C. The titration curves were obtained by fitting the experimental data to eq 1.

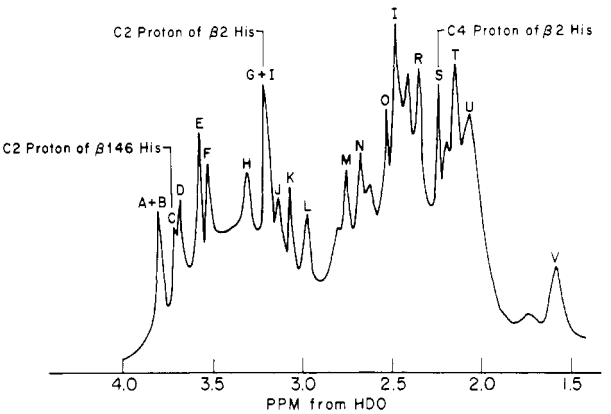

FIGURE 6: 250-MHz aromatic proton resonances of 10% HbCO A in 0.1 M Bis-Tris buffer in D_2O at pH 6.63 and at 27 °C.

same experimental conditions in Figure 7. Twenty-one proton resonances (labeled A–V in the spectra shown) can be observed individually as a function of pH in the aromatic proton resonance region of the ^1H NMR spectra of HbCO A. Resonance V has a constant chemical shift over the pH range examined. Resonance L can be monitored individually only in the range below 6.7, due to its strong overlap with adjacent proton resonances; thus, its complete ^1H NMR titration curve cannot be obtained. The experimental results for the rest of the aromatic proton resonances of HbCO A are shown in Figure 8 for resonances A–K and in Figure 9 for resonances M–U. The corresponding NMR titration parameters of these

Table II: ^{1}H NMR Titration of Histidyl Residues in 10% HbCO A in 0.1 M Bis-Tris and/or 0.1 M Tris Buffer in D_2O at 27 °C[a]

resonance	pK	δ^+	δ^0	$\delta^+ - \delta^0$	$V \times 10^4$
A	7.87 ± 0.02	3.86 ± 0.01	2.88 ± 0.01	0.98 ± 0.02	5.1
B	7.42 ± 0.02	3.93 ± 0.01	2.88 ± 0.01	1.04 ± 0.02	7.1
C (β146 C2)	7.85 ± 0.03	3.77 ± 0.01	2.90 ± 0.02	0.87 ± 0.03	8.0
D	7.65 ± 0.02	3.78 ± 0.01	2.80 ± 0.01	0.98 ± 0.02	8.8
E	7.15 ± 0.02	3.80 ± 0.01	2.85 ± 0.01	0.95 ± 0.2	7.1
F	7.00 ± 0.03	3.84 ± 0.01	2.85 ± 0.01	0.99 ± 0.02	6.1
G (β2 C2)	6.53 ± 0.02	3.77 ± 0.01	2.85 ± 0.01	0.92 ± 0.02	4.3
I	6.55 ± 0.03	3.70 ± 0.02	2.72 ± 0.01	0.98 ± 0.03	8.4
J	6.81 ± 0.03	3.45 ± 0.01	2.68 ± 0.01	0.77 ± 0.02	6.5
H	6.20 ± 0.05	3.88 ± 0.05	3.06 ± 0.01	0.82 ± 0.06	5.7
K	6.76 ± 0.06	3.42 ± 0.03	2.67 ± 0.01	0.75 ± 0.04	21.1
M	7.38 ± 0.05	2.81 ± 0.01	2.36 ± 0.01	0.45 ± 0.02	5.5
N	7.00 ± 0.06	2.70 ± 0.01	2.60 ± 0.01	0.10 ± 0.02	4.2
O	6.73 ± 0.06	2.58 ± 0.01	2.46 ± 0.01	0.12 ± 0.02	3.8
P	7.13 ± 0.06	2.61 ± 0.01	2.11 ± 0.01	0.50 ± 0.02	7.4
R	6.99 ± 0.05	2.47 ± 0.01	2.10 ± 0.01	0.37 ± 0.02	3.2
S (β2 C4)	6.68 ± 0.05	2.43 ± 0.01	2.09 ± 0.01	0.34 ± 0.02	3.4
T	6.50 ± 0.06	2.41 ± 0.01	2.04 ± 0.01	0.37 ± 0.02	3.6
U	7.38 ± 0.06	2.07 ± 0.01	1.96 ± 0.01	0.11 ± 0.02	4.3

[a] Nonlinear least-squares fitting of experimental data to the ^{1}H NMR equation for the proton dissociation equilibrium of a single ionizable group (eq 1). V is the variance of the nonlinear least-squares fit.

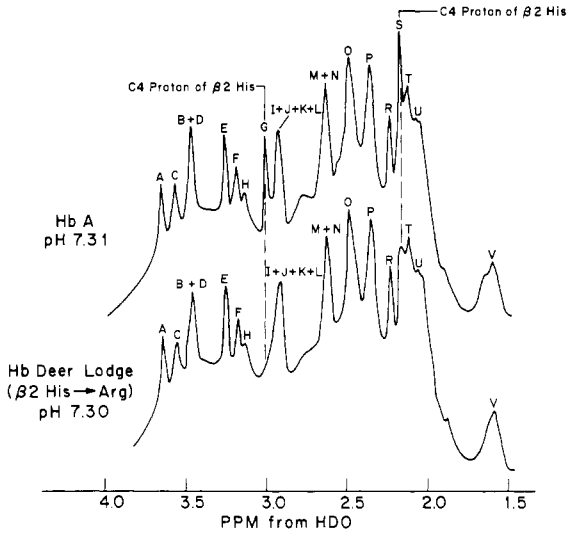

FIGURE 7: 250-MHz aromatic proton resonances of 10% HbCO A and HbCO Deer Lodge in 0.1 M Bis-Tris buffer in D_2O at 27 °C. Assignment of the C2 and C4 proton resonances of β2 His.

aromatic protons resonances of HbCO A, obtained by the nonlinear least-squares fit to eq 1, are presented in Table II.

Discussion

Origin and Assignment of Aromatic Proton Resonances of Hb A. Extensive ^{1}H NMR studies carried out during the last decade have indicated that the region of the ^{1}H NMR spectrum of a peptide or a protein molecule from ~1.5 to ~5.0 ppm downfield from the residual water proton resonance in D_2O originates, in general, from the proton resonances of aromatic amino acid residues [see, for example, Markley (1975) and Wüthrich (1976)]. In the present work, we have found that the large majority of these aromatic proton resonances of Hb A, in both deoxy and carbon monoxy forms, titrate in the pH range from 5.0 to 9.5 and their pK values are distributed around neutrality (Figures 4, 5, 8, and 9 and Tables I and II). This fact indicates that these resonances (i.e., resonances 1–10 and 12, 13, 17, and 18 in the spectra of deoxy-Hb A and those labeled A–U in the spectra of HbCO A) originate, most likely, from protons of His residues. The rest of the aromatic proton resonances of Hb A observed here (e.g., resonances 11 and 14 in the deoxy form and resonance

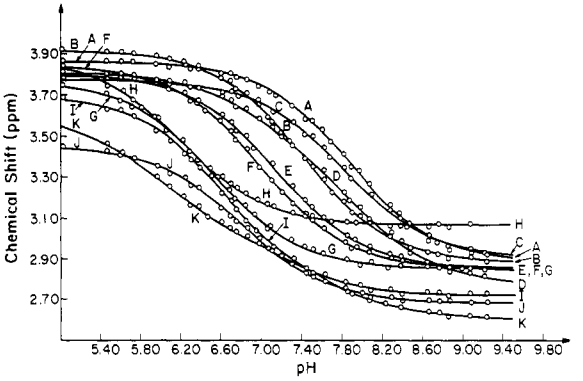

FIGURE 8: ^{1}H NMR titration of His C2 proton resonances in 10% deoxy-HbCO A in 0.1 M Bis-Tris and/or 0.1 M Tris buffer in D_2O at 27 °C. The titration curves were obtained by fitting the experimental data to eq 1 except for the resonance labeled K for which the experimental data were fitted to eq 2.

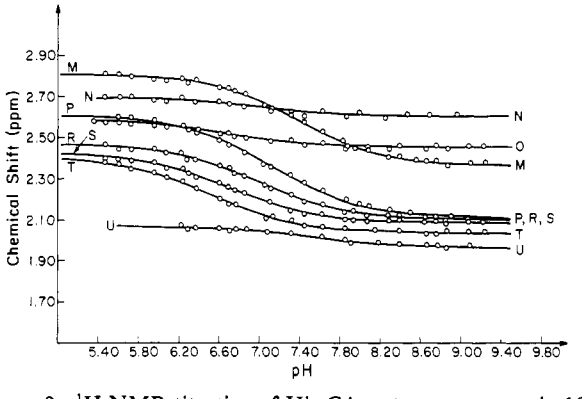

FIGURE 9: ^{1}H NMR titration of His C4 proton resonances in 10% HbCO A in 0.1 M Bis-Tris and/or 0.1 M Tris buffer in D_2O at 27 °C. The titration curves were obtained by fitting the experimental data to eq 1.

V in the CO form) could originate from protons of phenylalanine, tyrosine, and/or tryptophan residues as well as from His residues that are not accessible to solvent and thus do not titrate in a ^{1}H NMR experiment.

For a His residue of a peptide or a protein molecule, the His C2 proton resonance usually occurs between ~3.0 and ~5.0 ppm downfield from HDO, and the His C4 proton resonance usually occurs between ~1.5 and ~3.0 ppm

downfield from HDO (Markley, 1975; Wüthrich, 1976). Furthermore, the pH titration shift, $\delta^+ - \delta^0$, is about 0.9–1.0 ppm for the His C2 protons and about 0.5 ppm or less for the His C4 protons (Markley, 1975). According to these general trends and also on the basis of the data presented in Table I, we can conclude that in the ^{1}H NMR spectra of deoxy-Hb A, resonances 1–10 correspond to the His C2 protons, whereas resonances 12, 13, 17, and 18 correspond to the His C4 protons. Similarly, we can also conclude that, in the ^{1}H NMR spectrum of HbCO A, resonances A–K originate from the His C2 protons and resonances M–U from the His C4 protons.

The parallel ^{1}H NMR titrations carried out for Hb Deer Lodge (β2 His → Arg), des-His-Hb, and Hb Little Rock (β143 His → Gln) have allowed us to assign several resonances to specific His residues of Hb A. For instance, as shown in Figures 2 and 3, resonances 10 and 17 in the ^{1}H NMR spectra of deoxy-Hb A are missing in the corresponding spectra of deoxy-Hb Deer Lodge. Therefore, in deoxy-Hb A, these resonances should originate from the C2 and C4 protons of β2 His, respectively. Similarly, as illustrated in Figure 7, resonances G and S in the ^{1}H NMR spectrum of HbCO A are missing in the spectrum of HbCO Deer Lodge. Thus, in HbCO A, these two resonances should correspond to the C2 and C4 protons of β2 His, respectively. The assignment of the proton resonances of β146 His in the ^{1}H NMR spectra of deoxy-Hb A and HbCO A has been discussed in detail by Russu et al. (1980). We have shown that in the deoxy form resonances 3 and 12 originate from the C2 and C4 protons of β146 His, respectively (Figure 2). In the carbon monoxy form, we have shown that resonance C corresponds to the C2 proton of β146 His (Figure 6) and that resonance L could originate from the C4 proton of β146 His (Figure 6; Russu et al., 1980). As illustrated in Figure 3, resonance 9 in deoxy-Hb A is missing from the corresponding spectrum of deoxy-Hb Little Rock. This suggests that this resonance corresponds to the C2 proton of β143 His of deoxy-Hb A. This assignment should be regarded, at present, as tentative; since due to the limited amount of Hb Little Rock sample available to us, the ^{1}H NMR titration of Hb Little Rock was restricted to only a few pH values. No upfield resonance was consistently missing in the spectra of deoxy-Hb Little Rock compared to the corresponding ones of deoxy-Hb A. This may be due to a difference in the line width between the β143 His C2 proton resonance and the β143 His C4 proton resonance, which makes the observation of the latter more difficult. In the carbon monoxy form, we have found that the ^{1}H NMR spectra of Hb Little Rock differ significantly from the corresponding ones of Hb A in both the aromatic and the ring current shifted resonances (data not shown). These results indicate that the replacement of the β143 His residue by a Gln residue in Hb Little Rock results, in the ligated form, in significant changes in the conformation over the entire Hb Little Rock molecule. Consequently, the assignment of the proton resonances of β143 His in the ligated form requires further investigations of alternative mutant hemoglobins with substitutions at the β143 position.

The remaining histidine resonances observed in the present work have not yet been assigned to specific His residues of Hb A. Nevertheless, since these resonances have line widths on the order of 4–6 Hz at the observation frequency of 250 MHz, it is very likely that they all originate from His residues situated on the surface of the Hb molecule. For such a His residue, the C2 and C4 proton resonances are expected to be narrower due to (i) the internal motions of the His residue relative to the Hb molecule and (ii) the small number of neighboring protons surrounding the His C2 and the His C4 protons (i.e., in a deuterated solvent). The suggestion that the histidine resonances observed here originate from surface His residues of Hb A is confirmed by the specific assignments carried out thus far; namely, the His residues at the β2, β143, and β146 positions are all situated on the surface of the molecule according to the X-ray crystal structure of Hb. Our suggestion is also supported by the fact that all the His resonances observed in the present work are titratable by ^{1}H NMR. This implies that the His residues from which they originate are easily accessible to solvent so that their exchange rates with solvent protons are fast on the NMR time scale (Dwek, 1973). Inspection of the atomic model of the deoxy-Hb A molecule built in our laboratory according to the X-ray coordinates at 2.5-Å resolution (kindly provided to us by Dr. M. F. Perutz) reveals that, of the 19 His residues per $\alpha\beta$ dimer present in Hb, 13 of them are situated on the surface of the molecule. According to the X-ray diffraction data on horse HbCO (Heidner et al., 1976) and human oxy-Hb (Baldwin & Chothia, 1979), the same should be true for the ligated conformation of the Hb molecule. Two of these 13 His residues, α45 His and β97 His, are 6 Å or less from the porphyrin groups of the corresponding chains. Hence, their resonances could be shifted outside the spectral region 1.5–5.0 ppm from HDO by a ring current mechanism (Dwek, 1973). The rest of the 11 surface His residues of Hb should all give rise to proton resonances in the aromatic proton resonance region and should all be observed in the present experiments. At present, we may tentatively suggest that these His residues are at α20, α50, α72, α89, α112, β2, β77, β116, β117, β143, and β146. This suggestion awaits confirmation by further specific assignments of the histidine resonances of Hb A. We have found that, upon addition of 2,3-diphosphoglycerate (2,3-DPG) to deoxy-Hb A in 0.1 M Bis-Tris buffer at 27 °C, resonance 4 resolves into two resonances, 4 and 4′, of comparable intensity (results not shown). The same two resonances, 4 and 4′, can be observed separately at 27 °C in the 250-MHz ^{1}H NMR spectra of sickle deoxy-Hb over a pH range of ~0.5 unit around pH 7.35, even in the absence of 2,3-DPG [see Russu & Ho (1982)]. Moreover, when the ^{1}H NMR spectra of deoxy-Hb A in 0.1 M Bis-Tris buffer at neutral pH are observed at 360 MHz and at 37 °C, resonances 4 and 4′ can also be resolved (Russu & Ho, 1980). These findings indicate that resonance 4 could originate from the C2 protons of two His residues for which the difference in the pK values at 27 °C in deoxy-Hb A is below the resolution currently available. If this is the case, the number of His C2 protons that can be observed in the ^{1}H NMR spectra of deoxy-Hb A at 250 MHz is the same as that in ligated Hb A, i.e., 11 His residues per $\alpha\beta$ dimer or 22 His residues per tetramer.

The number of His C4 proton resonances resolved in the 250-MHz ^{1}H NMR spectra of Hb A, in both deoxy and carbon monoxy forms, is less than that of the His C2 proton resonances (Figures 2, 3, 6, and 7 and Tables I and II). This is due to the fact that the His-C4 proton resonances occur over the spectral region ~1.5 to ~3.0 ppm from HDO where they overlap strongly with the proton resonances of phenylalanine, tyrosine, and tryptophan residues. As a result of this overlap, the spectral resolution attainable for the His C4 proton resonances is much less than that of the His C2 proton resonances. A comparison of the pK values of the His C4 proton resonances observed here to those of the His C2 proton resonances allows us to suggest several C2 and C4 proton resonances originating from the same His residues of Hb. In the carbon monoxy form, these resonances are (i) resonances B

and M (or U, $pK_B = 7.42 \pm 0.02$, $pK_M = 7.38 \pm 0.05$, $pK_U = 7.38 \pm 0.06$), (ii) resonances E and P ($pK_E = 7.15 \pm 0.02$, $pK_P = 7.13 \pm 0.06$), (iii) resonances F and R (or N, $pK_F = 7.00 \pm 0.03$, $pK_R = 6.99 \pm 0.05$, $pK_N = 7.00 \pm 0.06$), (iv) resonances I and T ($pK_I = 6.55 \pm 0.03$, $pK_T = 6.50 \pm 0.06$), and (v) resonances K and O ($pK_K = 6.76 \pm 0.06$, $pK_O = 6.73 \pm 0.06$). For the β2 His residue, the C2 and the C4 proton resonances give pK values within 0.15 pH unit of each other: $pK_G = 6.53 \pm 0.02$ and $pK_S = 6.68 \pm 0.05$. In the deoxy form (Table I), the β146 His C4 proton (resonance 12) has a pK value of 8.03 ± 0.06, which is within 0.05 pH unit of the corresponding C2 proton (resonance 3, pK = 7.98 ± 0.03). The His C4 proton (resonance 17) has a pK value of 6.31 ± 0.05, which is within 0.04 pH unit of the corresponding C2 proton (resonance 10, pK = 6.35 ± 0.03). The His C4 proton resonance labeled 13 has a pK value of 7.49 ± 0.05. This value does not correspond to any of those observed for the His C2 proton resonances in the ^{1}H NMR spectra of deoxy-Hb A. Thus, it is likely that the ^{1}H NMR titration of resonance 13 observed here reflects the averaged titration of two or more single His C4 proton resonances. The His C4 proton resonance labeled 18 in the ^{1}H NMR spectra of deoxy-Hb A has a pK value of 7.24 ± 0.05, which is, within the experimental errors, the same as those of the His C2 proton resonances labeled 2 and 4 (Table I). However, the line width of resonance 18 ($\sim$35 Hz) is much larger than that of the rest of the histidine resonances (4–6 Hz). If one assumes comparable line widths for the C2 and C4 proton resonances of the same His residue, this finding raises the possibility that the C4 proton resonance labeled 18 could originate from a His residue whose C2 proton resonance was not observed in the present experiment (due to its large line width this His C2 proton resonance would not be resolved relative to the envelope of the broad aromatic proton resonances).

Environment of Surface Histidyl Residues in Hemoglobin. The ^{1}H NMR results presented in Tables I and II show a broad distribution in the pK values of the surface His residues of Hb as well as in the intrinsic chemical shifts, δ^+ and δ^0, of their C2 and C4 protons. This indicates the existence of a large variety of local environments of the surface His residues in the Hb molecule in both deoxy and ligated forms in solution. Further insights into the molecular origins of this variety of local environments could be obtained by comparing the present ^{1}H NMR results with those previously reported for histidine model compounds, such as *N*-acetyl-L-histidine methylamide (Ac-His-MA). Tanokura et al. (1978) reported that in D_2O solution, over a range of ionic strengths similar to that used in the present work (0–0.1 M), the pK value of the imidazole in Ac-His-MA varies from 6.52 to 6.57[4] and the intrinsic chemical shifts of the imidazole C2 and the C4 protons are $\delta^+ = 3.81$ ppm and $\delta^0 = 2.83$ ppm (C2 proton) and $\delta^+ = 2.48$ ppm and $\delta^0 = 2.10$ ppm (C4 proton), all downfield from HDO. We have found that, in deoxy-Hb A, the great majority of surface His residues have pK values higher than that observed in Ac-His-MA (Table I). The only exception is β2 His, which has, in deoxy-Hb A, a pK value lower than the model compound. We have attempted to interpret these ^{1}H NMR results in terms of the structural information available from the X-ray diffraction studies of Hb crystals. Inspection of the atomic model of the deoxy-Hb A molecule reveals that many of the surface His residues of deoxy-Hb A are situated in close proximity to negatively charged amino acid residues as a result of the folding of the polypeptide chains. These surface His residues of deoxy-Hb A are listed in Table III. The proximity of the negatively charged amino acid residues should raise the pK values of these His residues above that observed for Ac-His-MA. This prediction made on the basis of the X-ray diffraction data is in good agreement with our ^{1}H NMR results reported in Table I. We have found that at least six His residues of deoxy-Hb A have pK values higher than that observed in Ac-His-MA. As discussed in the previous section, the α45 His, which would also be expected to have a high pK value according to the data presented in Table III, was very likely not observed in the present study. Our ^{1}H NMR results also reveal that in solution the effect of adjacent negatively charged amino acid residues on the ionization properties of a His residue is largely dependent on the specific local conformation of the His residue in the Hb molecule. We have found that among the eleven surface His residues of deoxy-Hb A investigated here, only three, β146 His and the His residues whose C2 proton resonances are labeled 1 and 5, have pK values larger by more than 1 pH unit than the pK value of Ac-His-MA. This finding suggests that as a result of their local conformations in the Hb molecule, these three His residues of deoxy-Hb A form salt bridges with the adjacent negatively charged residues. This suggestion is in good agreement with the X-ray diffraction data, which show that as a result of the conformation of the carboxy-terminal part of the β chains of deoxy-Hb A, β146 His is held in a salt bridge with β94 Asp (Perutz, 1970; Fermi, 1975). The low pK value found in this work for β2 His in deoxy-Hb A can be explained as being due to the proximity of the β2 His residues to the β1 Val residues and to lysine residues such as β82 Lys, β132 Lys, and β144 Lys. All these amino acid residues have pK values higher by more than 1 pH unit than the pK value of a normal His residue [the pK of β1 Val is 7.7 (van Beek & de Bruin, 1980) and the pK of a Lys residue is 10.5 (Edsall & Wyman, 1958)], and they should, thus, lower the pK value of β2 His below that observed in Ac-His-MA.

Table III: Surface Histidyl Residues of Deoxy-Hb A Situated, according to X-ray Diffraction Data, in Close Vicinity of Negatively Charged Amino Acid Residues

surface histidyl residue	neighboring negatively charged residues
α20 His	α23 Glu
α45 His	α-chain porphyrin (propionic group of pyrrole ring III)
α50 His	α30 Glu
α89 His	α85 Asp
α112 His	α27 Glu and α23 Glu
β117 His	β22 Glu
β146 His	β94 Asp

In the carbon monoxy form, we have found that two surface His residues (β2 His and the His residue whose C2 proton resonance is labeled I) have pK values close to the normal value found in Ac-His-MA (Table II). Furthermore, an additional His residue (whose C2 proton resonance is labeled H) has, in HbCO A, a pK value lower than that of the model compound (Table II). The rest of the surface His residues of HbCO A have pK values higher than that of Ac-His-MA. Among them, β146 His has a pK value of 7.85 ± 0.03. This indicates that, under the experimental conditions used in the present work (i.e., 0.1 M Bis-Tris or 0.1 M Tris buffer with chloride ion concentrations ranging from 0.005 to 0.060 M), β146 His in the ligated form still forms a salt bridge with a neighboring negatively charged amino acid residue, which, very likely, is β94 Asp. This finding has been questioned recently by Kil-

[4] These pK values have been corrected from 37 to 27 °C by using the van't Hoff equation and an apparent enthalpy for the proton dissociation of imidazole in Ac-His-MA of 6.4 kcal/mol (Tanokura et al., 1978).

Table IV: ^{1}H NMR Titration of Histidyl Residues in 10% Deoxy-Hb A in 0.1 M Bis-Tris and/or 0.1 M Tris Buffer in D_2O at 27 °C[a]

resonance	pK	δ^+	δ^0	n	$V \times 10^4$
1	8.13 ± 0.06	3.97 ± 0.01	2.92 ± 0.04	0.87 ± 0.07	7.1
2	7.29 ± 0.02	4.00 ± 0.01	2.95 ± 0.01	0.91 ± 0.04	3.8
3 (β146 C2)	7.98 ± 0.05	3.88 ± 0.01	2.91 ± 0.03	0.80 ± 0.05	5.1
4	7.21 ± 0.03	3.81 ± 0.01	2.86 ± 0.01	0.85 ± 0.04	3.9
5	7.92 ± 0.05	3.70 ± 0.01	2.65 ± 0.04	0.63 ± 0.04	3.1
6	7.10 ± 0.03	3.79 ± 0.01	2.72 ± 0.01	0.81 ± 0.04	4.2
7	7.02 ± 0.08	3.76 ± 0.06	2.54 ± 0.05	0.48 ± 0.06	11.7
8	6.63 ± 0.09	3.77 ± 0.07	2.73 ± 0.03	0.59 ± 0.08	15.2
9 (β143 C2)	6.61 ± 0.07	3.84 ± 0.06	2.47 ± 0.03	0.52 ± 0.04	7.2
10 (β2 C2)	6.32 ± 0.05	3.80 ± 0.03	2.83 ± 0.01	0.88 ± 0.07	7.1

[a] Nonlinear least-squares fitting of the experimental data for the His C2 proton resonances to the modified titration equation (eq 2). V is the variance of the nonlinear least-squares fit.

Table V: ^{1}H NMR Titration of Histidyl Residues in 10% HbCO A in 0.1 M Bis-Tris and/or 0.1 M Tris Buffer in D_2O at 27 °C[a]

resonance	pK	δ^+	δ^0	n	$V \times 10^4$
A	7.90 ± 0.03	3.87 ± 0.01	2.84 ± 0.02	0.87 ± 0.05	4.5
B	7.42 ± 0.02	3.96 ± 0.01	2.84 ± 0.02	0.83 ± 0.04	4.8
C (β146 C2)	7.85 ± 0.04	3.79 ± 0.01	2.84 ± 0.03	0.82 ± 0.06	6.7
D	7.68 ± 0.03	3.82 ± 0.01	2.74 ± 0.02	0.82 ± 0.05	5.5
E	7.14 ± 0.03	3.86 ± 0.02	2.81 ± 0.01	0.81 ± 0.04	4.2
F	6.99 ± 0.03	3.86 ± 0.02	2.84 ± 0.01	0.91 ± 0.05	5.7
G (β2 C2)	6.49 ± 0.03	3.80 ± 0.03	2.84 ± 0.01	0.91 ± 0.05	4.1
I	6.59 ± 0.05	3.74 ± 0.04	2.70 ± 0.01	0.85 ± 0.06	7.3
J	6.78 ± 0.03	3.48 ± 0.02	2.66 ± 0.01	0.86 ± 0.05	5.6
K	6.22 ± 0.18	3.87 ± 0.02	2.55 ± 0.03	0.45 ± 0.06	6.0

[a] Nonlinear least-squares fitting of the experimental data for the His C2 proton resonances to the modified titration equation (eq 2). V is the variance of the nonlinear least-squares fit.

martin et al. (1980). They have found that, in horse met-Hb crystals prepared under salt-free conditions, the carboxyl-terminal salt bridge involving the β146 His is missing from the X-ray diffraction electron density maps. Kilmartin et al. (1980) argued that these X-ray diffraction results on crystals of horse met-Hb should be relevant to solutions of HbCO A under low ionic strength conditions because (i) human and horse hemoglobins have identical alkaline Bohr effects and they have the same amino acid sequences in the terminal region of the β chains and (ii) the absence of the salt bridge β146 His–β94 Asp from high-spin met-Hb makes very likely its absence from low-spin HbCO. The significance of the X-ray diffraction data on horse Hb for the local conformations of surface His residues, such as β146 His, in solutions of human Hb is questionable in view of the ^{1}H NMR results of Brown et al. (1976) and of Brown & Campbell (1976). They have shown that in the ^{1}H NMR spectra of human HbO_2 A at 37 °C, one can observe 11–14 surface His resonances whereas, in horse HbO_2, under the same experimental conditions, only nine surface His resonances are present. Among them, six surface His resonances have ^{1}H NMR titration curves that are, in horse HbO_2, significantly different from those in human HbO_2. These results, therefore, show that although in the X-ray diffraction studies human and horse Hb appear to have nearly identical overall structures (Kilmartin et al., 1980), the local conformations of the surface His residues in these two hemoglobins are quite different in solution. The relevance of the X-ray diffraction data on crystals of Hb in the high-spin ferric form to our present findings on solutions of Hb in the low-spin carbon monoxy form is also questionable based on the ^{1}H NMR results of Greenfield & Williams (1972). These authors have shown that the aromatic proton resonance region of low-spin oxy-Hb A differs greatly from that of high-spin met-Hb A. Furthermore, Greenfield & Williams (1972) have found that at least one His resonance in the ^{1}H NMR spectrum of met-Hb A has a pK value significantly different from the corresponding one in HbO_2 A. All these results clearly indicate that the absence of the β146 His–β94 Asp salt bridge observed by Kilmartin et al. (1980) in crystals of horse met-Hb does not represent sufficient experimental evidence to support the absence of this salt bridge in the solutions of HbCO A investigated in the present study.

Our conclusion that a large variety of local environments of the surface His residues exists in both deoxy-Hb A and HbCO A is also supported by the large variation in the intrinsic chemical shifts (δ^+ and δ^0) of their C2 and C4 proton resonances (Tables I and II) compared to the corresponding ones in Ac-His-MA. In this respect, it is interesting to note that the electrostatic interactions affecting the ionization properties of a His residue in Hb are not always responsible to the same extent for the specific shielding or deshielding of the histidine protons. For example, the δ^+ and δ^0 values of the His C2 proton resonance 2 are practically the same as the corresponding ones of the highly abnormal His C2 proton resonance 1 (Table I); yet, the pK value of resonance 2 is much closer to that of Ac-His-MA than is that of resonance 1. Conversely, the chemical shift of the His C2 proton resonance 5 in the protonated form (δ^+) is below that observed for the imidazole C2 proton in Ac-His-MA; yet, the pK value of this His residue is greatly raised above the pK value of Ac-His-MA.

Deviations of ^{1}H NMR Titration of Histidyl Residues of Hb from Simple Proton Dissociation Equilibrium. Inspection of the experimental data presented in Figures 4 and 5 reveals that the ^{1}H NMR titration curves of several surface His residues in both the deoxy and carbon monoxy forms of Hb A deviate greatly from the sigmoidal pattern predicted by a simple proton dissociation equilibrium of a single ionizable group (eq 1). To quantify these deviations, we have fitted the experimental data for the ^{1}H NMR titration of the His C2 protons to the modified titration equation (eq 2). The results of this analysis are presented in Tables IV and V for deoxy-Hb A and HbCO A, respectively. We have not carried out a similar analysis for the His C4 proton resonances because the characterization of an abnormal titration curve is less accurate

for these resonances due to their lower resolution and smaller pH titration shift ($\delta^+-\delta^0$).

Large deviations from unity of the titration coefficient,[3] n, in eq 2 are usually interpreted as indicating the presence of one or more groups in the vicinity of the His residue being observed, which titrate over the same pH range as the His residue (Markley, 1975). In the present work, we have found that the His C2 proton resonances labeled 5, 7, 8, and 9 (β143 His C2 proton) in deoxy-Hb A (Table IV) and the His C2 proton resonance labeled K in HbCO A (Table V) show the largest deviations of the titration coefficient from unity. These results suggest that the ^{1}H NMR titration of these His C2 proton resonances in Hb is strongly affected by the titration of neighboring amino acid residues. Our preliminary ^{1}H NMR results on Hb A_2 ($\alpha_2\delta_2$, β116 His → δ116 Arg, β117 His → δ117 Asn) indicate that the resonances labeled 7 and 8 in the spectra of deoxy-Hb A could originate from the C2 protons of β116 His and β117 His (results not shown). In view of these preliminary findings, we suggest that the large deviations from unity of the titration coefficients for resonances 7 and 8, observed here, reflect the mutual interaction between β116 His and β117 His residues. The deviation from unity of the titration coefficient of the resonance tentatively assigned to the β143 His C2 proton is not yet understood. According to the X-ray structure of deoxy-Hb A (Fermi, 1975), the β143 His is situated in the gap between the two β chains, being surrounded by the β2 His and β1 Val from the adjacent β chain and by the β82 Lys and β144 Lys from the same β chain. The possibility that a mutual interaction between β143 His and β2 His is responsible for the titration coefficient of the β143 His C2 proton resonance can be excluded at present since no corresponding deviation of the titration coefficient from unity has been detected for the β2 His C2 proton resonance (resonance 10, Table IV). This leads us to suggest a possible interaction between β143 His and the neighboring Lys and Val residues, perhaps mediated by the chloride ions present in the solution. This suggestion requires further investigation and should be regarded at present as tentative. An understanding of the structural basis for the deviation from unity of the titration coefficient of resonance 5 in deoxy-Hb A and resonance K in HbCO A awaits their assignments to specific His residues.

For the rest of the surface His residues of Hb A, in both deoxy and CO forms, the titration coefficient of their ^{1}H NMR titration curves ranges from 0.8 to 1. These small deviations from unity of the titration coefficient very likely reflect an overall electrostatic effect that results, as Matthew et al. (1979a,b) have clearly demonstrated, in the variation of the proton dissociation equilibrium constants of the His residues of Hb with pH and ionic strength. These variations have been shown to be the largest under low ionic strength conditions, similar to those used in the present work (Matthew et al., 1979a).

Comparison with Other Titration Studies of Histidyl Residues of Hb A. Several other ^{1}H NMR investigations of Hb A, in both deoxy and ligated forms, in excellent agreement with the results presented here, have shown that a large variety of local conformations and environments exist for the surface His residues in the Hb molecule (Greenfield & Williams, 1972; Brown et al., 1976; Brown & Campbell, 1976; Ferrige et al., 1979). Greenfield & Williams (1972) have found that at least nine His C2 proton resonances can be observed in the 100-MHz ^{1}H NMR spectrum of Hb A in 0.1 M phosphate buffer at 32 °C. The pK values of these His resonances range from 6.8 to 8.1 in the deoxy form and from 6.3 to 7.7 in the oxy form. Brown et al. (1976) and Brown & Campbell (1976) have observed in the 270-MHz ^{1}H NMR spectrum of Hb A 12 His C2 proton resonances in the deoxy form and 11–14 His C2 proton resonances in the oxy form, both in the presence of 0.067–0.1 M chloride, 0.021 M phosphate, and 0.03 M sulfate at 37 °C. In the oxy form, the pK values of these His residues range from 6.2 to 7.7. The greater number of His C2 proton resonances observed in these studies compared to that found here could be due to the difference in temperature between these two investigations as well as to the superior resolution attainable in the ^{1}H NMR spectra by using the $90°-\tau-180°-\tau$ spin-echo pulse sequence (Brown et al., 1976; Brown & Campbell, 1976). Furthermore, it is very likely that, in the ligated state, at the intermediate concentrations of anions used by Brown & Campbell (1976), the C2 proton of β146 His can give rise to two proton resonances in the aromatic proton region of the ^{1}H NMR spectrum as reported by Russu et al. (1980). Ferrige et al. (1979) have observed 11 proton resonances in the aromatic proton resonance region of the 90-MHz ^{1}H NMR spectrum of deoxy-Hb A in the presence of 0.1 M chloride ions at 37 °C. The pK values of these His resonances range from 6.67 to 8.10. They have also found that two of the His C2 proton resonances of deoxy-Hb A are affected by the presence of 2,3-DPG; accordingly, they have assigned these two resonances to the β2 and β143 His C2 protons. Their assignments appear to be consistent with those of the β2 His and β143 His resonances proposed in the present work.

In all these previous studies, the ^{1}H NMR titration of the His C2 protons in the Hb A molecule was assumed to be that predicted by a simple proton dissociation equilibrium of a single ionizable group, which can be described by eq 1, and the His titration curves were constructed from the experimental data accordingly. In the present work, by carrying out a parallel ^{1}H NMR titration of appropriate mutant and chemically modified hemoglobins, we are able to monitor individually the His C2 proton resonances of Hb. Thus, we have found that the assumption of an ideal ^{1}H NMR titration is not valid for all His C2 proton resonances of Hb and that, in fact, several His C2 proton resonances in both deoxy-Hb A and HbCO A have ^{1}H NMR titration curves that deviate greatly from that predicted by a simple proton dissociation equilibrium of a single charged group (see the previous section).

The ^{1}H NMR titration method has been used previously in our laboratory to measure the pK values of β146 His in both deoxy-Hb A and HbCO A (Kilmartin et al., 1973). We have found that the pK values of β146 His are 8.0 in the deoxy form in the presence of 0.1 M phosphate and 0.2 M chloride ions and 7.1 in the CO form in the presence of 0.2 M phosphate and 0.2 M chloride ions, both at 30 °C. Similar results have been reported subsequently by Kilmartin et al. (1978) for Hb A in the presence of a 2-fold excess of inositol hexaphosphate (IHP) per tetramer. The pK value of 7.98 found in the present work for β146 His in deoxy-Hb A in 0.1 M Bis-Tris buffer at 27 °C is in good agreement with both sets of measurements. However, in the CO form, the pK value of 7.85 found in the present work for β146 His in 0.1 M Bis-Tris buffer is different from that found previously in this laboratory in the presence of 0.2 M phosphate and 0.2 M chloride ions (Kilmartin et al., 1973) as well as from that found by Kilmartin et al. (1978) in the presence of a 2-fold excess of IHP per tetramer. We have previously shown that these differences can be explained by the specific effect of phosphate (both inorganic and organic) and chloride ions on the local conformation of β146 His in

HbCO A (Russu et al., 1980). We have proposed that in solution the β146 His residue is involved in an equilibrium between the two conformations inferred from the X-ray diffraction studies (Perutz, 1970): (i) the salt bridge conformation where β146 His is interacting electrostatically with β94 Asp and (ii) the free conformation where β146 His is floating freely into the solvent. Our previous ^{1}H NMR data have suggested that in the CO form the equilibrium constant for this change in the conformation of β146 His depends on various factors (Russu et al., 1980). Large concentrations of anions, such as phosphate (both inorganic and organic) and chloride ions, could greatly shift this equilibrium toward the free conformation of β146 His, and thus, its pK value in HbCO A could be lowered under these experimental conditions.

In the present work, we have found that the pK values of β2 His are 6.35 ± 0.03 in deoxy-Hb A and 6.53 ± 0.02 in HbCO A, both in 0.1 M Bis-Tris buffer at 27 °C. These values are in good agreement with the pK value of 6.4 measured by Nishikura (1978) using the hydrogen–tritium exchange for β2 His in deoxy and CO forms of Hb A in 0.05 M Bis-Tris buffer plus 0.01 M Cl^- at 37 °C. On the other hand, the pK values of β2 His found here are different from those measured by Ohe & Kajita (1977, 1980) using the technique of hydrogen–deuterium exchange. They reported that the pK value of β2 His in the deoxy and oxy forms of Hb A in 0.2 M Tris buffer at 36.5 °C is 6.9 and that the pK value of β2 His of Hb A in deoxy and CO forms in 0.1 M Bis-Tris buffer plus 0.1 M NaCl at 36.5 °C is 6.7. In addition, there are other differences in the pK values reported in the present work and those reported by Ohe & Kajita (1980). Of special interest to this work are the pK values of β146 His and β143 His. Ohe & Kajita (1980) reported that the pK values of β146 His of deoxy-Hb A and HbCO A in 0.1 M Bis-Tris buffer plus 0.1 NaCl at 36.5 °C are 8.1 and 7.0, respectively. The value for β146 His in the CO form reported by Ohe & Kajita (1980) is very different from our figure. They have also reported that in the presence of 0.1 M Bis-Tris plus 0.1 M NaCl at 36.5 °C, the pK value of β143 His in deoxy-Hb A is 5.6, which is again very different from our value of pK = 6.82 ± 0.07 for β143 His in 0.1 M Bis-Tris at 27 °C. The reasons for these differences are not clear at present. However, it should be mentioned that factors such as subunit dissociation and the incubation of the Hb solutions at 36 °C for 24 h in the presence of a 2-fold molar excess of dithionite may well have affected the exchange reactions used in these measurements. Furthermore, as clearly demonstrated by Gurd and co-workers (Matthew et al., 1979a,b; Flanagan et al., 1981), the surface His residues in Hb have different degrees of exposure to solvent, described, for example, in terms of their individual solvent accessibility parameters. Such variations in the extent of exposure or burial of a His residue could complicate the determination of the pK values from hydrogen–deuterium exchange data.

Contributions of β2 His and β146 His Residues to Bohr Effect of Hb A. The contributions of the β2 His and β146 *His* residues to the alkaline Bohr effect calculated on the basis of their pK values in the deoxy and CO forms of Hb A (Tables I and II, respectively) are shown in Figure 10. Also shown in this figure is the total Bohr effect of Hb A measured as described previously (Russu et al., 1980) under the same experimental conditions as those used in the ^{1}H NMR titration.

In the case of β2 His, we have found that when the Hb A molecule undergoes the transition from the deoxy to the CO form in the absence of phosphate ions and at low concentrations of chloride ions, the β2 His residue captures rather than

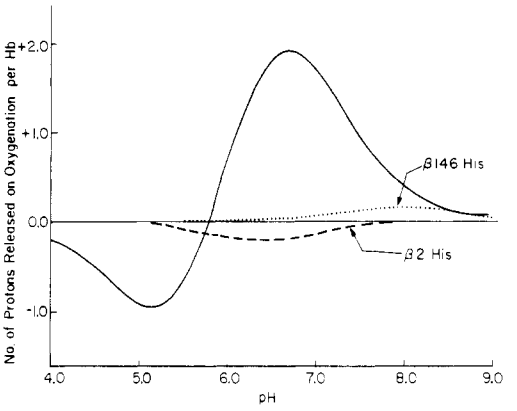

FIGURE 10: Contributions of β146 His and β2 His to Bohr effect of Hb A in 0.1 M Bis-Tris and/or 0.1 M Tris buffer in D_2O at 27 °C, calculated on the basis of corresponding pK values given in Tables I and II. The solid line is the Bohr effect measured experimentally from the variation of the oxygen affinity with pH as described previously (Russu et al., 1980).

releases protons. Thus, under these experimental conditions, the β2 His has a slight negative contribution to the alkaline Bohr effect of Hb A.

In the case of β146 His, we have found that, under the experimental conditions used in the present work, namely, 0.1 Bis-Tris and/or 0.1 M Tris buffer with chloride ion concentrations ranging from 0.005 to 0.060 M, the contribution of β146 His to the alkaline Bohr effect of Hb is less than 5%. On the other hand, according to the previous ^{1}H NMR results of this laboratory in the presence of 0.2 M potassium phosphate plus 0.2 M NaCl, the change in the pK value of β146 His between deoxy-Hb A and HbCO A can account for ~50% of the alkaline Bohr effect (Kilmartin et al., 1973; Russu et al., 1980). Furthermore, in the presence of 1.5–2-fold excess of IHP per Hb tetramer the change in the pK value of β146 His upon ligation can account for ~25% of the observed alkaline Bohr effect (Kilmartin et al., 1978).

All these results clearly indicate that the contributions of specific His residues, such as β146 His and β2 His, to the alkaline Bohr effect on Hb are dependent upon medium factors, such as the amounts of phosphate (both inorganic and organic) and chloride ions present in solution. The experimental evidence accumulated thus far suggests that this dependence is the result of two general effects. First, the pK values of the Bohr groups of Hb, such as the surface His residues, can be changed specifically in the deoxy and ligated Hb by the binding of chloride ions (de Bruin et al., 1974; Rollema et al., 1975; van Beek et al., 1979; van Beek & de Bruin, 1980; Matthew et al., 1979b, 1981), 2,3-DPG (de Bruin et al., 1974; Kilmartin, 1974), and IHP (Kilmartin, 1973). Second, the quantity and the nature of the anions present in solution can also influence the nature of the conformational changes occurring in the Hb molecule upon ligation. For example, the ^{1}H NMR studies of Viggaino & Ho (1979) and Viggiano et al. (1979) have shown that the tertiary structure changes around the heme pockets of the α and β chains of Hb A upon ligation are dependent upon the presence of 2,3-DPG or IHP and they are not always simply linked to the changes in the quaternary structure as manifested by the breaking and forming of the hydrogen bond between α42 Tyr and β99 Asp. Furthermore, Lindstrom & Ho (1973) have found that the detailed tertiary structures around the heme pockets in ligated Hb are strongly dependent upon the nature of the ligand as well as upon the pH and the presence of chloride and inorganic and organic phosphate ions. For a review of all this experi-

mental evidence, the reader is referred to Ho & Russu (1981). Our ^{1}H NMR results on β146 His indicate that these factors can also affect the stability of the intrasubunit salt bridge between β146 His and β94 Asp in ligated Hb (Russu et al., 1980). On the basis of available experimental evidence, we can thus conclude that the molecular mechanism of the alkaline Bohr effect could vary substantially depending on the experimental conditions.

An alternative method for investigating the role of a given amino acid residue in the Bohr effect is the study of mutant and chemically modified hemoglobins in which the amino acid residue of interest is either completely removed by enzymatic digestion or altered by mutation. This approach has been used to demonstrate the role of several surface His residues, such as β146 His and β143 His, in the Bohr effect of Hb (Kilmartin & Wootton, 1970; Perutz et al., 1980). Our present ^{1}H NMR results raise several questions about the validity of using mutant or chemically modified hemoglobins as general model systems for the Bohr effect of Hb A. For instance, in view of our present ^{1}H NMR findings, the reduction in the Bohr effect of des-His-Hb found by Kilmartin et al. (1980) in 0.1 M Bis-Tris buffer and at low concentrations of chloride ions cannot be interpreted as conclusive evidence for a 60% contribution of the β146 His to the Bohr effect. As discussed above, under these experimental conditions, β146 His does not significantly change the electrostatic environment upon ligation, and thus, its contribution to the Bohr effect is quite small. These results, therefore, suggest that the reduction of the alkaline Bohr effect in des-His-Hb cannot be interpreted at the molecular level as simply being due to the removal of β146 His in this Hb. In fact, it is very likely that the reduction of the alkaline Bohr effect in des-His-Hb also results from additional subtle changes in the conformation of the Hb molecule, generated by the removal of the β146 His residue. Such changes in the conformation of the des-His-Hb molecule are consistent with the different kinetic properties observed by Moffat et al. (1973) for the binding of oxygen, carbon monoxide, and *n*-butyl isocyanide to this modified hemoglobin compared to Hb A. Disturbances of the conformation of the des-His-Hb molecule in solution have been detected as in alterations in the chemical shifts of several surface His residues in des-His-Hb compared to the corresponding ones in Hb A (Kilmartin et al., 1973, 1978). It has been argued that similar changes in the chemical shift of the His resonances could account for the absence of resonance C in the ^{1}H NMR spectrum of des-His-Hb in the CO form (Russu et al., 1980), and thus, the present assignment of the β146 His C2 proton resonance in the spectrum of HbCO A is not the correct one (Kilmartin et al., 1980). Our ^{1}H NMR results clearly show that this is not the case. First, no additional His resonance has been detected in the ^{1}H NMR spectrum of des-His-Hb compared to the corresponding spectrum of Hb A; this should be the case if one assumes that resonance C does not originate from β146 His and is simply shifted to a different spectral position in the spectrum of des-His-Hb. Second, in the pH range of the β146 His titration, no His resonances other than resonance C were missing in the spectra of des-His-Hb compared to the spectra of Hb A; since β146 His is a surface His residue, its absence in des-His-Hb should be reflected by at least one missing resonance in the ^{1}H NMR spectrum of des-His-Hb compared to the corressponding spectrum of Hb A.

Our suggestion that there is no simple or direct relationship between the replacement or the deletion of β146 His and the corresponding reduction in the alkaline Bohr effect is also supported by the Bohr effect of Hb Cochin Port-Royal (β146 His → Arg). Kilmartin and co-workers have found that the Bohr effect of Hb Cochin Port-Royal in 0.1 M KCl at 25 °C is reduced by only about 25% relative to that in Hb A (Wajcman et al., 1975). According to Perutz's model (1970) and the results on other mutant or chemically modified hemoglobins where β146 His is absent or prevented from forming its salt bridge with β94 Asp (Kilmartin et al., 1980; Perutz et al., 1980), the Bohr effect of Hb Cochin Port-Royal should be reduced by about 50%. This is not the case, indicating that, in these mutant or chemically modified hemoglobins, additional mechanisms for the reduction of the Bohr effect should be considered. Another example of the limited validity of using mutant hemoglobins as models for the Bohr effect of Hb A is provided by the recent studies of Hb Little Rock (β143 His → Gln) carried out by Perutz et al. (1980). They have found that in 0.1 M KCl at 25 °C, the Bohr effect of Hb Little Rock is reduced. As mentioned under Results, our ^{1}H NMR data show that the surface His resonances as well as the ring current shifted resonances of Hb Little Rock in the CO form are quite different from the corresponding ones of Hb A, both in 0.1 M Bis-Tris buffer at 27 °C. These results indicate that, at low concentrations of chloride ions, the surface conformation and the conformation of the heme pockets of the α and β chains in Hb Little Rock in the CO form are different from those in Hb A. These differences in conformation between Hb A and Hb Little Rock in the CO form suggest that the mechanism for the reduction of the Bohr effect in Hb Little Rock is more complex than the simple replacement of the β143 His residue by a glutamine residue.

Total Contribution of Surface Histidyl Residues to Bohr Effect of Hb A. The total contribution, $\Delta\bar{X}$, of the 22 surface histidyl residues observed in the present study of the Bohr effect can be expressed by the following equation:

$$\Delta\bar{X} = 2\sum_{i=1}^{11}[f^+_i(\text{deoxy}) - f^+_i(\text{CO})] \qquad (3)$$

where f^+_i(deoxy) and f^+_i(CO) are the fractions of charged His residues in the deoxy and CO forms, respectively. The factor 2 was introduced due to the presence of two His residues of one kind per Hb tetramer. Equation 3 is based on the assumption that the same 22 His residues of Hb A are observed in the deoxy form as in the CO form.

The fractions f^+_i of charged His residues can be calculated from the Henderson–Hasselbalch equation as follows:

$$f^+_i = [H^+]/([H^+] + K_i) \qquad (4)$$

where K_i is the proton dissociation constant of a given His residue. In the present work, we have found that the ^{1}H NMR titration of the His C2 protons in Hb cannot always be described by a simple proton dissociation equilibrium of a single ionizable group. These deviations from the ideal ^{1}H NMR titration can be accounted for by (i) a thermodynamic interaction in which the titration of the neighboring group affects the acid–base equilibrium of the His residue and (ii) a spectroscopic interaction in which the titration of the neighboring group changes the intrinsic chemical shifts (δ^+ and δ^0) of the His C2 proton (Shrager et al., 1972). We have previously shown that although the physical nature of these two interactions is quite different, each of them can account for the ^{1}H NMR titration curves that deviate from the simple dissociation of a single ionizable group (Russu, 1979).

In order to determine how these interactions affect the fractions f^+_i of chaged His residues, we have fitted the data for each of the 11 His C2 proton resonances in both the deoxy

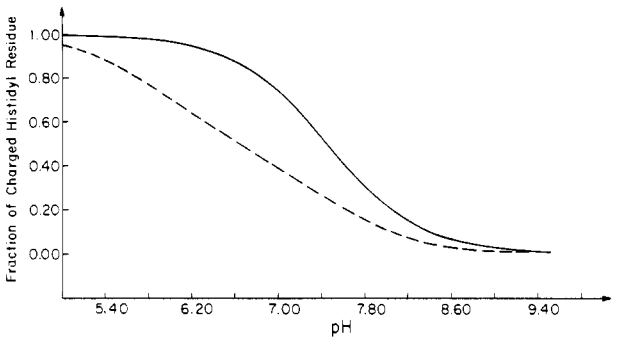

FIGURE 11: Fraction of charged β143 His residues as a function of pH: (solid curve) spectroscopic interaction model; (dashed curve) thermodynamic interaction model.

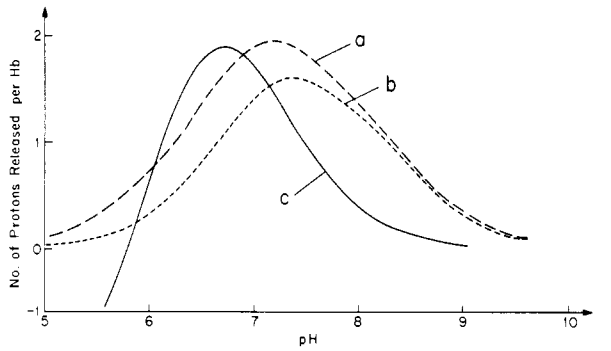

FIGURE 12: Total contribution of surface His residues to Bohr effect in 0.1 M Bis-Tris and/or 0.1 M Tris buffer in D_2O at 27 °C. Curve a is the contribution of the surface His residues calculated on the basis of a spectroscopic interaction model for the His resonances labeled 5 and 7–9 in the deoxy form and K in the CO form. Curve b is the contribution of the surface His residues calculated on the basis of a thermodynamic interaction model for the His resonances labeled 5 and 7–9 in the deoxy form and K in the CO form. Curve c is the Bohr effect measured as described previously (Russu et al., 1980).

and CO forms to the equations given by Shrager et al. (1972) for the thermodynamic and the spectroscopic perturbations of the ^{1}H NMR titration curves of His residues. We have found that for the His C2 proton resonances whose titration coefficients range from 0.8 to 1.0 (Tables III and IV), the fractions f^+_i of charged His residues calculated under the assumption of a spectroscopic interaction are larger by only 1–3% than those calculated under the assumption of a thermodynamic interaction. Therefore, for these His residues, the small deviations from unity of their titration coefficients do not affect significantly the values for the fractions f^+_i as compared to those calculated with the Henderson–Hasselbalch equation (eq 4). On the other hand, for the His C2 proton resonances whose titration coefficients are much smaller than 1 (i.e., the β143 His C2 proton resonance and the resonances labeled 5, 7, and 8 in the deoxy form and resonance K in the CO form), the fraction f^+_i calculated under assumption of a spectroscopic interaction is significantly larger than that calculated under assumption of a thermodynamic interaction. This finding is illustrated in Figure 11 where we show the fraction of charged His residues corresponding to resonance 9 (β143 His C2 proton) as a function of pH in these two cases. Given these facts, we have calculated the total contribution of the 22 His residues investigated here to the Bohr effect using eq 3 and assuming for the His resonances whose titration coefficient deviates greatly from unity either a thermodynamic or a spectroscopic interaction. The results are presented in Figure 12 in comparison to the Bohr effect of Hb A measured experimentally under the same conditions as those used in the ^{1}H NMR experiments. From this figure, it is clear that, for both thermodynamic and spectroscopic interactions, the present ^{1}H NMR data predict a number of protons released upon ligation that is comparable to that observed experimentally. This finding suggests that many of the 22 surface His residues investigated in the present study could contribute to the alkaline Bohr effect of Hb A through small individual changes in their pK values upon ligation. This suggestion is consistent with the theoretical results for the Bohr effect obtained by Matthew et al. (1979b). They have shown that, under low ionic strength conditions close to those used in the present work, 10–28 ionizable groups of Hb A could contribute to the Bohr effect. Their contribution was found to result from the unique charge distribution of ionizable groups in the deoxy and the ligated quaternary states of Hb. Our suggestion that many surface His residues of Hb A could contribute to the Bohr effect is also in good agreement with the ^{1}H NMR results of Brown & Campbell (1976). These authors have used a NMR chemical exchange method to cross-correlate the His resonances in deoxy- and oxy-Hb A at various values of the oxygen partial pressure. They have found that at least five His residues change their electrostatic environment during ligation, indicating that they all could contribute to the Bohr effect of Hb.

The contribution of several His residues to the Bohr effect of Hb A under low ionic strength conditions has also been recently investigated by Ohe & Kajita (1980). They have found that α20 His, α89 His, β143 His, and β146 His are the histidyl residues primarily responsible for the Bohr effect of Hb A. Our present results suggest that the changes in the pK values of these four His residues upon ligation, measured by the hydrogen–deuterium exchange method by Ohe & Kajita (1980), are overestimated. This suggestion is also supported by the finding that the total contribution of these four histidyl residues to the Bohr effect calculated from the experimental data reported by Ohe & Kajita (1980) exceeds by ~50% the total Bohr effect measured experimentally (Ohe & Kajita, 1980; Russu et al., 1980).

Conclusions

The ^{1}H NMR results obtained in the present study clearly indicate that, in solution, the surface His residues of Hb A can exist in a large variety of local electrostatic environments in both deoxy and CO states. This variation of the local environments is reflected in the ^{1}H NMR measurements by a broad range of pK values and intrinsic chemical shifts of the His C2 proton resonances as well as by the specific shapes of the ^{1}H NMR titration curves of several His residues. The pH titration properties of His residues, such as β2 His and β146 His, in deoxy and CO forms, indicate that under "stripped" conditions (i.e., in the absence of phosphate and at low concentrations of chloride ions), these His residues undergo only small changes in their electrostatic environments upon the binding of ligand to Hb. As a result, their contribution to the Bohr effect is negligible, suggesting that under stripped experimental conditions, the Bohr effect originates from subtle changes in the environments of a large number of surface residues. Therefore, although under these experimental conditions the Hb molecule undergoes the normal change in its quaternary structure (Baldwin, 1975; Viggiano & Ho, 1979), the corresponding changes in the conformations and environments of the surface His residues are not the same as those in the presence of relatively high concentrations of phosphate and chloride ions (Kilmartin et al., 1973, 1978; Russu et al., 1980). Thus, our present ^{1}H NMR results show clearly that medium factors, such as the anions present in solution, can

greatly affect the molecular mechanism of the Bohr effect of hemoglobin.

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