Role of the β146 Histidyl Residue in the Alkaline Bohr Effect of Hemoglobin†

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ABSTRACT: High-resolution proton nuclear magnetic resonance spectroscopy has been used to investigate the effects of inorganic anions, such as phosphate or chloride, on the alkaline Bohr effect of normal human adult hemoglobin. By monitoring the chemical shift of the C2 proton of the β146 histidyl residue as a function of pH, we have determined its pK values in both ligated and unligated forms. In the presence of 0.1 M Bis-Tris buffer (with chloride ion concentration ranging from 0.005 to 0.06 M) in D_2O at 27 °C, the pK value of the β146 histidine of deoxyhemoglobin is 7.98 ± 0.03 and that of (carbon monoxy)hemoglobin is 7.85 ± 0.03. However, in the presence of 0.2 M phosphate and 0.2 M NaCl in D_2O at 27 °C, the corresponding pK values are 8.08 and 7.14, as previously reported by this laboratory [Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246–1249]. This large difference in the pK value between the deoxy and carbon monoxy forms in the presence of 0.2 M phosphate and 0.2 M NaCl was interpreted as direct support for (1) the breaking of an intrasubunit salt bridge between β146 histidine and β94 aspartate when the hemoglobin molecule undergoes the quaternary structural transition as proposed by Perutz [Perutz, M. F. (1970) *Nature (London) 228*, 726–739] and (2) Perutz's suggestion that the β146 histidine is one of the amino acid residues responsible for the alkaline Bohr effect. The absence of a large change in the pK value of the β146 histidine in the presence of 0.1 M Bis-Tris buffer implies that (1) the above-mentioned intrasubunit salt bridge is not broken in going from the deoxy to the carbon monoxy form and (2) the β146 histidyl residue does not contribute significantly to the alkaline Bohr effect under these conditions. We have also found that in measuring the oxygen affinity of hemoglobin as a function of pH in the presence of 0.1 M Bis-Tris or 0.2 M phosphate plus 0.2 M NaCl (both in D_2O), there is no significant difference in the alkaline Bohr effect in these two media. Hence, our results suggest that the detailed molecular mechanism for the Bohr effect depends on the experimental conditions.

The ability of the hemoglobin (Hb)[1] molecule to release protons during the transition from the deoxy to the oxy form at a pH above 6.0 is known as the alkaline Bohr effect (Bohr et al., 1904). The Bohr effect is due to a structural change occurring in the Hb molecule upon oxygenation. This results in an alteration of the environment of certain amino acid residues such that their ionization properties are affected and protons are either released or captured. Thus, any amino acid residue can, in principle, contribute to the Bohr effect if its pK value is altered in going from deoxy- to oxyhemoglobin.

On the basis of the X-ray structural information, Perutz (1970) proposed that the carboxyl-terminal histidine-β146-(HC3) could be one of the residues involved in the alkaline Bohr effect. In deoxy-Hb, the imidazole group of β146 histidine forms a salt bridge with the carboxyl group of β94(FG1) aspartate. This would greatly raise the pK value of β146 histidine. In the ligated Hb molecule, this salt bridge is broken. Thus, β146 histidine is expected to float freely in solution and its pK value should be normal (Perutz, 1970; Perutz & Ten Eyck, 1971). This idea was first confirmed when it was found that either the enzymatic removal of the β146 histidine in des-β146-histidine-hemoglobin (des-His-Hb) (Kilmartin & Wootton, 1970) or its replacement by aspartic acid in Hb Hiroshima (β146 His → Asp) (Perutz et al., 1971) results in a substantial reduction in the alkaline Bohr effect. More exact quantification of the role of β146 His in the alkaline Bohr effect was provided by a direct measurement of its pK values in both ligated and unligated forms of hemoglobin by a proton nuclear magnetic resonance (^{1}H NMR) titration of the β146 histidine carried out in this laboratory (Kilmartin et al., 1973). We found that in the presence of 0.2 M potassium phosphate and 0.2 M NaCl in D_2O, the pK values of the β146 histidine of normal human adult hemoglobin (Hb A) are 7.1 in the CO form and 8.0 in the deoxy form at 30 °C. A recent investigation of the pK values of the β146 histidine of Hb A in both deoxy and CO forms in the presence of inositol hexaphosphate gives similar results (Kilmartin et al., 1978). This large change in the pK values of β146 histidine has confirmed the prediction made by Perutz (1970) that at physiological pH this amino acid residue releases protons (≈0.8 proton/Hb tetramer) upon oxygenation of the Hb molecule.

On the other hand, the X-ray crystallographic findings of Anderson (1975) on a mutant hemoglobin, Hb Kansas (β102 Asn → Thr), in the carbon monoxy form, suggest that the Bohr group salt bridges in Perutz's (1970) stereochemical model for the Bohr effect (including the one between β146 His and β94 Asp) remain intact in the ligated T state. More recently, Kilmartin et al. (1978) reported that the pK value of β146 histidine remains unchanged on ligation of the T state of Hb Kansas with NO and that there is little T state Bohr effect with either NO or O_2. These results have confirmed

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[1] Abbreviations used: Hb, hemoglobin; HbCO, (carbon monoxy)-hemoglobin; HbO_2, oxyhemoglobin; Hb A, human adult hemoglobin; des-His-Hb, des-β146-histidine-hemoglobin; T and R, symbols for deoxy and oxy quaternary structures in Perutz's (1970) stereochemical mechanism of cooperative oxygenation of hemoglobin; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane; P_{O_2}, partial pressure of oxygen; P_{50}, partial pressure of oxygen at 50% saturation; ppm, parts per million.

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Anderson's (1975) suggestion that the Bohr group salt bridges are not broken on ligation of the T state of Hb Kansas. In order to reconcile these seemingly contradictory results in Hb A and Hb Kansas, Kilmartin et al. (1978) concluded that the stereochemical mechanism for the cooperative oxygenation of hemoglobin as proposed by Perutz (1970) is not unique. They further stated that Perutz's (1970) mechanism is only applicable to the T state of stripped Hb A and not to the T states of hemoglobins with low ligand affinity, whether it is caused by mutation, crystallization in the deoxy form, the presence of a strong allosteric effector like inositol hexaphosphate, or ligation with a ligand like NO. It should be pointed out that their generalization on the validity of the Bohr effect was based on indirect evidence and that there were several experimental limitations in their study. First, they were not able to measure the effect of inositol hexaphosphate on the pK values of β146 His in the T and R states of Hb Kansas. They stated that this is due to the fact that HbCO Kansas is switched to the T state at low pH (Ogawa et al., 1972). Second, it was not possible for them to prepare large enough quantities of des-His-Hb Kansas needed for all their experiments. They were forced to measure the pK value of β146 His of Hb Kansas in the NO form in the presence of inositol hexaphosphate (pK = 8.0 ± 0.1 at 30 °C), which is in the T state up to pH 8.0 and is converted to the R state between pH 8.0 and 9.0 (Salhany et al., 1975).

Several studies have shown that anions, such as phosphate or chloride, have a large effect on the oxygen affinity and the alkaline Bohr effect of Hb A (Antonini et al., 1962, 1972; Benesch et al., 1969). At increasing salt concentration, the oxygen affinity decreases and the Bohr effect is strongly suppressed, but there is no influence on the cooperativity of the oxygenation process as measured by the Hill coefficient. Recently, de Bruin and co-workers (de Bruin et al., 1974a,b; Rollema et al., 1975) have carried out an investigation of the effects of chloride and phosphate on the Bohr effect of Hb A. They have found that at low chloride ion concentration (5×10^{-3} M), the alkaline Bohr effect is smaller than at a concentration of 0.1 M. On the other hand, at a higher concentration of chloride ions (0.1–2.0 M), there is also a suppression of the alkaline Bohr effect. The effect of Cl^- on the Bohr effect shows a striking similarity to the effect of 2,3-diphosphoglycerate on the Bohr effect (Riggs, 1971; de Bruin et al., 1974a). These results strongly suggest that part of the alkaline Bohr effect is due to a difference in the binding of chloride or phosphate ions to the Hb molecule in the oxy and deoxy states and that the suppression of the alkaline Bohr effect by high concentrations of neutral univalent salt is not caused by a weakening of the salt bridges in deoxyhemoglobin as suggested by Perutz et al. (1969). The chlorine-35 NMR results have confirmed that deoxy-Hb A has a higher affinity for chloride ions than ligated Hb (Chiancone et al., 1972, 1975; Bull et al., 1973).

High-resolution ^{1}H NMR spectroscopy is a unique technique for investigating the structure–function relationship in hemoglobin [for example, see Ho et al. (1973, 1975, 1978), Johnson & Ho (1974), Shulman et al. (1975), Fung et al. (1976, 1977), Viggiano & Ho (1979), and Viggiano et al. (1979). Previous work in this laboratory has shown that anions, such as organic phosphate, inorganic phosphate, or chloride, have an important effect on the hyperfine-shifted, ring current shifted, exchangeable, and aromatic proton resonances of Hb (Lindstrom & Ho, 1973; Kilmartin et al., 1973; Ho et al., 1973, 1976; Fung & Ho, 1975; Fung et al., 1975, 1976, 1977). Of special interest to this work is the effect of anions on the aromatic proton resonances, most of which originate from surface histidyl residues of the Hb molecule (Kilmartin et al., 1973; Fung et al., 1975; Ho et al., 1976; Ho & Russu, 1978).

In view of the important role that the β146 histidyl residue plays in the stereochemical mechanism for the cooperative oxygenation of Hb as proposed by Perutz (1970) and the fact that its proton resonances are very sensitive to experimental conditions, we have carried out an investigation of the effects of various experimental variables (such as anions and pH) on the proton resonances of the β146 histidyl residue of Hb A in both deoxy and carbon monoxy forms. In addition, we have also measured the oxygen dissociation curves of Hb A under the same experimental conditions as those of the ^{1}H NMR studies. An analysis of these data allows us to assess the contribution of the β146 histidyl residue to the Bohr effect in hemoglobin. In the present study, we have made an assumption that the Bohr effect due to the binding of CO to Hb A is the same as that due to O_2. This assumption is supported by the experimental results of Antonini et al. (1963) which showed that there is no difference between HbO_2 and HbCO in the amount of hydrogen ions bound by hemoglobin at any pH studied. The decision to use the carbon monoxy form of Hb A rather than its oxy form is dictated by the fact that HbCO A is more stable than its oxy analogue (against acid and alkaline denaturation, oxidation to methemoglobin, etc). over the wide range of pH used in this work.

Experimental Section

Materials. Hb A was prepared by standard procedure from fresh blood samples obtained from the local Blood Bank (Lindstrom & Ho, 1972). Des-His-Hb was prepared as described by Kilmartin et al. (1975) and was kindly supplied to us by Janice Fogg. Organic phosphates in Hb samples were removed by passage through a column of Sephadex G-25 (Pharmacia) equilibrated with 0.01 M tris(hydroxymethyl)-aminomethane (Tris) (obtained from Aldrich) containing 0.1 M NaCl at pH 7.5 (Berman et al., 1971). The Hb samples were then exchanged 4 times with D_2O (99.8% in deuterium content, obtained from Bio-Rad) through an ultrafiltration membrane so as to suppress the intense water proton signal in the ^{1}H NMR spectra. Appropriate amounts of 1 M bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris) (obtained from Aldrich) or 1 M Tris buffer in D_2O, titrated to appropriate pH values with 2 N HCl or 2 N NaOH, were added to the stripped Hb solution in order to obtain the desired pH values. As a result of this pH adjustment of the Bis-Tris buffer, the chloride ion concentration in the Hb samples varied from 0.005 to 0.06 M in the pH range studied. The samples for pH below 6 were obtained by dialyzing the stripped Hb solutions against 0.1 M Bis-Tris buffer in D_2O at the appropriate pH. The deoxygenation process was performed as described previously (Lindstrom & Ho, 1972).

The pH values of the deoxy samples were measured anaerobically by using a Radiometer microelectrode unit (E5021a) connected to a Beckman pH meter (Model 3500). The pH values given here are direct pH meter readings and are reproducible to ±0.02 pH unit. They were not corrected for the deuterium isotope effect on the glass electrode, namely, pD = pH + 0.4 (Glasoe & Long, 1960).

Methods. Oxygen dissociation curves of Hb A were measured by using an Aminco Hem-O-Scan oxygen dissociation analyzer. The concentration of Hb A in these measurements was 10%. The partial pressure of oxygen (P_{O_2}) was monitored by an oxygen electrode and the fractional O_2 saturation by dual wavelength spectroscopy at 390 and 480 nm. The partial

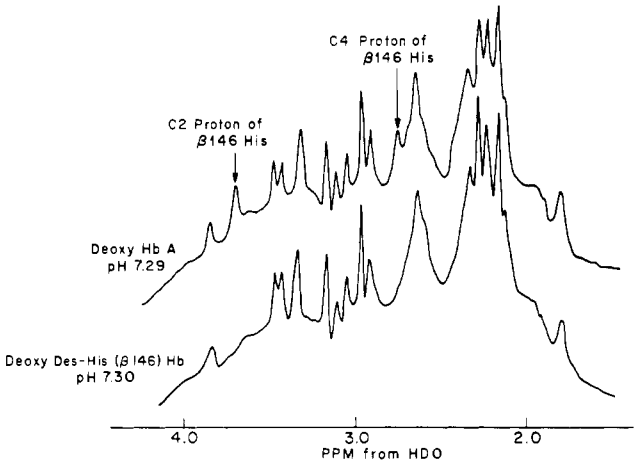


FIGURE 1: The 250-MHz aromatic proton resonances of 10% deoxy-Hb A and deoxy-des-Hisβ146-Hb in 0.1 M Bis-Tris plus 0.01 M chloride ions in D_2O at 27 °C, showing the assignment of the C2 and C4 proton resonances of the β146 histidyl residue of hemoglobin.

pressure of O_2 at 50% saturation (P_{50}) was obtained directly from the plots of fractional O_2 saturation vs. P_{O_2}. The gas mixtures (obtained from Lif-O-Gen) used were 25% O_2–75% N_2 for oxygenation and 100% N_2 for deoxygenation. D_2O instead of H_2O was used in the humidifying chamber of the instrument to minimize exposure of the samples to atmospheric conditions.

High-resolution ^{1}H NMR spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer interfaced with a Sigma 5 computer. The signal to noise ratio was improved by the technique of NMR correlation spectroscopy (Dadok & Sprecher, 1974). The spectrometer was locked on the residual water signal in each sample, and the ambient temperature inside the probe was 27 °C. The proton chemical shifts are expressed as parts per million (ppm) with respect to the residual water proton signal (HDO), which is 4.83 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate at 27 °C. The chemical-shift scale is presently defined as positive in the low-field direction with respect to the residual water proton signal.[2] The sweep width was 1460 Hz with a sweep time of 3 s, and each spectrum is the result of 50–100 scans. The resolution was improved by using the resolution enhancement technique, which provides a better spectral resolution in the aromatic proton resonance region and permits us to monitor the chemical shifts of individual proton resonances as a function of experimental conditions to an accuracy of ±0.02 ppm. However, the intensity of individual resonances is somewhat distorted by this technique so that accurate intensity measurements cannot be made directly from these spectra.

Results

Assignment of the β146 Histidine Proton Resonance. Following the procedure used in our earlier work (Kilmartin et al., 1973), we have assigned the resonances of the C2 and C4 protons of the β146 histidyl residue through a comparison of the ^{1}H NMR spectra of Hb A and des-His-Hb at the same pH in 0.1 M Bis-Tris buffer. Figure 1 shows the 250-MHz ^{1}H NMR spectra of deoxy-Hb A and deoxy-des-His-Hb in 0.1 M Bis-Tris in the presence of 0.01 M Cl^- in D_2O at pH 7.3 and 27 °C. Two resonances are missing in the spectrum of des-His-Hb, one at 3.68 ppm and the other one at 2.72 ppm. These two resonances are consistently missing in the spectra of des-His-Hb over the pH range from 6 to 10. Therefore, these resonances should correspond to the C2 and C4 protons of β146 His in the Hb A spectrum.

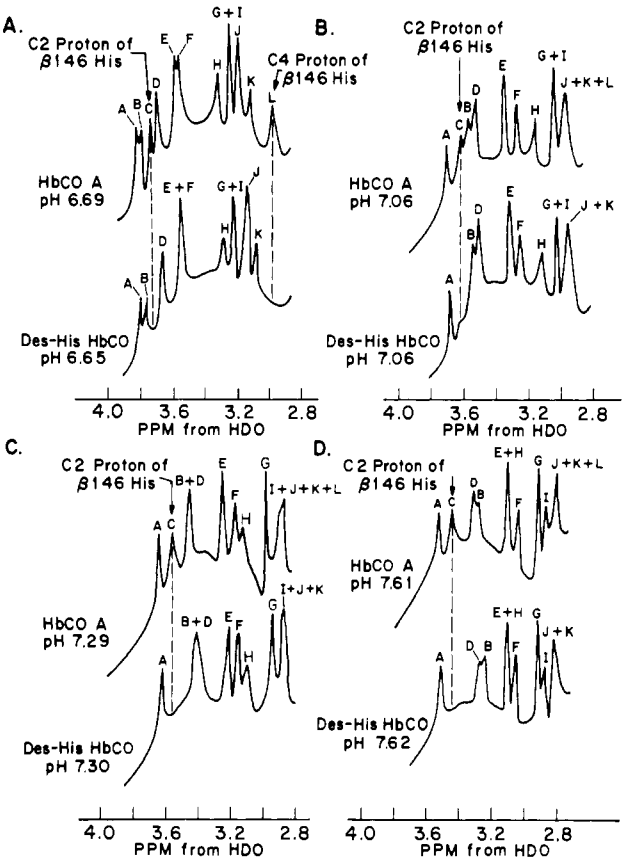


FIGURE 2: The 250-MHz aromatic proton resonance spectra of 10% Hb A and des-Hisβ146-Hb in the carbon monoxy form in the presence of 0.1 M Bis-Tris in D_2O as a function of pH at 27 °C, showing the assignment of the C2 proton resonances of the β146 histidyl residue of hemoglobin. The resonance labeled C is the C2 proton of β146 histidine.

Figure 2 shows the assignment of the C2 and C4 protons of β146 histidine in the carbon monoxy form. At pH 6.69 (Figure 2A), the resonances at 3.72 (labeled C) and 2.95 ppm (labeled L) in the spectrum of HbCO A are missing in the corresponding spectrum of des-His-HbCO; therefore they should correspond respectively to the C2 and C4 protons of β146 His. The resonance labeled C was consistently missing in the spectra of des-His-HbCO over the entire pH range examined (for example, see Figures 2B–D). The resonance labeled L overlaps in the spectrum of HbCO A with one or more aromatic resonances and, as a consequence, its absence in the des-His-HbCO spectrum is more difficult to observe over the entire pH range studied.

NMR Titration of the β146 Histidine. We have followed the position of the C2 proton resonance of β146 histidine as a function of pH in both deoxy- and (carbon monoxy)-hemoglobin solutions. Our titration of this proton in deoxy-Hb A follows the same pattern as the one reported by Kilmartin et al. (1973); hence, it is not reproduced here.

[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. Previously, we had used the negative sign to indicate that the chemical shift of a given resonance is downfield from the resonance of a standard, such as the proton resonance of the residual water (HDO) signal. Hence, this change in the sign of the chemical-shift scale should be noted when referring to earlier publications reported by this laboratory.

The NMR titration of the C2 proton of β146 histidine in the carbon monoxy form is shown in Figure 3. At pH 6.36, we have labeled the three downfield resonances as A + B, C + D, and E + F as shown in Figure 3A. The fact that each of them is a sum of two single resonances becomes clear as the pH is gradually increased (e.g., at pH 6.63 and 6.69). Each of these six protons starts to titrate differentially; i.e., B shifts faster than A, D faster than C, etc. Hence, at pH 6.69 all six of them can be separately observed. At this stage, a comparison with the des-His-HbCO spectrum (Figure 2A) reveals that the resonance labeled C corresponds to the C2 proton of β146 histidine. When the pH is further increased, we make an implicit assumption (based on the continuity of the titration curve) that the resonance labeled B titrates not only faster than resonance A but also faster than resonance C such that at pH ~6.9 resonances B and C overlap and at higher pH values (e.g., pH 7.06) resonance B is shifted upfield from resonance C. A comparison with the des-His-HbCO spectrum at pH 7.06 (Figure 2B) shows that this "switch" in position between resonances B and C is correct; the missing resonance in the des-His-HbCO spectrum is the downfield one, namely, resonance C. Therefore, through resonance C, we can monitor the C2 proton of β146 histidine.

For pH values higher than 7.06, as shown in parts A and B of Figure 3, the titration of resonance C becomes straightforward, with the only change occurring in the downfield part of the spectrum being a constant upfield shift of resonances A, C, and B (or B + D) with no further overlapping. For instance, at pH 7.30 (Figure 2C) and at pH 7.62 (Figure 2D), resonance C is consistently missing in the des-His-Hb spectra, showing that its origin is the C2 proton of β146 histidine.

The chemical shift for the C2 proton of β146 histidine, obtained as indicated above, has been fitted to the standard Henderson–Hasselbalch equation (using a nonlinear least-squares computer program) as a function of pH in both deoxy and carbon monoxy forms:

$$\delta = \frac{\delta_{A+}[H^+] + \delta_{AO}K}{[H^+] + K} \qquad (1)$$

where δ is the observed chemical shift, δ_{A+} and δ_{AO} are, respectively, the chemical shifts in the protonated and unprotonated forms, $[H^+]$ is the concentration of hydrogen ions, and K is the ionization constant. The experimental points and the fitted curves are shown in Figure 4. Using this procedure, we have found that in a 10% Hb A solution in 0.1 M Bis-Tris buffer in D_2O, β146 histidine has a pK of 7.98 ± 0.03 in deoxyhemoglobin and a pK of 7.85 ± 0.03 in HbCO at 27 °C. Since appropriate amounts of HCl have to be added to various Hb solutions to adjust to the desired pH values, the concentration of chloride ions varies from 0.005 to 0.06 M in our Hb samples.

Alkaline Bohr Effect Measurement. From the individual pK values of β146 histidine obtained by using ^{1}H NMR titration, it is possible to calculate the concentration of charged β146 histidyl residues at a given pH and thus the number of protons released by β146 histidines when the Hb molecule undergoes the transition from the deoxy to the carbon monoxy state (Figure 5). A knowledge of the total Bohr effect under these experimental conditions is necessary in order to relate these findings and to evaluate the contribution of β146 His to the alkaline Bohr effect. We have measured the variation of P_{50} of Hb A as a function of pH in 10% Hb A in 0.2 M phosphate buffer + 0.2 M NaCl at 30 °C and in 0.1 M Bis-Tris buffer at 27 °C, both in D_2O, i.e., under the same

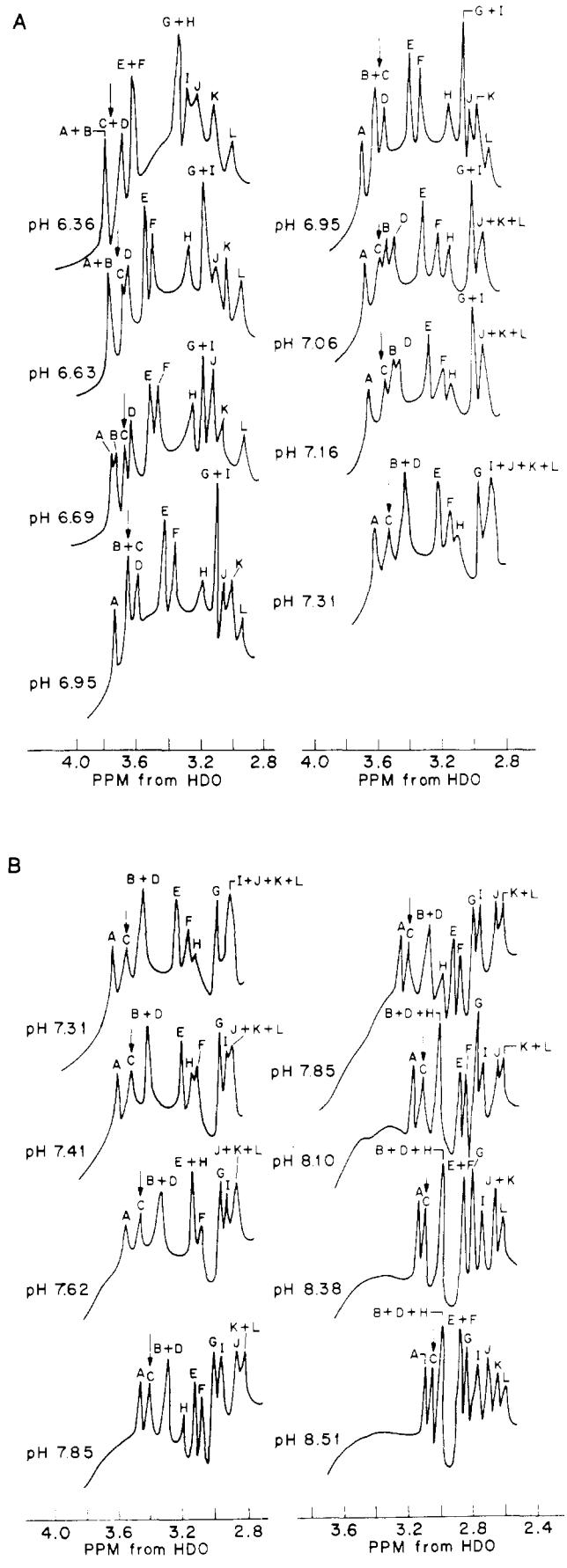


FIGURE 3: The 250-MHz aromatic proton resonance spectra of HbCO A in 0.1 M Bis-Tris in D_2O as a function of pH at 27 °C: (A) from pH 6.36 to 7.31; (B) from pH 7.31 to 8.51. The resonance labeled C is the C2 proton of β146 histidine.

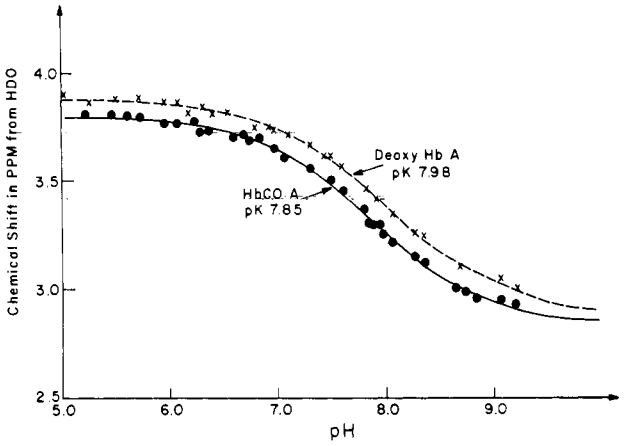


FIGURE 4: Chemical shift of the C2 proton of β146 histidine of Hb A in both deoxy and CO forms as a function of pH in 0.1 M Bis-Tris in D_2O at 27 °C. The solid and dashed lines are obtained by the least-squares fitting to eq 1.

conditions as our NMR experiments. [It should be mentioned that this difference in temperature is due to the fact that the ambient temperature of the probe was 30 °C when the NMR experiments were carried out by Kilmartin et al. (1973), whereas the probe temperature in the present NMR experiments was 27 °C. The MPC-HF 250-MHz NMR spectrometer does not have a variable temperature accessory. Hence, one cannot control the probe temperature readily.] These results are shown in Figure 6. As was first suggested by Wyman (1948) and thereafter confirmed by Antonini et al. (1962), the Bohr effect can be fitted according to the hypothesis of two independent oxygen-linked groups per heme, one responsible for the alkaline effect and the other for the acid effect. Accordingly, the experimental data, log P_{50} vs. pH, have been fitted in both cases to the linkage equation given by Wyman (1948)

$$\log P_{50} = \text{constant} + \log \frac{([H^+] + K_1')([H^+] + K_2')}{([H^+] + K_1'')([H^+] + K_2'')} \quad (2)$$

where K_1 and K_2 are the ionization constants of the two hypothetical oxygen-linked groups. The single prime mark refers to deoxyhemoglobin and the double prime mark to HbCO. The pK values obtained by using this fitting procedure are given as follows: (1) in the presence of 0.1 M Bis-Tris at 27 °C, $pK_1' = 7.10$, $pK_2' = 5.09$, $pK_1'' = 5.89$, and $pK_2'' = 5.88$; (2) in the presence of 0.2 M phosphate + 0.2 M NaCl at 30 °C, $pK_1' = 7.98$, $pK_2' = 4.98$, $pK_1'' = 7.13$, and $pK_2'' = 5.62$.

The Bohr effect, expressed as the total number of protons released upon oxygenation vs. pH, has been reconstructed theoretically in both cases, using the equation

$$\Delta\bar{X} = 4\left(\frac{K_1''}{[H^+] + K_1''} + \frac{K_2''}{[H^+] + K_2''} - \frac{K_1'}{[H^+] + K_1'} - \frac{K_2'}{[H^+] + K_2'} \right) \quad (3)$$

where $\Delta\bar{X}$ is the number of protons released upon oxygenation per Hb molecule. Antonini et al. (1962) have shown that the agreement between the number of protons released upon oxygenation obtained from this method and that obtained from direct differential titration is excellent. The results obtained by this method for the total Bohr effect in 0.1 M Bis-Tris buffer at 27 °C and in 0.2 M phosphate buffer + 0.2 M NaCl at 30 °C are given in Figure 5.

Discussion

Contribution of the β146 Histidine to the Alkaline Bohr Effect. Figure 5A compares the total Bohr effect and that contributed by β146 histidine in 0.2 M phosphate plus 0.2 M NaCl at 30 °C. The latter is calculated by using the pK values reported by Kilmartin et al. (1973). One can see that in the presence of inorganic phosphate and Cl^- ions, the variation in the pK values for β146 histidine can account for as much as 50% of the observed alkaline Bohr effect as first pointed out by Kilmartin et al. (1973). On the other hand, as shown in Figure 5B, in 0.1 M Bis-Tris buffer and a low concentration of Cl^- at 27 °C, the contribution of β146 histidine to the alkaline Bohr effect is less than 5%. Despite the absence of a significant contribution from the β146 histidine in 0.1 M Bis-Tris buffer, the total Bohr effect has essentially the same magnitude as that in the presence of 0.2 M phosphate + 0.2 M chloride, the only difference being a shift toward lower pH values. These findings suggest that the Hb molecule retains its full Bohr effect capacity in both sets of experimental

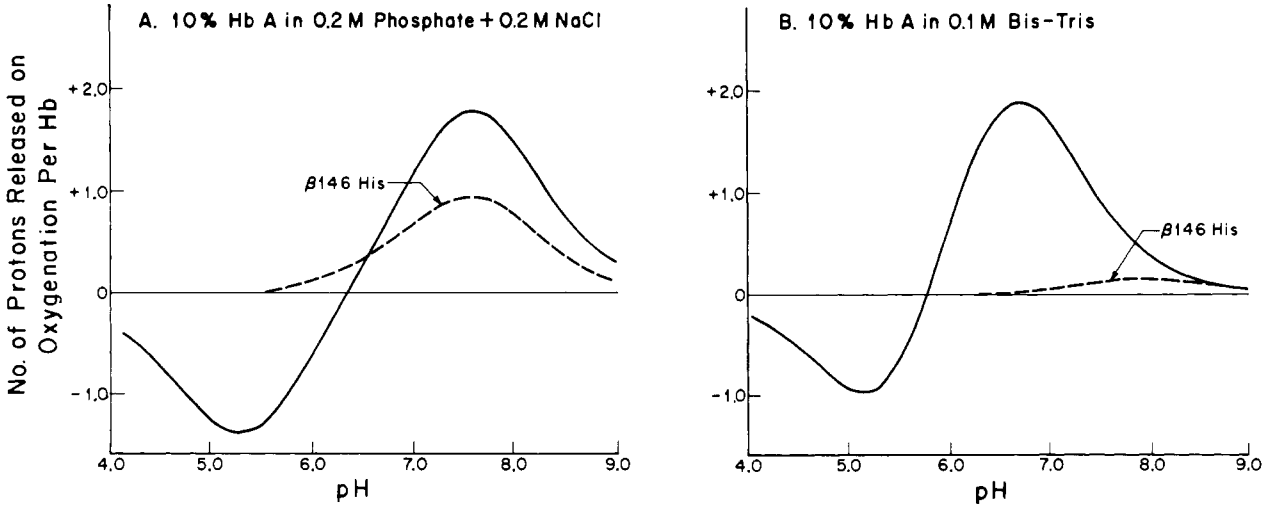


FIGURE 5: Contribution of the β146 histidyl residue of Hb A to the Bohr effect. (A) 10% Hb A in 0.2 M phosphate plus 0.2 M NaCl in D_2O at 30 °C. The solid line is the Bohr effect obtained from log P_{50} vs. pH as given in Figure 6. The dashed line is the calculated contribution of β146 histidine based on the pK values of 8.0 and 7.1 for deoxy- and (carbon monoxy)hemoglobin reported by Kilmartin et al. (1973). (B) 10% Hb A in 0.1 M Bis-Tris in D_2O at 27 °C. The solid line is the Bohr effect obtained from log P_{50} vs. pH as given in Figure 6. The dashed line is the calculated contribution of β146 histidine based on the pK values of 7.98 and 7.85 for deoxy- and (carbon monoxy)hemoglobin obtained from Figure 4.

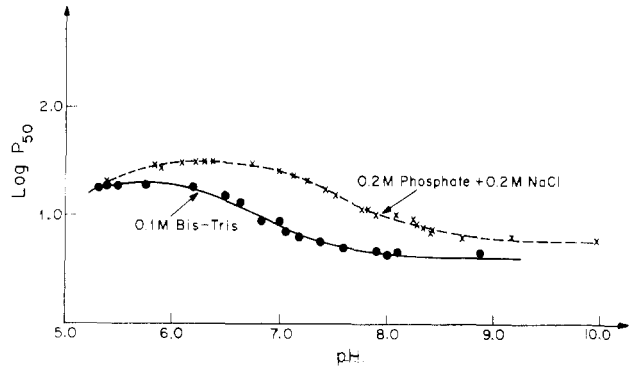


FIGURE 6: Variation of oxygen affinity of 10% Hb A as a function of pH in D_2O: (×) in 0.2 M phosphate plus 0.2 M NaCl at 30 °C; (●) in 0.1 M Bis-Tris at 27 °C. The solid and dashed lines are obtained by the least-squares fitting to eq 2.

conditions, but the molecular mechanism responsible for it could differ substantially in the stripped condition as compared to that in the presence of relatively large amounts of phosphate and chloride ions.

According to the results of de Bruin and co-workers (de Bruin et al., 1974b; Rollema et al., 1975), the alkaline Bohr effect of Hb A is enhanced when the concentration of Cl^- is increased from 5×10^{-3} to 0.1 M and thereafter is decreased when the chloride ion concentration is elevated further. These findings suggest that low concentrations of Cl^- (0.005–0.1 M) make a significant contribution to the alkaline Bohr effect. This is most likely due to a differential interaction between deoxy- and oxyhemoglobin with chloride ions (Van Beek et al., 1979).[3] Thus, under the present experimental conditions of 0.1 M Bis-Tris, the presence of a small (but not negligible) amount of Cl^- (from 0.005–0.06 M) can make a substantial contribution to the alkaline Bohr effect. On the other hand, under the experimental conditions used by Kilmartin et al. (1973), the presence of relatively high concentrations of anions (0.2 M phosphate plus 0.2 M chloride) should have greatly suppressed the alkaline Bohr effect. However, the total Bohr effect under these conditions is the same as that using 0.1 M Bis-Tris buffer as shown in Figure 5. This emphasizes the important role that the β146 histidyl residue can play in the alkaline Bohr effect (~50%) under certain experimental conditions. Thus, these results show clearly that there is not a unique mechanism for the Bohr effect, a conclusion in agreement with that made by Kilmartin et al. (1978). On the other hand, our results in the presence of 0.1 M Bis-Tris with low concentrations of chloride ions (the so-called "stripped condition") disagree with a conclusion made by Kilmartin et al. (1978) that Perutz's mechanism for the Bohr effect is applicable to the T state of "stripped" Hb A. The available experimental results appear to suggest that there is an excellent balance in the Bohr effect due to medium or environmental factors and the change in the pK values of certain amino acid residues upon the binding of a ligand. However, the exact role that the medium factors, such as anions, play in the Bohr effect awaits further investigation. In order to investigate the role that the β146 histidyl residue plays in the Bohr effect, we have also measured the P_{50} of des-His-Hb as a function of pH. Our results show that the total Bohr effect of des-His-Hb in both 0.1 M Bis-Tris and 0.2 M phosphate + 0.2 M KCl is less than that of Hb A (results not shown). This conclusion is in agreement with the differential titration data on des-His-Hb previously reported by Kilmartin & Wootton (1970) as well as the recent findings in 0.01 M Cl^- by Kilmartin, Fogg, and Perutz (M. F. Perutz, personal communication). The reduction of the alkaline Bohr effect in des-His-Hb has been interpreted as being due to the removal of one-half of the alkaline Bohr groups (Kilmartin & Wootton, 1970). However, this interpretation has been questioned by Saroff (1972), who pointed out that, even within the model proposed by Wyman (1948), the differential titration data for des-His-Hb cannot be accounted for without assuming additional perturbation of the des-His-Hb molecule. Hence, the exact molecular mechanism responsible for the reduction of the alkaline Bohr effect in des-His-Hb requires further research.

Environment of the β146 Histidine in Deoxy- and (Carbon monoxy)hemoglobin. The pK value for β146 histidine in deoxy-Hb A reported in this work (pK = 7.98 at 27 °C) is in very good agreement with that (pK = 8.08 at 27 °C) obtained previously in 0.2 M phosphate buffer + 0.2 M NaCl (Kilmartin et al., 1973), after correcting for the temperature difference (from 30 to 27 °C) by using the observed heat of ionization of the Bohr groups (Rossi-Bernardi & Roughton, 1967). This suggests that the environment of β146 histidine in the deoxy form is independent of the solvent composition. In both cases, β146 histidine exhibits an anomalously high pK value which is the result of its participation in the salt bridge with β94 aspartate. The existence of this salt bridge has been "seen" in an X-ray diffraction study of deoxy-Hb A at 2.5-Å resolution (Fermi, 1975). In the carbon monoxy form, the pK value for β146 histidine obtained here in 0.1 M Bis-Tris buffer (pK = 7.85 at 27 °C) is very different from that (pK = 7.14 at 27 °C) obtained by Kilmartin et al. (1973) in 0.2 M phosphate buffer + 0.2 M NaCl, even after correcting for the temperature difference between these two measurements. Moreover, in 0.1 M Bis-Tris buffer, the pK value of β146 histidine in the carbon monoxy form is very close to the value obtained in the deoxy form (i.e., pK changes from 7.98 to 7.85). These results suggest that the intrasubunit salt bridge between β146 histidine and β94 aspartate can exist in the ligated form of hemoglobin.[4] On the basis of X-ray crystallographic results (Perutz, 1970; Heidner et al., 1976; Baldwin & Chothia, 1979), β146 histidine is generally thought to be floating freely in solution in the ligated form of Hb. This was, in fact, the explanation given in our previous paper for its "much lower or almost normal" pK value in the carbon monoxy form in 0.2 M phosphate and 0.2 M NaCl (Kilmartin et al., 1973). However, it should be pointed out that there are large differences in the experimental conditions between our present NMR experiments and those of X-ray diffraction. The Hb solutions used in our NMR experiments were conditioned in 0.1 M Bis-Tris with a low concentration of Cl^- and no phosphate. In the X-ray diffraction studies, Hb crystals were grown in the presence of very high concentrations of salts [such as 2–4 M solutions of ammonium sulfate and sodium phosphate (Perutz, 1968)]. This difference in the solvent composition could affect the stability of salt bridges in protein molecules (for details, see Appendix).

In order to gain a better understanding of the salt bridge β146 His–β94 Asp, we have monitored the aromatic proton resonance region of HbCO A in 0.1 M Bis-Tris in the presence

[3] It should be pointed out that, according to our ^1H NMR results, the intrinsic, chloride-independent part of the Bohr effect cannot be solely attributed to the contribution of β146 His as in the model proposed by Van Beek et al. (1979).

[4] Examination of the atomic model of oxy-like methemoglobin indicates that there is no stereochemical restriction on the existence of the salt bridge between β146 His and β94 Asp, even after the expulsion of the penultimate tyrosine-β145 from the pocket between helices F and H in the ligated hemoglobin molecule (M. F. Perutz, personal communication).

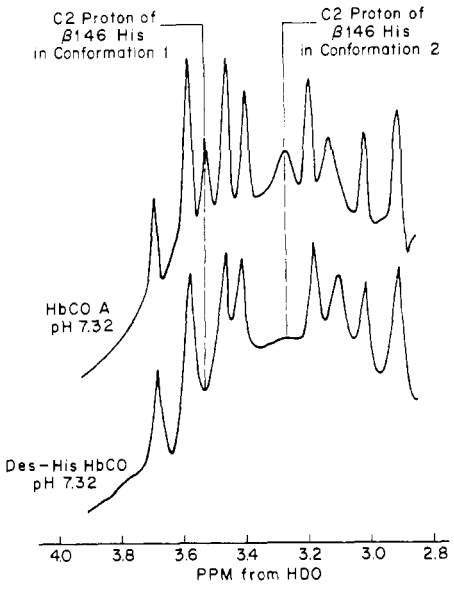


FIGURE 7: The 250-MHz aromatic proton resonance spectra of HbCO A and des-Hisβ146-HbCO in 0.1 M Bis-Tris plus 0.15 M phosphate in D_2O at 27 °C: effect of phosphate on the conformation of β146 histidine in HbCO A. For details, see the text and Appendix.

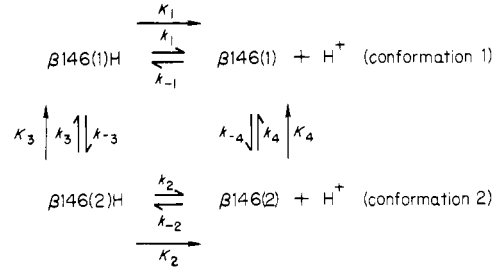
of increasing amounts of chloride or phosphate ions, keeping the pH constant. The same experiment has also been carried out by using des-His-Hb in the carbon monoxy form as a control. For phosphate concentrations less than 0.04 M (approximately 25-fold molar in excess of the Hb concentration), no appreciable effect on the β146 histidine resonance has been observed, except for a slight upfield shift (~0.05 ppm), as compared with the spectrum in 0.1 M Bis-Tris alone. When the phosphate concentration is increased above 0.04 M, the main change in the spectrum is the occurrence of a new upfield resonance at 3.26 ppm. Figure 7 shows the results for a 10% Hb A solution in the carbon monoxy form in 0.1 M Bis-Tris + 0.15 M phosphate ions at pH 7.32. Comparing with the spectrum of des-His-HbCO solution under the same conditions, one can see that two resonances are missing, one resonance at 3.51 ppm and another at 3.26 ppm. Both of them originate from the C2 proton of β146 histidine.[5]

We believe that the two resonances observed for the C2 proton of β146 histidine in the intermediate concentration range of phosphate ions (i.e., 0.04–0.2 M) are the result of a slow exchange (slow on the NMR time scale) between two species of the HbCO molecule: one in which β146 histidine is held in a salt bridge with β94 aspartate ("conformation 1" for β146 His), thus having a higher pK and its resonance being shifted downfield, and another one in which β146 histidine is floating free in solution ("conformation 2" for β146 His) and its pK is reverting to a lower value. For details, see Appendix.

The addition of increasing concentrations of chloride ions to the Hb solution in 0.1 M Bis-Tris has a similar effect on the Hb molecule, the only difference being that this anion is less effective in breaking the β146 His–β94 Asp salt bridge; i.e., a 0.4 M Cl^- concentration is necessary in order to observe the same effect. At chloride ion concentrations higher than 0.4 M, the presence of both C2 proton resonances of β146 histidine indicates the existence of a similar equilibrium between the two conformations of the β146 histidyl residue (results not shown).

[5] It should be mentioned that the upfield resonance at 3.26 ppm cannot be due to the C4 proton of the β146 histidine since in HbCO A at pH 7.32 this proton should occur at a position upfield from 2.95 ppm.

Scheme I: Conformational Equilibria of the β146 Histidine of Hb A



The present results reemphasize the remarkable features of the hemoglobin molecule in carrying out its physiological functions. Its structure is designed with such great flexibility and ingenuity that it can adjust or adapt to various environmental perturbations without compromising its functional obligations. An excellent demonstration of these features is the role of β146 histidine in the alkaline Bohr effect. In the presence of moderate amounts of anions, like phosphate and chloride (such as under physiological conditions), the β146 histidyl residue can contribute up to 50% of the alkaline Bohr effect as discussed earlier. On the other hand, in the absence or in low concentrations of such anions, the intrasubunit salt bridge between β146 histidine and β94 aspartate can remain intact when the Hb molecule changes from the deoxy (T) to the oxy (R) quaternary structure. Under such conditions, even though the β146 histidyl residue plays an insignificant role, the Hb molecule still has its full alkaline Bohr effect (presumably due, at least in part, to the contribution of the anions present in the system). The implication of these findings is that there is no unique molecular mechanism for the Bohr effect. Regarding the cooperativity of the oxygen binding, a preliminary analysis of our O_2 saturation data indicates only small differences in the Hill coefficient between the two extreme experimental conditions (the Hill coefficient varies from 2.5 to 2.8 over the pH range from 6 to 9 in both buffer systems). We know from our own ^{1}H NMR results (Ho et al., 1975; Fung & Ho, 1975; Viggiano & Ho, 1979; Viggiano et al., 1979) and O_2 saturation studies, as well as the results of other investigators [for example, see Baldwin (1975) and the references therein], that in going from the deoxy to the oxy state the Hb molecule undergoes the usual quaternary structural transition in 0.1 M Bis-Tris as well as in 0.2 M phosphate plus 0.2 M NaCl. Thus, there are no obvious links among the tertiary structural change as manifested by the breaking and forming of the intrasubunit salt bridge between β146 histidine and β94 aspartate, the Bohr effect, and the quaternary structural change as manifested by the breaking and forming of the intersubunit hydrogen bonds between α42 tyrosine and β98 aspartate [based on our ^{1}H NMR data and on Perutz's (1970) X-ray crystallographic data]. The recent ^{1}H NMR results on Hb A strongly suggest that there are complex tertiary and quaternary structural changes upon oxygenation of hemoglobin and that the cooperative oxygenation of hemoglobin cannot be adequately described by two-state allosteric models (Viggiano & Ho, 1979; Viggiano et al., 1979).

Finally, since the present ^{1}H NMR results are obtained in D_2O rather than in H_2O, one must ask whether or not the experimental results obtained in D_2O are relevant to those under physiological conditions, namely, in H_2O. More recently, we have made a direct comparison between the oxygen dissociation curves of Hb A and ^{1}H NMR studies of the binding of O_2 to Hb A in D_2O and H_2O (Viggiano & Ho, 1979; Viggiano et al., 1979). We have found that these results are very comparable. Hence, the results obtained in D_2O are

indeed relevant to those in H_2O.

Acknowledgments

We are grateful to Janice Fogg of the MRC Laboratory of Molecular Biology, Cambridge, England, for sending us a sample of des-β146-histidine-hemoglobin needed for this work. We thank Drs. Susan R. Dowd, John V. Kilmartin, Max F. Perutz, E. Ann Pratt, and Guilio Viggiano for helpful discussions.

Appendix

^{1}H NMR Investigation of Conformational Equilibria of the β146 Histidine in Hemoglobin

On the basis of the experimental results presented in this paper, the environment of the β146 histidine of Hb A can be described in terms of the conformational equilibria as given in Scheme I, where β146(1)H and β146(1) refer to the conformation in which the β146 histidine is close to β94 aspartate, such that they can form a salt bridge, i.e., conformation 1, and β146(2)H and β146(2) refer to the conformation in which the β146 histidine is floating free in the solvent, i.e., conformation 2. The k's are the rate constants and the K's are the equilibrium constants.

The type of ^{1}H NMR spectrum obtained for the C2 proton of histidine from such a system depends on the magnitude of the rate constants k_i and k_{-i} (i = 1–4) relative to each other and relative to the NMR time scale. In the protonation equilibrium, the exchange of protons (i.e., on and off the nitrogens of an imidazole ring) is fast compared to the NMR time scale (Eigen et al., 1960; Markley, 1975). Therefore, for each of the protonation equilibria in Scheme I, one should observe a single resonance with a chemical shift which is a weighted average of the chemical shifts in the protonated and unprotonated forms. When the exchange rates between the two conformations of β146 histidine (i.e., k_3^{-1}, k_{-3}^{-1}, k_4^{-1}, and k_{-4}^{-1}) are slow on the NMR time scale, two resonances should be present in the ^{1}H NMR spectrum, one for each conformation, as shown in Figure 7. In the following model, we have assumed that the equilibrium constant between the two conformations of the β146 His, i.e., K_3, is pH independent.

Conformation 1. For the conformation in which β146 histidine is held close to β94 aspartate, its observed chemical shift (δ_1) is given by

$$\delta_1 = \delta_{\beta146(1)H} f_{\beta146(1)H} + \delta_{\beta146(1)} f_{\beta146(1)} \qquad (A1)$$

and its intensity is proportional to $f_{\beta146(1)H} + f_{\beta146(1)}$, where $f_{\beta146(1)H}$ and $f_{\beta146(1)}$ denote, respectively, the fraction of β146(1)H and β146(1) species and $\delta_{\beta146(1)H}$ and $\delta_{\beta146(1)}$ are the C2 proton chemical shifts corresponding, respectively, to these two states in conformation 1.

Conformation 2. For the conformation in which β146 histidine is floating free in solution, its observed chemical shift (δ_2) is given by

$$\delta_2 = \delta_{\beta146(2)H} f_{\beta146(2)H} + \delta_{\beta146(2)} f_{\beta146(2)} \qquad (A2)$$

and its intensity is proportional to $f_{\beta146(2)H} + f_{\beta146(2)}$, where $f_{\beta146(2)H}$ and $f_{\beta146(2)}$ denote, respectively, the fraction of β146(2)H and β146(2) species and $\delta_{\beta146(2)H}$ and $\delta_{\beta146(2)}$ are their respective C2 proton chemical shifts in these two states in conformation 2.

By use of the equilibrium constants K_1 to K_3, the fraction of each species can be expressed as

$$f_{\beta146(1)H} = \frac{K_3[H^+]}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \qquad (A3)$$

$$f_{\beta146(1)} = \frac{K_1K_3}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \qquad (A4)$$

$$f_{\beta146(2)H} = \frac{[H^+]}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \qquad (A5)$$

$$f_{\beta146(2)} = \frac{K_2}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \qquad (A6)$$

For the resonance of β146 His in conformation 1, we have

$$\delta_1 = \frac{\delta_{\beta146(1)H}K_3[H^+] + \delta_{\beta146(1)}K_1K_3}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \qquad (A7)$$

and for the corresponding resonance in conformation 2, we have

$$\delta_2 = \frac{\delta_{\beta146(2)H}[H^+] + \delta_{\beta146(2)}K_2}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \qquad (A8)$$

It can be shown that in a general case these two resonances should titrate with *the same pK value* despite the fact that they originate from two different environments. Each of the resonances in conformations 1 and 2 behaves as a single titratable group resonance in a NMR titration experiment. Mathematically, this means that eq A7 and A8 are each equivalent to a simple Henderson–Hasselbalch equation of the type given in eq 1.

For the resonance in conformation 1, this equivalence is

$$\delta_1 = \frac{\delta_{\beta146(1)H}K_3[H^+] + \delta_{\beta146(1)}K_1K_3}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \equiv \frac{\delta_{\beta146(1)H}'[H^+] + \delta_{\beta146(1)}'K^{(1)}}{[H^+] + K^{(1)}} \qquad (A9)$$

where $\delta_{\beta146(1)H}'$ and $\delta_{\beta146(1)}'$ are the higher and the lower asymptotes and $K^{(1)}$ is the ionization constant for the titration curve that one "sees" for the resonance in conformation 1 in a NMR experiment. The sign ≡ means that eq A9 should be valid at any pH value or, in other words, that eq A9 should be a mathematical identity for any $[H^+]$. Through simple algebraic manipulations, equating the coefficients for the same powers of $[H^+]$ on both sides of eq A9, one obtains

$$\delta_{\beta146(1)H}' = \left(\frac{K_3}{1 + K_3}\right)\delta_{\beta146(1)H} \qquad (A10)$$

$$\delta_{\beta146(1)}' = \left(\frac{K_1K_3}{K_2 + K_1K_3}\right)\delta_{\beta146(1)} \qquad (A11)$$

$$K^{(1)} = \frac{K_2 + K_1K_3}{1 + K_3} \qquad (A12)$$

These three expressions relate the *observed* titration parameters for the resonance in conformation 1 [i.e., $\delta_{\beta146(1)H}'$, $\delta_{\beta146(1)}'$, and $K^{(1)}$] to the parameters which characterize the *intrinsic* conformation of the histidyl residue [i.e., $\delta_{\beta146(1)H}$, $\delta_{\beta146(1)}$, K_1, K_2, and K_3].

For the resonance in conformation 2, using the same procedure, the identity relationship is

$$\delta_2 = \frac{\delta_{\beta146(2)H}[H^+] + \delta_{\beta146(2)}K_2}{K_3[H^+] + [H^+] + K_2 + K_1K_3} \equiv \frac{\delta_{\beta146(2)H}'[H^+] + \delta_{\beta146(2)}'K^{(2)}}{[H^+] + K^{(2)}} \qquad (A13)$$

Similarly, one can readily obtain the relations

$$\delta_{\beta146(2)H}' = \left(\frac{1}{1 + K_3}\right)\delta_{\beta146(2)H} \quad \text{(A14)}$$

$$\delta_{\beta146(2)}' = \left(\frac{K_2}{K_2 + K_1K_3}\right)\delta_{\beta146(2)} \quad \text{(A15)}$$

$$K^{(2)} = \frac{K_2 + K_1K_3}{1 + K_3} \quad \text{(A16)}$$

where $\delta_{\beta146(2)H}'$, $\delta_{\beta146(2)}'$, and $K^{(2)}$ are the observed titration parameters for the resonance in conformation 2.

One should notice that the apparent pK value for each of these two resonances is the same, as shown in eq A12 and A16. Therefore, despite the fact that these two resonances originate from two environments in which the histidyl residue has very different ionization properties, they both exhibit the same apparent pK value in a NMR titration experiment. Moreover, the chemical shifts for the protonated and unprotonated forms that one could obtain from the NMR data for the two resonances do not entirely reflect the real shielding in the two conformations. This is because the chemical shifts depend on the ionization constants K_1 and K_2 as well as on the conformational equilibrium constant K_3. Thus, the equality of the apparent pK values of these two resonances could, in the most general case, be mistakenly interpreted as a lack of contribution of a certain group to the Bohr effect.

In the present context, two extreme cases should be considered.

(1) For the case of $K_3 \gg 1$, the great majority of the β146 histidyl residues is in conformation 1 [this situation may be equated to Perutz's (1970) T structure of the Hb molecule].

In this case, $f_{\beta146(1)H} + f_{\beta146(1)}$ is practically 1. Inspection of eq A10–A12 reveals that for $K_3 \gg 1$, $\delta_{\beta146(1)H}' = \delta_{\beta146(1)H}$, $\delta_{\beta146(1)}' = \delta_{\beta146(1)}$, and $K^{(1)} = K_1$. Hence, the titration of the resonance in conformation 1 reflects in this case the intrinsic environment of β146 histidine in this conformation. This situation has been observed experimentally by Kilmartin et al. (1973) in deoxy-Hb A solution and by us in both deoxy- and (carbon monoxy)hemoglobin solutions. In all these cases, only the resonance of β146 His in conformation 1 is observed.

(2) For the case of $K_3 \ll 1$, the great majority of the β146 histidine residues is floating free in solution [this situation may be equated to Perutz's (1970) R structure]. In this case, $f_{\beta146(2)H} + f_{\beta146(2)}$ is practically 1. From eq A14–A16, one gets for $K_3 \ll 1$, $\delta_{\beta146(2)H}' = \delta_{\beta146(2)H}$, $\delta_{\beta146(2)}' = \delta_{\beta146(2)}$, and $K^{(2)} = K_2$. Hence, the titration of the resonance of β146 His reflects only the second conformation for this histidine. This situation has been observed experimentally by Kilmartin et al. (1973) in 0.2 M phosphate + 0.2 M NaCl buffer in the carbon monoxy form, where only the resonance in conformation 2 is observed.

In the most general case, a detailed analysis of this type of NMR data is able to provide not only the pK values but also information on the dynamics of the β146 His in the Hb molecule. For instance, one can obtain the specific equilibrium constant for the formation of the β146 His–β94 Asp salt bridge. In the present work, we have found that this equilibrium constant is strongly dependent not only on the quaternary and tertiary structure of the Hb molecule but also on the solvent environment. The physical basis for such a dependence is not fully understood. Nevertheless, in the following paragraph, we suggest a simple hypothesis for this behavior.

Based on X-ray crystallographic data, Perutz (1970) pointed out that the conformation of the carboxy-terminal region of the β chain in deoxy-Hb is such that β146 His is "forced" to a position close to β94 Asp. We shall express this conformational constraint on the position of β146 His in the deoxy-Hb molecule as a decrease in the free energy of β146 His (i.e., $\Delta G^\circ_{\text{conformation}}$ is negative) when it goes from a random surface conformation to the position found by X-ray crystallography, i.e., close to β94 Asp. Once the β146 His is close to β94 Asp, this position is further stabilized by the formation of the salt bridge β146 His–β94 Asp (Perutz, 1970). This electrostatic interaction will further decrease the free energy of β146 His by an amount that we shall call $\Delta G^\circ_{\text{salt bridge}}$. It should be pointed out that it is this $\Delta G^\circ_{\text{salt bridge}}$ term that is mainly responsible for the abnormally high pK value observed for β146 His in deoxy-Hb.

The results obtained in this work indicate that the conformation of β146 His is also determined by its interaction with the solvent. We shall take into account this effect by assuming that the transfer of this histidyl residue from a position "inside" the protein molecule (such as in conformation 1) to a position where it is in contact with a polar solvent (such as in conformation 2) involves a decrease in its free energy, which we shall call $\Delta G^\circ_{\text{solvent}}$.

As shown above in the conformation equilibrium scheme involving β146 His (Scheme I), if the pK values in the two conformations are given, the equilibrium distribution will be determined solely by K_3, the equilibrium constant for the change in conformation involving two *charged* β146 histidines. This is a consequence of the fact that K_4 is not an independent constant in the above equilibrium scheme, but it is related to the other three by the relation $K_1K_3 = K_2K_4$.

In our model, K_3 can be written as $RT \ln K_3 = -(\Delta G^\circ_{\text{conformation}} + \Delta G^\circ_{\text{salt bridge}} + \Delta G^\circ_{\text{solvent}})$. The NMR results of Kilmartin et al. (1973) as well as those reported here show that in the deoxy form the great majority of β146 histidyl residues is in the first conformation, i.e., $K_3 \gg 1$. In our model, this means that $|\Delta G^\circ_{\text{conformation}}(\text{deoxy}) + \Delta G^\circ_{\text{salt bridge}}| \gg |\Delta G^\circ_{\text{solvent}}|$. In the carbon monoxy form, since the conformation is such that $\Delta G^\circ_{\text{conformation}}$ could be positive or if negative it has a smaller absolute value than that in the deoxy form, the solvent effect, expressed here as $\Delta G^\circ_{\text{solvent}}$, becomes much more important in determining the K_3 value. In the "stripped" condition (i.e., 0.1 M Bis-Tris) used in this work, the $\Delta G^\circ_{\text{solvent}}$ contribution should not be large enough to compensate for the total decrease in the free energy as a result of the salt bridge formation; i.e., $|\Delta G^\circ_{\text{conformation}}(\text{CO}) + \Delta G^\circ_{\text{salt bridge}}| \gg |\Delta G^\circ_{\text{solvent}}|$ and K_3 is still much greater than 1. Once we start adding phosphate and/or chloride ions to the hemoglobin solution, $\Delta G^\circ_{\text{solvent}}$ becomes more important. For intermediate concentrations of these ions, $|\Delta G^\circ_{\text{solvent}}|$ can be in the same range of magnitude as $|\Delta G^\circ_{\text{conformation}}(\text{CO}) + \Delta G^\circ_{\text{salt bridge}}|$. In this case, β146 histidines in both conformations are present in solution, a situation shown in Figure 7. In the experimental conditions used by Kilmartin et al. (1973), $\Delta G^\circ_{\text{solvent}}$ is increased greatly so that $|\Delta G^\circ_{\text{solvent}}| \gg |\Delta G^\circ_{\text{conformation}}(\text{CO}) + \Delta G^\circ_{\text{salt bridge}}|$, K_3 becomes much less than unity, and most of the β146 histidines are then in the "floating" free conformation in the carbon monoxy form.

The above formulation, although very schematic, could provide a way to correlate the X-ray data with the situation observed in solution.

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