Biol. Chem. 263, 16209–16217.
Tanaka, K., Yoshimura, T., Ichihara, A., Ikai, A., Nishigai, M., Morimoto, M., Sato, M., Tanaka, N., Katsube, Y., Kameyama, K., & Takagi, T. (1988b) *J. Mol. Biol. 203*, 985–996.
Tanaka, K., Kumatori, A., Ii, K., & Ichihara, A. (1989) *J. Cell. Physiol. 139*, 34–41.
Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayers, E. L. V., Whittle, N., Waterfield, M. D., & Seeburg, P. H. (1984) *Nature 309*, 418–425.
Waxman, L., Fagan, J. M., Tanaka, K., & Goldberg, A. L. (1985) *J. Biol. Chem. 260*, 1194–1200.
Yarden, Y., Escobedo, J. A., Kuang, W.-J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Fried, V. A., Ullrich, A., & Williams, L. T. (1986) *Nature 323*, 226–232.
Yoneda, Y., Imamoto-Sonobe, N., Matsuoka, Y., Iwamoto, R., Kiho, Y., & Uchida, T. (1988) *Science 242*, 275–278.

^1H and ^{31}P Nuclear Magnetic Resonance Investigation of the Interaction between 2,3-Diphosphoglycerate and Human Normal Adult Hemoglobin†

Irina M. Russu,‡ Shing-Shing Wu, Keith A. Bupp,§ Nancy T. Ho, and Chien Ho*
Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213
Received June 28, 1989; Revised Manuscript Received November 22, 1989

ABSTRACT: High-resolution ^1H and ^{31}P nuclear magnetic resonance spectroscopy has been used to investigate the binding of 2,3-diphosphoglycerate to human normal adult hemoglobin and the molecular interactions involved in the allosteric effect of the 2,3-diphosphoglycerate molecule on hemoglobin. Individual hydrogen ion NMR titration curves have been obtained for 22–26 histidyl residues of hemoglobin and for each phosphate group of 2,3-diphosphoglycerate with hemoglobin in both the deoxy and carbonmonoxy forms. The results indicate that 2,3-diphosphoglycerate binds to deoxyhemoglobin at the central cavity between the two β chains and the binding involves the β2-histidyl residues. Moreover, the results suggest that the binding site of 2,3-diphosphoglycerate to carbonmonoxyhemoglobin contains the same (or at least some of the same) amino acid residues responsible for binding in the deoxy form. As a result of the specific interactions with 2,3-diphosphoglycerate, the β2-histidyl residues make a significant contribution to the alkaline Bohr effect under these experimental conditions (up to 0.5 proton/Hb tetramer). 2,3-Diphosphoglycerate also affects the individual hydrogen ion equilibria of several histidyl residues located away from the binding site on the surface of the hemoglobin molecule, and, possibly, in the heme pockets. These results give the first experimental demonstration that long-range electrostatic and/or conformational effects of the binding could play an important role in the allosteric effect of 2,3-diphosphoglycerate on hemoglobin. The ^{31}P nuclear magnetic resonance titration data for each phosphate group of 2,3-diphosphoglycerate have been used to calculate the pK values of the phosphate groups in 2,3-diphosphoglycerate bound to deoxy- and carbonmonoxyhemoglobin and the proton uptake by 2,3-diphosphoglycerate upon ligand binding to hemoglobin.

2,3-Diphosphoglycerate (2,3-DPG)[1] is the predominant phosphorylated metabolite inside red blood cells. The allosteric effect of this compound on hemoglobin (Hb) leads to a dramatic decrease in the oxygen affinity of the Hb molecule and thus facilitates the unloading of oxygen to the tissues (Benesch & Benesch, 1969, 1974). 2,3-DPG also influences the pH dependence of the oxygen affinity of Hb by increasing both the alkaline and the acid Bohr effect (de Bruin et al., 1971, 1973, 1974; de Bruin & Janssen, 1973; Kilmartin, 1974). The allosteric effect of 2,3-DPG upon Hb function results from the higher binding affinity of 2,3-DPG for the deoxy form of the Hb molecule. The site of the binding of 2,3-DPG to human normal adult Hb (Hb A) in the deoxy form has been identified by X-ray crystallography to be at the central cavity between the two β chains on the 2-fold symmetry axis of the molecule (Arnone, 1972). The site of the binding of 2,3-DPG to the ligated form of Hb is not known, although it has been suggested that it may be close to that in the deoxy form (Gupta et al., 1979).

An understanding of the allosteric effect of 2,3-DPG on Hb requires a characterization of the roles played by individual groups of Hb and 2,3-DPG in the binding process. Nuclear magnetic resonance (NMR) spectroscopy is uniquely suited to obtain such a characterization, since it is the only technique

† Preliminary results were presented at the 27th Annual Meeting of the Biophysical Society, Feb 14–17, 1983, San Diego, CA. This research was supported by a research grant from the National Institutes of Health (HL-24525).
* Address correspondence for this author at the Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213.
‡ Present address: Department of Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06457.
§ Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

[1] Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; Hb, hemoglobin; Hb A, human normal adult hemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; deoxy-Hb, deoxyhemoglobin; NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/90/0429-3785$02.50/0

presently available which can monitor single functional groups of proteins in the solution state. Several spectroscopic probes in Hb are of special value for an investigation of its interactions with 2,3-DPG. The surface histidyl residues of Hb can be observed individually and their hydrogen ion equilibria can be measured by ^{1}H NMR spectroscopy. Hence, these amino acid residues can be used as markers for the binding site(s) of 2,3-DPG. Moreover, the changes in their H^+ equilibria upon the binding of 2,3-DPG to Hb provide direct information on the molecular basis of the linkage between the allosteric effects of H^+ and 2,3-DPG. Complementary information on this linkage can be obtained by monitoring the ^{31}P resonances of each phosphate group in 2,3-DPG (Huestis & Raftery, 1972). The latter method has also been employed extensively to monitor the metabolism of phosphorylated compounds in normal and diseases erythrocytes [see Moon and Richards (1973), Lam et al. (1979), and Tehrani et al. (1982) and references cited therein]. An investigation of the interaction of 2,3-DPG with Hb in solution by ^{31}P NMR spectroscopy is, thus, of interest for further studies of the intracellular environment of red blood cells and of metabolism inside intact red blood cells.

Materials and Methods

Materials

Hb A was prepared by the procedure of Drabkin (1946) as described previously (Lindstrom & Ho, 1972). The organic phosphates were removed by passing the Hb solutions through a Sephadex G-25 column equilibrated with 0.01 M Tris buffer plus 0.1 M NaCl at pH 7.6 (Berman et al., 1971). In preliminary experiments, we have found that the concentration of 2,3-DPG in Hb A solutions, prepared as described, decreased in time, probably due to the presence of 2,3-DPG phosphatase. Hb A was further purified on a DE-52 column (30 × 2.5 cm) equilibrated with 0.05 M Tris buffer at pH 8.1 (pH measured at room temperature) (D. Elbaum, private communication). The column was run at 4 °C, and Hb A was eluted with the same buffer plus 0.2 M NaCl. The concentration of 2,3-DPG in Hb A solutions purified by this method remained constant for at least 72 h after the addition of 2,3-DPG. The Hb A solutions in H_2O were exchanged with D_2O and concentrated by repeated centrifugation in Centriflo (Amicon) membrane cones. Concentrated stock solutions in D_2O of Bis-Tris buffer (for the pH range from 6.0 to 8.0) or of Tris buffer (for pH values higher than 8.0) were added to the Hb solutions such that the final buffer concentration was 0.1 M. For the pH range below 6.0, the Hb solutions were dialyzed against 0.1 M Bis-Tris buffer in D_2O. As a result of the titration of the stock buffer solutions with DCl, the concentration of chloride ions in our NMR samples varied from 0.005 to 0.06 M over the pH range investigated.

Stock solutions of 2,3-DPG in the protonated form were obtained from the pentacyclohexylammonium salt (Calbiochem) by stirring it in Bio-Rad cation-exchange resin (50–100 mesh, H^+ form) for 5 min at room temperature. The resin was removed by filtration, and the 2,3-DPG solutions were lyophilized overnight. A volume of 40–50 μL of the concentrated solution of 2,3-DPG in D_2O was added to each Hb sample in 0.1 M Bis-Tris (or Tris) buffer in D_2O 1–4 h prior to the NMR experiment. The final pH values of the Hb samples are reported as direct pH-meter readings since the deuterium isotope effect on glass electrodes (namely, pD = pH + 0.4; Glasoe & Long, 1960) is compensated by its effect on the imidazole pK value (Tanokura et al., 1978). The deoxygenation of the Hb A samples was carried out in a rotary evaporator under nitrogen at 4 °C as described previously (Lindstrom & Ho, 1972).

Immediately following the NMR measurements, the Hb samples were added to trichloroacetic acid and stored at −70 °C. Within a week, the final concentration of 2,3-DPG in the Hb A samples was measured by using an enzymatic assay (Sigma, 35-UV bulletin). Each NMR sample was also assayed for the concentration of inorganic phosphate by measuring the optical absorption at 820 nm following reaction with ascorbic acid and ammonium molybdate. For all the experiments, the molar ratio of 2,3-DPG to Hb was between 0.9 and 1.1.

Methods

The NMR spectra were obtained on a WH-300 Bruker spectrometer operating at 300 MHz for ^{1}H and at 121.48 MHz for ^{31}P. Each ^{1}H NMR spectrum was the result of averaging 200–400 transients at a repetition rate of 0.25 s^{-1}. The ^{1}H chemical shifts are expressed relative to the proton resonance of residual water in D_2O solvent. The water resonance is 4.73 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 27 °C. The ^{31}P NMR spectra were obtained with broad-band proton decoupling, and each spectrum was the result of averaging 1600 transients at a repetition rate of 0.3 s^{-1}. The ^{31}P chemical shifts are expressed relative to the ^{31}P resonance of 85% phosphoric acid. The chemical shifts are defined as positive if they are downfield from the reference signal.

Data Analysis. The ionizable groups of interest to the present work are the surface histidyl residues of Hb A and the phosphate groups of 2,3-DPG. Under the conditions of our experiments, these groups can exist in the free state or in the complex between 2,3-DPG and Hb. In each of these states, the group of interest can be protonated or unprotonated (deuterated or undeuterated in D_2O). As shown under Results, the exchange between these states (i.e., free vs bound and protonated vs unprotonated) is fast on the NMR chemical shift scale (for both ^{1}H and ^{31}P). Consequently, in the NMR titration, an averaged resonance is observed, and its chemical shift is

$$\delta = \delta_B^+ f_B^+ + \delta_B^0 f_B^0 + \delta_F^+ f_F^+ + \delta_F^0 f_F^0 \quad (1)$$

where f_B^+ and f_F^+ are the fractions of the protonated group in the bound and free state, respectively. Similarly, f_B^0 and f_F^0 are the fractions of the unprotonated group in the two states. δ_B^+, δ_F^+, δ_B^0, and δ_F^0 are the intrinsic chemical shifts in the corresponding states.

To simplify the data analysis, we have assumed that the intrinsic chemical shifts do not change upon binding of 2,3-DPG to Hb; i.e., $\delta_B^+ = \delta_F^+ = \delta^+$ and $\delta_B^0 = \delta_F^0 = \delta^0$. Under this assumption, eq 1 becomes

$$\delta = \delta^+ f^+ + \delta^0 f^0 \quad (2)$$

where $f^+ = f_B^+ + f_F^+$ and $f^0 = f_B^0 + f_F^0$

In analogy to the protonation equilibrium of a single ionizable group, f^+ and f^0 can be expressed as (Markley, 1975; Russu et al., 1982)

$$f^+ = \frac{[H^+]}{[H^+] + K} \text{ and } f^0 = \frac{K}{[H^+] + K} \quad (3a)$$

or

$$f^+ = \frac{[H^+]^n}{[H^+]^n + K^n} \text{ and } f^0 = \frac{K^n}{[H^+]^n + K^n} \quad (3b)$$

where K is the apparent equilibrium constant for the protonation of the ionizable group of interest (pK = −log K) and n

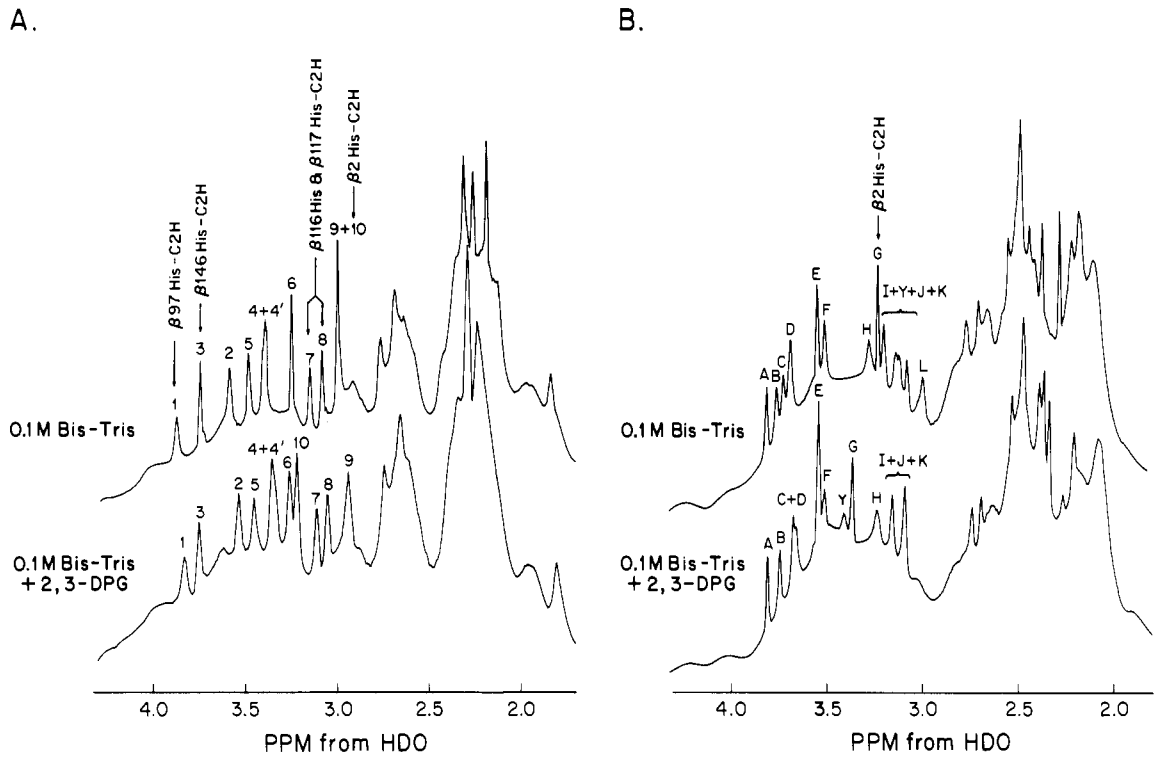

FIGURE 1: 300-MHz ^{1}H NMR spectra of 10% Hb A solutions in 0.1 M Bis-Tris buffer in D_2O at 27 °C: (A) deoxy form at pH 7.10; (B) CO form at pH 6.70. Effects of 2,3-DPG (1:1 molar ratio to Hb) upon the aromatic proton resonances.

is the apparent NMR titration coefficient.[2] With these expressions, eq 2 becomes

$$\delta = \frac{\delta^+[H^+] + \delta^0 K}{[H^+] + K} \quad (4)$$

or

$$\delta = \frac{\delta^+[H^+]^n + \delta^0 K^n}{[H^+]^n + K^n} \quad (5)$$

Under the experimental conditions used, the fractions of Hb A and 2,3-DPG in the complex change as a function of pH (especially for the ligated form at pH above 7.0, where the binding constant is greatly decreased). Consequently, the titration parameters obtained from eq 4 or 5 do not reflect solely the ionization properties of the group in the complex of 2,3-DPG with Hb A, but are averaged by the exchange between the free and the bound state. Nevertheless, under the assumption used in eq 2 (i.e., in the absence of spectroscopic effects), the NMR titration curves do reflect the individual H^+ binding equilibrium for the group of interest under the experimental conditions used.

The NMR titration curves of the phosphate groups in a 2,3-DPG molecule bound to Hb, δ_B, were obtained from eq 1 as

$$\delta_B = \delta_F + \frac{\delta - \delta_F}{f_B} \quad (6)$$

where δ_F is the ^{31}P NMR titration curve of the corresponding phosphate group in a free 2,3-DPG molecule and f_B is the fraction of 2,3-DPG molecules in the Hb-bound state. The ^{31}P NMR titration curves for free 2,3-DPG, δ_F, are assumed to be the same as those measured for a solution of 2,3-DPG in Bis-Tris buffer in the absence of Hb. The observed ^{31}P NMR titration curves of 2,3-DPG, δ, were corrected in deoxy-Hb A for the effect of the magnetic susceptibility according to Fabry and San George (1983) (i.e., the chemical shifts were reduced by 0.35 ppm over the entire pH range investigated, as indicated for solenoidal magnets and 0.002 M deoxy-Hb A solutions). The fractions of 2,3-DPG molecules in the bound state, f_B, were calculated, at each pH value, by using the equilibrium binding constants determined by Hobish and Powers (1986) for the binding of 2,3-DPG to deoxy-Hb A and ligated Hb A in 0.05 M Bis-Tris (or Tris) buffer in the presence of 0.1 M chloride ions and 0.001 M EDTA. By using these values for the binding constants, we have neglected any effects of the buffer and chloride ions upon binding.

Results

In the absence of 2,3-DPG, 11–13 surface histidyl residues per $\alpha\beta$ dimer of Hb A can be individually observed in the ^{1}H NMR spectra. In Figure 1, the C2-proton resonances of these histidyl residues are labeled 1–10 in the deoxy form and A–L in the CO form as in our previous publications (Russu et al., 1982). Several of these resonances have been assigned to specific histidyl residues of Hb A as follows: in the deoxy form, resonance 1 has been assigned to β97His, resonance 3 to β146His, resonances 7 and 8 to β116His and β117His (tentatively), and resonance 10 to β2His; in the carbonmonoxy form, resonances J and K have been assigned to β116His and β117His (tentatively) and resonance G to β2His (Kilmartin et al., 1973; Russu et al., 1980, 1982, 1984; Perutz et al., 1985a,b).

The addition of an equimolar concentration of 2,3-DPG to Hb A solutions specifically affects several His-C2 proton resonances in both the deoxy and CO forms. The largest effect

[2] The coefficient n in eqs 3b and 5 is normally called the Hill coefficient for the ^{1}H NMR titration of the histidyl residue. In the case of Hb, this nomenclature could be confusing due to the use of the same term to describe the cooperativity in the oxygen binding. For this reason, we have chosen to call the coefficient n in eqs 3b and 5 the titration coefficient for the ^{1}H NMR titration of the histidyl residue.

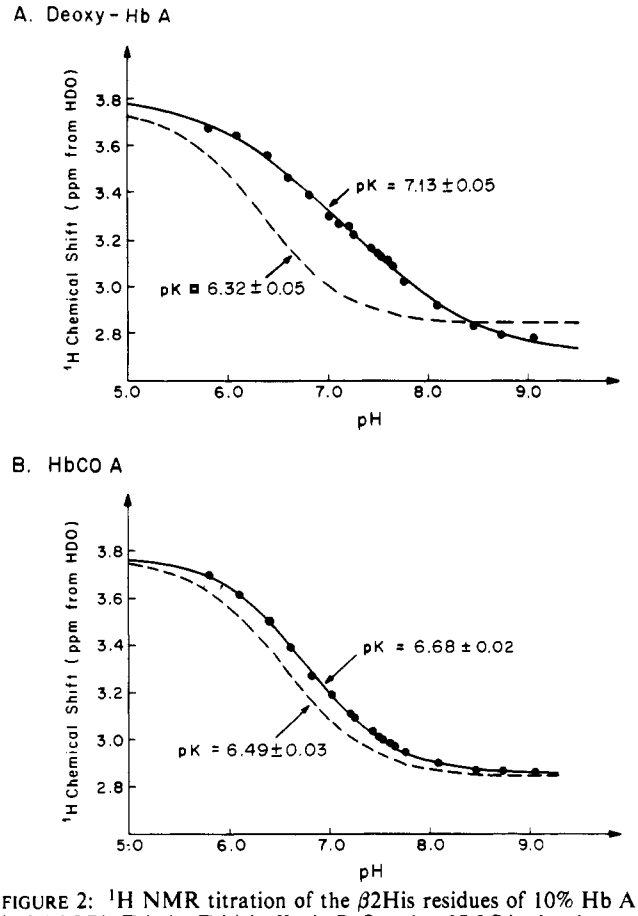

FIGURE 2: ^{1}H NMR titration of the β2His residues of 10% Hb A in 0.1 M Bis-Tris (or Tris) buffer in D_2O and at 27 °C in the absence (---) and presence (—) of 2,3-DPG (1:1 molar ratio to Hb): (A) deoxy-Hb A; (B) HbCO A. The experimental data (●) have been fitted to eq 5. The ^{1}H NMR titration data of the β2His residues in the same buffer in the absence of 2,3-DPG are reproduced from Russu et al. (1982).

is observed for the C2-proton resonances of the β2His residues (resonance labeled 10 in the deoxy form and that labeled G in the CO form) which shift downfield by 0.1–0.5 ppm in the presence of the allosteric effector. As the concentration of 2,3-DPG increases, the β2His-C2 proton resonance shifts as a single resonance (results not shown), indicating that the exchange of the 2,3-DPG molecule between its free and Hb-bound forms is fast on the NMR chemical shift time scale [i.e., exchange rates greater than approximately 10^3 s^{-1}; also see Huestis and Raftery (1972)]. The complete pH titration of the β2His residues reveals that this downfield shift of the β2His-C2 proton resonances originates from an increase in the p*K* value of this histidyl residue in the presence of 2,3-DPG (vide infra, Tables I and II, Figure 2). In the CO form, the addition of 2,3-DPG to the Hb solution also results in resonance L being broadened beyond detection and in the occurrence of a new His-C2 proton resonance in the spectrum (labeled Y in Figure 1B). Resonance Y has been previously assigned in HbCO A in the presence of inorganic phosphate ions to the β146His residues in a conformation in which the β146His–β94Asp salt-bridge is broken (Kilmartin et al., 1973; Russu et al., 1980; Russu & Ho, 1986). In the absence of phosphate ions and at low anion concentrations, resonance Y is difficult to monitor in the ^{1}H NMR spectra of HbCO A over the entire pH range of interest.

The chemical shifts of each of the His-C2 proton resonances of Hb A in both deoxy and CO forms have been fitted as a function of pH to eqs 4 and 5, and the results are summarized in Tables I and II.[3] Tables I and II also include the p*K* values

Table I: pK Values of Surface Histidyl Residues in 10% Deoxy-Hb A and HbCO A Solutions in 0.1 M Bis-Tris (or Tris) Buffer in D_2O in the Absence and Presence of 0.0015 M 2,3-DPG at 27 °C[a]

	p*K*	
resonance[b]	0.1 M Bis-Tris[c]	0.1 M Bis-Tris + 2,3-DPG
1 (β97)	8.07 ± 0.04	7.85 ± 0.02
2	7.28 ± 0.02	7.17 ± 0.01
3 (β146)	7.98 ± 0.03	7.99 ± 0.02
4	7.20 ± 0.03	7.10 ± 0.01
4′	7.20 ± 0.03	7.03 ± 0.02
5	7.76 ± 0.04	7.65 ± 0.03
6	7.10 ± 0.03	7.02 ± 0.01
7	7.07 ± 0.06	7.12 ± 0.02
8	6.71 ± 0.06	6.74 ± 0.04
9	6.82 ± 0.07	6.86 ± 0.02
10 (β2)	6.35 ± 0.03	7.25 ± 0.03
A	7.87 ± 0.02	7.87 ± 0.01
B	7.42 ± 0.02	7.35 ± 0.02
C	7.85 ± 0.03	7.82 ± 0.03
D	7.65 ± 0.02	7.66 ± 0.01
E	7.15 ± 0.02	7.13 ± 0.02
F	7.00 ± 0.03	7.03 ± 0.01
G (β2)	6.53 ± 0.02	6.75 ± 0.01
I	6.55 ± 0.03	6.41 ± 0.05
J	6.81 ± 0.03	6.95 ± 0.04
K	6.76 ± 0.06	6.79 ± 0.03
H	6.20 ± 0.05	6.02 ± 0.05
Y	*d*	6.82 ± 0.02

[a] Results of nonlinear least-squares fitting to eq 4. [b] Resonances labeled 1–10 correspond to the deoxy form of Hb A, and those labeled A–Y correspond to the carbonmonoxy form. [c] Taken from Tables I and II in Russu et al. (1982). [d] Not readily observable under these experimental conditions.

Table II: pK Values and NMR Titration Coefficients of Surface Histidyl Residues in 10% Deoxy-Hb A and HbCO A Solutions in 0.1 M Bis-Tris (or Tris) Buffer in D_2O in the Absence and Presence of 0.0015 M 2,3-DPG at 27 °C[a]

	0.1 M Bis-Tris[c]		0.1 M Bis-Tris + 2,3-DPG	
resonance[b]	p*K*	*n*	p*K*	*n*
1 (β97)	8.13 ± 0.06	0.87 ± 0.07	7.88 ± 0.03	0.94 ± 0.06
2	7.29 ± 0.02	0.91 ± 0.04	7.18 ± 0.01	1.09 ± 0.03
3 (β146)	7.98 ± 0.05	0.80 ± 0.05	8.12 ± 0.04	0.80 ± 0.04
4	7.21 ± 0.03	0.85 ± 0.04	7.09 ± 0.01	0.95 ± 0.02
4′	7.21 ± 0.03	0.85 ± 0.04	7.05 ± 0.02	1.05 ± 0.04
5	7.92 ± 0.05	0.63 ± 0.04	7.71 ± 0.03	0.74 ± 0.04
6	7.10 ± 0.03	0.81 ± 0.04	6.98 ± 0.01	0.89 ± 0.02
7	7.02 ± 0.08	0.48 ± 0.06	7.04 ± 0.02	0.78 ± 0.03
8	6.63 ± 0.09	0.59 ± 0.08	6.30 ± 0.20	0.57 ± 0.06
9	6.61 ± 0.07	0.52 ± 0.04	6.80 ± 0.02	0.87 ± 0.02
10 (β2)	6.32 ± 0.05	0.88 ± 0.07	7.13 ± 0.05	0.62 ± 0.05
A	7.90 ± 0.03	0.87 ± 0.05	7.90 ± 0.01	0.91 ± 0.02
B	7.42 ± 0.02	0.83 ± 0.04	7.33 ± 0.02	0.78 ± 0.03
C	7.85 ± 0.04	0.82 ± 0.06	8.01 ± 0.09	0.62 ± 0.06
D	7.68 ± 0.03	0.82 ± 0.05	7.74 ± 0.03	0.71 ± 0.04
E	7.14 ± 0.03	0.81 ± 0.04	7.04 ± 0.02	0.72 ± 0.03
F	6.99 ± 0.03	0.91 ± 0.05	7.01 ± 0.01	0.93 ± 0.02
G (β2)	6.49 ± 0.03	0.91 ± 0.05	6.68 ± 0.02	0.87 ± 0.02
I	6.59 ± 0.05	0.85 ± 0.06	5.60 ± 0.40	0.56 ± 0.07
J	6.78 ± 0.03	0.86 ± 0.05	6.48 ± 0.09	0.52 ± 0.03
K	6.20 ± 0.20	0.45 ± 0.06	6.51 ± 0.04	0.66 ± 0.02
H	6.23 ± 0.05	0.98 ± 0.07	5.80 ± 0.20	0.88 ± 0.09
Y	*d*	*d*	6.87 ± 0.02	1.20 ± 0.05

[a] Results of nonlinear least-squares fitting to eq 5. [b] Resonances labeled 1–10 correspond to the deoxy form of Hb A, and those labeled A–Y correspond to the carbonmonoxy form. [c] Taken from Tables IV and V in Russu et al. (1982). [d] Not readily observable under these experimental conditions.

and the titration coefficients (*n*) of the surface His residues in 0.1 M Bis-Tris (or Tris) buffer in the absence of 2,3-DPG

[3] A preliminary report from this laboratory (Ho & Russu, 1987) included the p*K* values of the histidyl residues of Hb A in the presence of 2,3-DPG which were obtained by fitting the experimental data to eq 5.

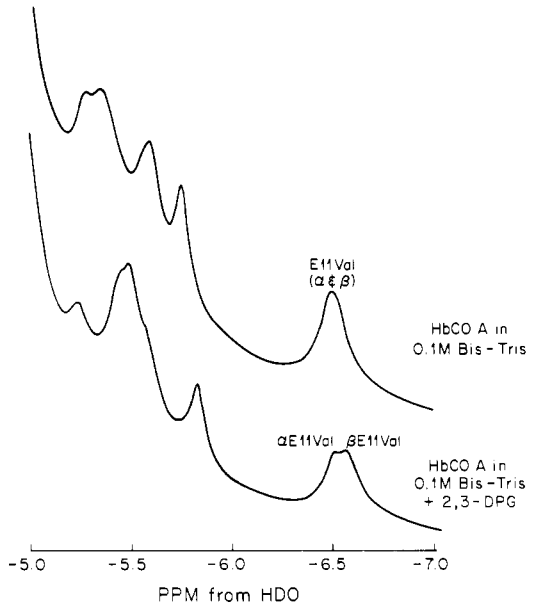

FIGURE 3: 300-MHz ^{1}H NMR spectra of 10% HbCO A solutions in 0.1 M Bis-Tris (or Tris) buffer in D_2O at pH 7.0 and at 27 °C: effect of 2,3-DPG (1:1 molar ratio to Hb) upon the ring-current shifted resonances.

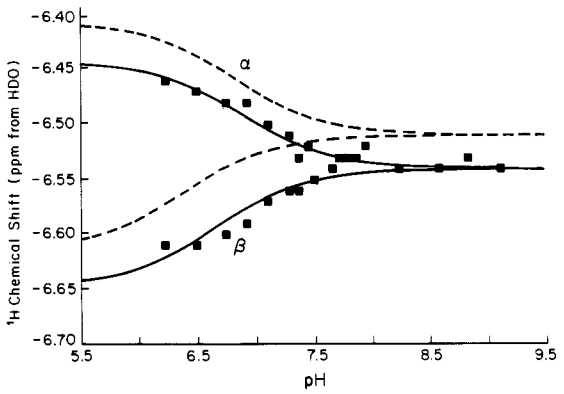

FIGURE 4: pH dependence of the ^{1}H chemical shift of the γ_2-CH_3 resonances of E11Val in the α and β chains of HbCO A in 0.1 M Bis-Tris buffer in D_2O at 27 °C in the absence (---) and presence (—) of a 1:1 molar ratio of 2,3-DPG to Hb.

previously obtained in our laboratory (Russu et al., 1982). The ^{1}H NMR titration curves of the histidyl residues of Hb A in the presence of 2,3-DPG are illustrated in Figure 2 by the titration of β2His.

To investigate the effects of 2,3-DPG upon the conformation of the heme pockets in Hb A, we have used the ring-current shifted proton resonances of HbCO A between −5.0 and −7.0 ppm upfield from HDO (Figure 3). Previous work from this laboratory has shown that these resonances originate from the heme protons and/or from the protons of amino acid residues located in the heme pockets of the α and β chains (Lindstrom et al., 1972; Lindstrom & Ho, 1973; Dalvit & Ho, 1985). The resonance at −6.55 ppm in the ^{1}H NMR spectrum of HbCO A in the absence of 2,3-DPG has been assigned to the γ_2-CH_3 groups of the E11Val in the α and β chains (Lindstrom & Ho, 1972; Dalvit & Ho, 1985). We have carried out the ^{1}H NMR titration of the γ_2-CH_3 proton resonances of the α- and βE11Val residues of HbCO A in the presence of an equimolar concentration of 2,3-DPG (Figure 4). The chemical shift of each resonance has been fitted as a function of pH to eq 4. The apparent pK values of the α- and βE11Val resonances obtained by this analysis are 6.47 ± 0.03 and 6.0 ± 0.1, respectively, in the absence of 2,3-DPG (at 29 °C), and 6.55 ± 0.08 and 6.6 ± 0.1, respectively, in the presence of 2,3-DPG (at 27 °C). These pK values should be regarded as upper limit estimates of the actual values since the ^{1}H NMR titration of these resonances is not complete at the lowest pH value investigated.

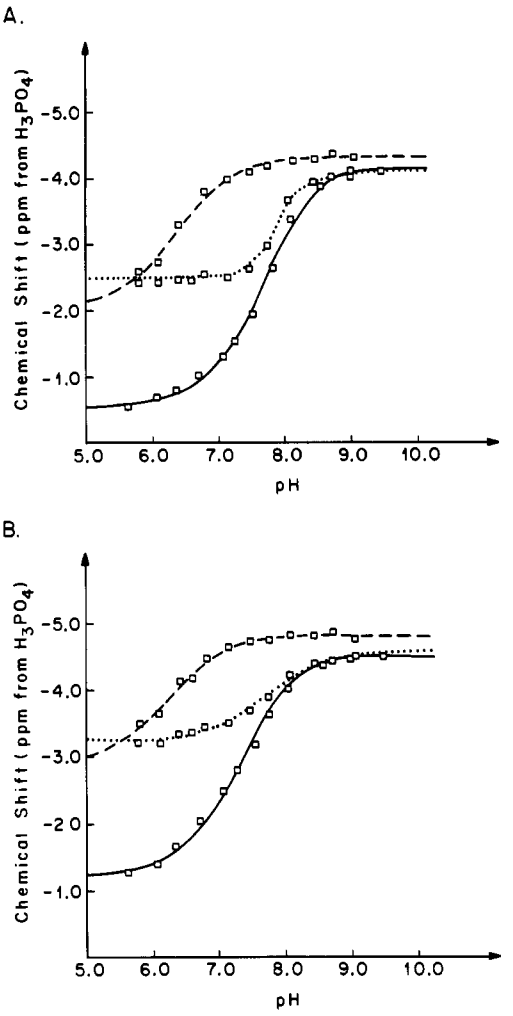

FIGURE 5: ^{31}P NMR titration of 2,3-DPG in 0.1 M Bis-Tris (or Tris) buffer in D_2O at 27 °C in the absence (—) and in the presence of deoxy-Hb A (---) or HbCO A (···) (1:1 molar ratio to 2,3-DPG): (A) C2-phosphate; (B) C3-phosphate. The curves correspond to the nonlinear least-squares fit to eq 5.

To obtain further insight into the molecular mechanism involved in the interaction of Hb A with 2,3-DPG, we have also monitored the ^{31}P resonances of 2,3-DPG as a function of pH in Hb A solutions in both deoxy and CO forms. Each phosphate group in 2,3-DPG gives rise to a single ^{31}P resonance over the entire pH range investigated (results not shown), confirming that the exchange rates of the 2,3-DPG molecule between its free and Hb-bound states are fast on the NMR chemical shift time scale. The assignment of the ^{31}P resonances to the C2- and C3-phosphate is taken from Moon and Richards (1973). The ^{31}P NMR titration of 2,3-DPG in deoxy-Hb A and HbCO A solutions is shown in Figure 5. This figure also includes the corresponding titrations in 0.0015 M 2,3-DPG solutions in 0.1 M Bis-Tris (or Tris) buffer. The chemical shifts of each ^{31}P resonance of the 2,3-DPG molecule have been fitted as a function of pH to eq 5, and the results are shown in Table III.

Discussion

Effects of 2,3-DPG upon the H^+ Equilibria of Surface Histidyl Residues of Hb A. In deoxy-Hb A, the pK value of

Table III: pK Values and NMR Titration Coefficients for the Phosphate Groups of 2,3-DPG in 0.1 M Bis-Tris (or Tris) Buffer in D_2O at 27 °C[a]

	observed experimentally			calculated for Hb bound	
	2,3-DPG alone	2,3-DPG + deoxy-Hb A	2,3-DPG + HbCO A	2,3-DPG + deoxy-Hb A	2,3-DPG + HbCO A
C2-phosphate					
pK	7.68 ± 0.04	6.4 ± 0.1	7.87 ± 0.01	6.48	6.97
n	1.4 ± 0.1	1.0 ± 0.2	2.6 ± 0.1	0.95	2.28
C3-phosphate					
pK	7.29 ± 0.05	6.43 ± 0.07	7.75 ± 0.02	6.30	6.88
n	0.85 ± 0.08	1.4 ± 0.2	1.4 ± 0.1	0.97	2.51

[a] The observed and the calculated values of the chemical shifts were fitted as a function of pH to eq 5. For details about calculation of the titration curves for 2,3-DPG bound to Hb A, see Data Analysis.

the β2His residues increases in the presence of 2,3-DPG by 0.8–0.9 pH unit (Figure 2, Tables I and II). This pK change clearly results from the direct interaction of the β2His with the negatively charged C2-phosphate (or C3-phosphate for the symmetry-related second half of the binding site) of the 2,3-DPG molecule bound to Hb. This interaction is also reflected by the shape of the ^{1}H NMR titration curve of the β2His residues in the presence of 2,3-DPG (Figure 2). The titration coefficient (n) for the ^{1}H NMR titration of β2His decreases in the presence of the allosteric effector from 0.88 to 0.62 (Table II). A large deviation of the titration coefficient from unity usually reflects the interaction of the amino acid residue of interest with neighboring charged groups which ionize over the same pH range (Russu et al., 1982). In the present case, the group interacting with β2His is the C2-phosphate (or C3-phosphate) of the bound 2,3-DPG molecule, since the pH range for the second ionization of 2,3-DPG in the presence of deoxy-Hb A overlaps that for the deprotonation of β2His (Table III and Figure 5). According to the X-ray diffraction results (Arnone, 1972), 2,3-DPG binds to deoxy-Hb A in the central cavity between the two β chains on the 2-fold symmetry axis of the Hb molecule. The negatively charged groups of 2,3-DPG form salt-bridges with β1Val, β2His, and β143His of both β chains and with β82Lys of one β chain. Our present results on β2His confirm the binding of 2,3-DPG to this site for deoxy-Hb A in the solution state.

In ligated Hb A, the pK value of β2His increases in the presence of 2,3-DPG by about 0.2 pH unit (Tables I and II, and Figure 2). This increase is one of the largest among all surface histidyl residues of HbCO A and suggests that 2,3-DPG binds to ligated Hb A at or near β2His. The site for binding 2,3-DPG to HbCO A is also suggested by our finding that, in the presence of 2,3-DPG, resonance L in the ^{1}H NMR spectra of HbCO A is broadened beyond detection (Figure 1, panel B). Resonance L is missing from the spectra of HbCO Abruzzo (β143His → Arg) and HbCO Little Rock (β143His → Gln) as well as from the spectra of mutant hemoglobins containing single amino acid substitutions in the carboxyl-terminal domain of the β chains (Ho & Russu, 1985). These previous results suggested that resonance L could originate from the C2-proton of the β143His residue. Thus, the effect of 2,3-DPG upon this resonance is consistent with the involvement of this histidyl residue in the binding of 2,3-DPG to HbCO A.

Our present suggestion that the binding of 2,3-DPG to HbCO A involves the same (or at least some of the same) amino acid residues responsible for its binding to deoxy-Hb A is supported by the findings of Gupta et al. (1979). They have measured the enhancement in the relaxation rates of the ^{31}P resonances of 2,3-DPG in solutions of Hb A spin-labeled at the β93Cys residues. From these measurements, they have inferred that the 2,3-DPG molecule bound to ligated Hb A is at the same distance from the spin label as when bound to deoxy-Hb A.

Several other histidyl residues are affected by the interaction of 2,3-DPG with Hb A (Tables I and II). For example, in the deoxy form, the pK values of several histidyl residues whose C2-proton resonances are labeled 1 (β97His), 2, 4, 4′, 5, 6, and 8 are lowered by the binding of 2,3-DPG. Similarly, in the CO form, several histidyl residues (such as the histidyl residues whose C2-proton resonances are labeled B, E, I, and H) show a decrease in their pK values upon binding of 2,3-DPG to Hb. According to the crystal structure of Hb A, it is very likely that these histidyl residues are situated away from the 2,3-DPG binding sites. However, the changes induced by 2,3-DPG in their H^+ equilibria cannot be due to a nonspecific ionic strength effect for the following reason. We have recently identified the histidyl residues whose NMR titrations change in the presence of 0.18 M chloride ions or 0.1 M inorganic phosphate ions (Russu et al., 1989). The effects observed under these conditions and/or the identity of the histidyl residues affected are different from those observed in the present work for 2,3-DPG. Hence, the changes observed here are specific to the interaction of 2,3-DPG with Hb A.

One long-range mechanism for the changes in the H^+ equilibria of histidyl residues in the presence of 2,3-DPG is indicated by the electrostatic calculations carried out by Matthew et al. (1981, 1985) and Matthew (1985). They have found that the binding of 2,3-DPG to the central cavity of the β chains in deoxy-Hb A induces electrostatic perturbations throughout the entire Hb structure even at an ionic strength of 0.1 M. Some of the histidyl residues affected by the binding of 2,3-DPG (apart from those at the binding site) are α45His, α50His, α112His, β77His, β117His, and β146His [Table III in Matthew et al. (1981)]. However, according to these calculations, the pK values of these histidyl residues (except for α112His) should increase in the presence of 2,3-DPG. In contrast, the present ^{1}H NMR results indicate that the long-range electrostatic effects of 2,3-DPG can result in lower H^+ affinities for histidyl residues outside the binding site. Such effects could make significant contributions to the electrostatic free energy of stabilization of the Hb A molecule by 2,3-DPG.

The changes in the histidyl protonation equilibria could also result from the long-range changes in the conformation of the Hb A molecule induced by the binding of 2,3-DPG. The X-ray diffraction studies indicate that, upon binding of 2,3-DPG to deoxy-Hb A, the A helix is pulled closer to the H helix and the EF corner. Moreover, the H helix moves away from the β-chain central cavity and closer to the heme pockets (Arnone, 1972). Conformational changes in the heme pockets upon binding of 2,3-DPG have also been detected by ^{1}H NMR spectroscopy as shifts in the hyperfine-shifted resonances in the deoxy form (Ho et al., 1978; Ho & Russu, 1981) and in the ring-current shifted resonances in the ligated form (Lindstrom & Ho, 1973). In the present work, we have found that 2,3-DPG affects the pH dependence of the γ_2-CH_3 proton resonances of α- and βE11Val residues (Figure 4). The apparent pK value of βE11Val increases in the presence of

2,3-DPG from 6.0 ± 0.1 to 6.6 ± 0.1. The pH dependence of the γ_2-CH_3 proton resonances of the E11Val residues could reflect the ionization of the distal E7 histidyl residues in the α and β chains. According to various investigators, the pK values of the distal histidyl residues vary from 4.1 to 6.0 (Ikeda-Saito et al., 1977; Fuchsman & Appleby, 1979; Asher et al., 1981; Doster et al., 1982; Dalvit & Ho, 1985). Thus, our results suggest that the long-range conformational changes induced by the binding of 2,3-DPG may affect the ionization properties of the distal histidyl residues in the β chains.

Our present results on the effects of 2,3-DPG upon the ^{1}H NMR titration of the histidyl residues in Hb A are in good agreement with previous findings by other investigators. For example, Ferrige et al. (1979) have found that, in deoxy-Hb A, the pK values of three His-C2 proton resonances are affected by the presence of 2,3-DPG. Due to the lack of specific assignments for these resonances, no direct information on the 2,3-DPG binding site was obtained in this study. Similarly, Nieto (1981) has observed that three His-C2 proton resonances of Hb A within oxygenated red blood cells are shifted by increasing concentrations of 2,3-DPG. These shifts were interpreted as being due to the binding of 2,3-DPG to the corresponding histidyl residues in oxy-Hb A. Our present results show that, although this is true for the β2His residues, the origin of the shifts of other resonances may be more complex.

Molecular Mechanism of the Bohr Effect in the Presence of 2,3-DPG. Extensive experimental evidence has demonstrated that the binding of 2,3-DPG to Hb A enhances the alkaline and the acid Bohr effect (de Bruin et al., 1971, 1973; de Bruin & Janssen, 1973; Kilmartin, 1974). Under the conditions of our experiments (namely, a 1:1 ratio of 2,3-DPG to Hb, which is the same as that in red blood cells), the enhancement in the Bohr effect is approximately 75% of the maximum observed (de Bruin et al., 1971, 1973). The total extra Bohr effect due to the binding of 2,3-DPG results from the differences in the number of protons bound by Hb and by 2,3-DPG between the deoxy and ligated states (de Bruin & Janssen, 1973).

According to our present results, β2His is one histidyl residue responsible for the difference in charge between deoxy- and HbCO A in the presence of 2,3-DPG. We have calculated the contribution of this residue to the Bohr effect based on the equation:

$$\Delta X = 2[f^+(\text{deoxy}) - f^+(\text{CO})] \quad (7)$$

where f^+(deoxy) and f^+(CO) are the fractions of protonated β2His in the deoxy and CO forms, respectively. These fractions have been obtained on the basis of eq 3b and the titration parameters of the β2His residues given in Table II. The results indicate that, in the presence of 2,3-DPG, the β2His residues make a positive contribution to the alkaline Bohr effect of up to 0.5 proton/Hb tetramer (Figure 6). In contrast, under "stripped" conditions, the β2His residues capture H^+ upon ligand binding to Hb (i.e., up to 0.5 proton/Hb tetramer; Russu et al., 1989), and, thus, they make a negative contribution to the alkaline Bohr effect. These results add further support to our previous suggestion that the contribution of a given amino acid residue to the Bohr effect of Hb A can change depending upon solvent conditions and the presence of allosteric effectors other than H^+ (Russu et al., 1980, 1982, 1989; Russu & Ho, 1986; Ho & Russu, 1987).

Other histidyl residues in the 2,3-DPG binding site and away from it could be expected to make contributions to the enhancement of the Bohr effect of Hb by 2,3-DPG. Their contributions cannot be evaluated at the present time since

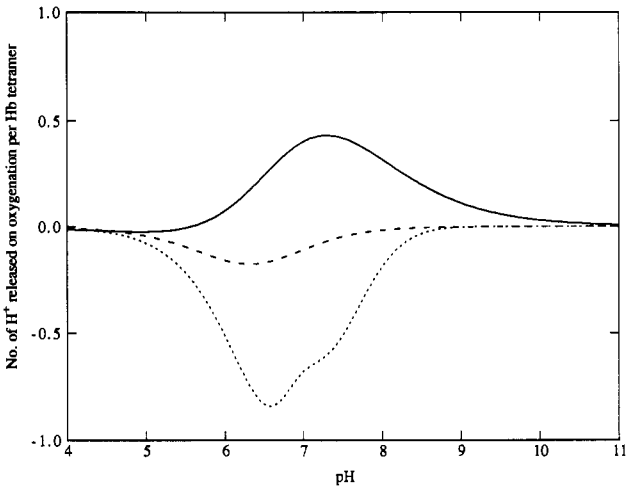

FIGURE 6: Proton release/uptake by β2His and 2,3-DPG upon ligand binding to Hb in 10% Hb A solutions in 0.1 M Bis-Tris (or Tris) buffer in D_2O at 27 °C: (—) β2His in the presence of 2,3-DPG (1:1 molar ratio to Hb); (- - -) β2His in the absence of 2,3-DPG; (···) 2,3-DPG alone.

the corresponding resonances have not yet been definitely assigned in the ^{1}H NMR spectra. Moreover, in the presence of 2,3-DPG, several His-C2 proton resonances of interest (e.g., resonance L in HbCO A, Figure 1) are broadened beyond detection, and, thus, their NMR titration cannot be monitored.

The number of protons bound by 2,3-DPG varies between deoxy and ligated states due to two effects. First, the release of 2,3-DPG from Hb upon oxygenation results in an uptake of protons since the pK values of the phosphate groups are higher in the free than in the Hb-bound form (Table III). Second, the difference in the pK values of 2,3-DPG bound to deoxy-Hb A and HbCO A (Table III) also results in an uptake of protons by 2,3-DPG since the pK values of the phosphate groups are lower when 2,3-DPG is bound to deoxy-Hb A. Accordingly, the proton uptake by each phosphate group of 2,3-DPG upon ligand binding to Hb can be expressed as

$$\Delta X = f_B^+(\text{deoxy}) - f_B^+(\text{CO}) + f_F^+(\text{deoxy}) - f_F^+(\text{CO}) \quad (8)$$

where symbols have the same meaning as in eq 1. Equation 8 extends the thermodynamic models previously proposed by Riggs (1971) and de Bruin and Janssen (1973) by taking into account the differences in the ionization of *each* phosphate group of 2,3-DPG between (i) free and Hb-bound states and (ii) 2,3-DPG bound to deoxy and ligated Hb. The pK values of the 2,3-DPG bound to Hb A were calculated as described under Data Analysis and are shown in Table III. They are lower than the corresponding ones in free 2,3-DPG as expected from the electrostatic interactions with the positively charged amino acid residues in the binding site. The decrease in the pK values of 2,3-DPG bound to HbCO A is less than that for deoxy-Hb A. This difference reflects the electrostatic contribution to the differences in the binding affinities of 2,3-DPG between the deoxy and the ligated forms of Hb.

The calculated proton uptake by the C2- and C3-phosphate groups of 2,3-DPG is shown in Figure 6. Up to 0.85 H^+ (per Hb tetramer) is captured by 2,3-DPG as a result of changes in its binding affinity and ionization properties upon the oxygenation of Hb. The maximum uptake occurs around pH 6.5, suggesting that the changes in 2,3-DPG ionization could be involved in the enhancement of the acid Bohr effect by 2,3-DPG. This suggestion is in agreement with the results obtained previously by de Bruin and Janssen (1973) using thermodynamic modeling. The small inflection in the calculated proton uptake curve results from the different pK values of the C2-

and C3-phosphate groups in both free and Hb-bound states.

Summary and Conclusions

The results obtained in this paper characterize the changes in the individual hydrogen ion equilibria of histidyl residues of Hb A and phosphate groups of 2,3-DPG upon the binding of this allosteric effector to Hb. The localized interactions at the binding site greatly increase the pK value of β2His in deoxy-Hb A. As a result of this increase, β2His makes a significant contribution to the Bohr effect of Hb in the presence of 2,3-DPG. The binding of 2,3-DPG also affects the local electrostatic environment of histidyl residues removed from the binding site. These findings suggest that the molecular mechanisms responsible for the allosteric effects of hydrogen ions and 2,3-DPG involve specific long-range interactions between ionizable groups of Hb. The changes in the hydrogen ion equilibria of 2,3-DPG upon binding to Hb are found to be different between the deoxy and carbonmonoxy forms. These differences contribute to the proton uptake by 2,3-DPG upon ligand binding to Hb.

References

Arnone, A. (1972) *Nature 237*, 146–149.

Asher, S. A., Adams, M. L., & Schuster, T. M. (1981) *Biochemistry 20*, 3339–3346.

Benesch, R., & Benesch, R. E. (1969) *Nature 221*, 618–622.

Benesch, R. E., & Benesch, R. (1974) *Adv. Protein Chem. 28*, 211–237.

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

Dalvit, C., & Ho, C. (1985) *Biochemistry 24*, 3398–3407.

de Bruin, S. H., & Janssen, L. H. M. (1973) *J. Biol. Chem. 248*, 2774–2777.

de Bruin, S. H., Janssen, L. H. M., & Van Os, G. A. J. (1971) *Biochem. Biophys. Res. Commun. 45*, 544–550.

de Bruin, S. H., Janssen, L. H. M., & Van Os, G. A. J. (1973) *Biochem. Biophys. Res. Commun. 55*, 193–199.

de Bruin, S. H., Rollema, H. S., Janssen, L. H. M., & Van Os, G. A. J. (1974) *Biochem. Biophys. Res. Commun. 58*, 204–209.

Doster, W., Beece, D., Bowne, S. F., Dilorio, E. E., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., Winterhalter, K. H., & Yue, K. T. (1982) *Biochemistry 21*, 4831–4839.

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

Fabry, M. E., & San George, R. C. (1983) *Biochemistry 22*, 4119–4125.

Ferrige, A. G., Lindon, J. C., & Paterson, R. A. (1979) *J. Chem. Soc., Faraday Trans. 1*, (*75*), 2851–2864.

Fuchsman, W. H., & Appleby, C. A. (1979) *Biochemistry 18*, 1309–1321.

Glasoe, P. K., & Lang, F. A. (1960) *J. Phys. Chem. 64*, 188–190.

Gupta, R. K., Benovic, J. L., & Rose, Z. B. (1979) *J. Biol. Chem. 254*, 8250–8255.

Ho, C., & Russu, I. M. (1981) *Methods Enzymol. 76*, 275–312.

Ho, C., & Russu, I. M. (1985) in *New Methodologies in Studies of Protein Configuration* (Wu, T. T., Ed.) pp 1–35, Van Nostrand Reinhold, New York.

Ho, C., & Russu, I. M. (1987) *Biochemistry 26*, 6299–6305.

Ho, C., Fung, L. W.-M., & Wiechelman, K. J. (1978) *Methods Enzymol. 54*, 192–223.

Hobish, M. K., & Powers, D. A. (1986) *Proteins: Struct., Funct., Genet. 1*, 164–175.

Huestis, W. H., & Raftery, M. A. (1972) *Biochem. Biophys. Res. Commun. 49*, 428–433.

Ikeda-Saito, M., Iizuka, T., Yamamoto, H., Kayne, F. J., & Yonetani, T. (1977) *J. Biol. Chem. 252*, 4882–4887.

Kilmartin, J. V. (1974) *FEBS Lett. 38*, 147–148.

Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246–1249.

Lam, Y.-F., Lin, A. K.-L. C., & Ho, C. (1979) *Blood 54*, 196–209.

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

Lindstrom, T. R., & Ho, C. (1973) *Biochemistry 12*, 134–139.

Lindstrom, T. R., Noren, I. B. E., Charache, S., Lehman, H., & Ho, C. (1972) *Biochemistry 11*, 1677–1681.

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

Matthew, J. B. (1985) *Annu. Rev. Biophys. Chem. 14*, 387–417.

Matthew, J. B., Friend, S. H., & Gurd, F. R. N. (1981) *Biochemistry 20*, 571–580.

Matthew, J. B., Gurd, F. R. N., Garcia-Moreno, E. B., Flanagan, M. A., March, K. L., & Shire, S. J. (1985) *CRC Crit. Rev. Biochem. 18*, 91–197.

Moon, R. B., & Richards, J. H. (1973) *J. Biol. Chem. 248*, 7276–7278.

Nieto, J. L. (1981) *FEBS Lett. 136*, 85–88.

Perutz, M. F., Gronenborn, A. M., Clore, G. M., Shih, D. T.-b., & Craescu, C. T. (1985a) *J. Mol. Biol. 186*, 471–490.

Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H., & Shih, D. T.-b. (1985b) *J. Mol. Biol. 183*, 491–498.

Riggs, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2062–2065.

Russu, I. M., & Ho, C. (1986) *Biochemistry 25*, 1706–1716.

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

Russu, I. M., Ho, N.-T., & Ho, C. (1982) *Biochemistry 21*, 5031–5043.

Russu, I. M., Lin, A. K.-L. C., Ferro-Dosch, S., & Ho, C. (1984) *Biochim. Biophys. Acta 785*, 123–131.

Russu, I. M., Wu, S.-S., Ho, N. T., Kellogg, G. W., & Ho, C. (1989) *Biochemistry 28*, 5298–5306.

Tanokura, M., Tasumi, M., & Miyazawa, T. (1978) *Chem. Lett.*, 739–742.

Tehrani, A. Y., Lam, Y.-F., Lin, A. K.-L. C., Dosch, S. F., & Ho, C. (1982) *Blood Cells 8*, 245–261.