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Effects of anions on the molecular basis of the Bohr effect of hemoglobin

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High-resolution ^{1}H-NMR spectroscopy has been used to investigate the molecular basis of the Bohr effect in human normal adult hemoglobin in the presence of anions which serve as heterotropic effectors, i.e., Cl^-, P_i, and 2,3-diphosphoglycerate. The individual H^+ equilibria of 22–26 histidyl residues of hemoglobin in both deoxy and carbonmonoxy forms have been measured under buffer conditions chosen to demonstrate the effects of anion binding. The results indicate that β2His residues are binding sites for Cl^- and P_i in both deoxy and carbonmonoxy forms, and that the affinity of this site for these anions is greater in the deoxy form. Recently assigned, the resonance of β146His does not show evidence of involvement in anion binding. The results also indicate that the binding of 2,3-diphosphoglycerate at the central cavity between the two β-chains in deoxyhemoglobin involves the β2His residues, and that the 2,3-diphosphoglycerate-binding site in carbonmonoxyhemoglobin may remain similar to that in deoxyhemoglobin. The interactions of Cl^-, P_i and 2,3-diphosphoglycerate also result in changes in the pK values for other surface histidyl residues which vary in both magnitude and direction. The array of pK changes is specific for the interaction of each effector. The participation of β2His in the Bohr effect demonstrates that this residue can release or capture protons, depending on its protonation properties and its linkage to anion binding, and therefore provides an excellent illustration of the variable roles of a given amino acid. Although β146His does not bind anions, its contributions to the Bohr effect are substantially affected by the presence of anions. These results demonstrate that long-range electrostatic and/or conformational effects of anions binding play significant roles in the molecular basis of the Bohr effect of hemoglobin.

1. Introduction

Functional properties of the hemoglobin (Hb) molecule are mediated both by homotropic interactions between its oxygen-binding sites and by heterotropic interactions of individual amino acid residues with solvent components. Heterotropic effectors include hydrogen ions, chlorides and inorganic phosphates, and organic polyanions such as 2,3-diphosphoglycerate (2,3-DPG) [1,2]. The functional consequences of these heterotropic effectors imply differential interaction of each with the ligated forms of the Hb molecule [3,4]. The higher affinity of deoxyhemoglobin (deoxy-Hb) for H^+ leads to the thermodynamic consequence of increasing oxygen affinity as pH increases above pH 6.5, with oxygenation of Hb resulting in a release of H^+ by the Hb molecule. This physiologically relevant effect is known as the alkaline Bohr effect and is fundamentally important in the ability of the Hb molecule to deliver oxygen to the tissues. Below pH 6.0, ligated Hb has a higher affinity for H^+, oxygen affinity increases with decreasing pH, and the Hb molecule absorbs H^+ upon oxygenation. This phenomenon is known as the acid Bohr effect.

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On the molecular level, the alkaline Bohr effect requires that additional H^+ ions bind to deoxy-Hb. Potential sites for additional H^+ binding include the α-amino groups from the amino termini and the imidazole groups from the histidyl residues, due to their pK values which are likely to be in the physiologically relevant range. The macroscopically measured Bohr effect is a summation of the effects of changes in the pK values which occur upon changing the ligation state of the Hb molecule. This was expressed by German and Wyman [5], assuming i specific oxygen-linked groups with ionization constants changing from K_i to K_i' upon deoxygenation:

$$\Delta H^+ = \sum_i \left(\frac{K_i}{[H] + K_i} - \frac{K_i'}{[H] + K_i'} \right) \quad (1)$$

Any group which changes its ionization constant between the deoxy and oxy forms of the Hb molecule participates in the Bohr effect. It is inappropriate to assume that all sites have pK changes which result in positive contributions to the macroscopically observed effect. The microscopic behavior of a specific group will be defined by its site-specific chemical and electrostatic environments, and its resulting microscopic behavior may oppose the macroscopic behavior seen for the Hb molecule.

Changes in the pK values resulting from the oxygenation-linked conformational transitions of Hb are not independent of interactions with other heterotropic effectors, especially anions. Anions such as chloride can stabilize the protonation of a histidyl residue or a terminal α-amino group, and thereby can raise its pK value. The number of protons released upon oxygenation of Hb has been shown to be strongly dependent on the concentration of chloride [6–9]. An analysis of the normal and differential titration curves of oxy- and deoxy-Hb has demonstrated that the release of about half of the Bohr protons is due to the difference in chloride binding to the oxy and deoxy forms [8]. In this model, the chlorides bind to positively charged groups whose pK values are around neutral pH, such as the imidazoles of histidyl residues and the α-amino group of α1 Val, and increase the binding of protons. The release of chlorides upon oxygenation of Hb should be accompanied by the release of protons, and an enhancement of the experimentally observed Bohr effect [8–10].

The differential affinity of the Hb molecule for anions with resulting changes in H^+ binding (pK changes) is known as the anion Bohr effect, to distinguish this effect from the pK changes resulting solely from the conformational transitions induced by oxygenation in the absence of anions, i.e., the intrinsic Bohr effect. Possible modulation of the intrinsic Bohr effect, in both magnitude and mechanism, by the nature and concentration of anions in solution has been suggested on the basis of ^{1}H-NMR measurements and electrostatic calculations [11–14].

Extensive investigation aimed at identifying the oxygenation-linked anion-binding sites has been fueled by the functional significance of anion binding in the modulation of the oxygen affinity of Hb and the Bohr effect. Two oxygenation-linked chloride-binding sites at α1Val have been located by X-ray crystallographic analyses of Hb A and its carbamylated derivatives, namely, an intrachain site between the α-amino group of α1Val and the β-hydroxyl group of α131Ser, and an interchain site between the α-amino group of α1Val and the guanidinium group of the α141Arg of the opposite chain [15]. These findings have provided a structural basis for the contribution of the α1Val residue to the anion Bohr effect which has been demonstrated by titration and functional studies [9,15,16].

Bonaventura and co-workers [17–20] have investigated the functional properties of a series of mutant and chemically modified Hbs for the identification of the oxygenation-linked chloride-binding sites in Hb. By extensive characterization of these Hbs containing single-site structural modifications, specific differences in the allosteric effects of anions and the linkages between oxygen, proton, and anion binding have been suggested. The differences have localized the chloride-binding sites within a cluster of positively charged amino acid residues in the central cavity between the two β-chains. β82Lys is the most likely candidate among those residues to be an oxygenation-linked chloride-binding site. Nigen et al. [21] reached a

similar conclusion on the basis of their studies of hybrids between carbamylated Hb A and Hb Providence I (β82Lys → Asn).

The allosteric effect of 2,3-DPG on Hb results in a dramatic decrease in the oxygen affinity, and therefore, facilitates the delivery of oxygen to the tissues [1,22]. 2,3-DPG increases both the alkaline and acid Bohr effects [6,23–26]. The higher affinity of 2,3-DPG for the deoxy form of Hb gives rise to the observed allosteric effect. The binding site in deoxy-Hb A has been identified by X-ray crystallography to be in the central cavity of the molecule between the two β-chains [27].

^{1}H-NMR spectroscopy is particularly suited to studying the involvement of histidyl residues in the anion Bohr effect, in that the technique allows the observation of individual C2 (Cϵ1) and C4 (Cδ2) hydrogens of histidyl residues in the solution state and under experimental conditions relevant to Hb function [12,13,28]. Histidyl residues are of particular interest in the investigation of the molecular basis of the anion Bohr effect, due to their likely involvement as the Bohr groups and as the oxygenation-linked chloride- and phosphate-binding sites [8,9]. This laboratory has previously shown that 22–26 C2 (Cϵ1) protons of the surface histidyl residues can be resolved in the ^{1}H-NMR spectra of the deoxy and carbonmonoxy forms of human normal adult hemoglobin (Hb A), and that the surface histidyl residues can exist in a wide variety of local environments in both deoxy and carbonmonoxy forms [12,28].

In this article, we shall summarize our recent efforts using ^{1}H-NMR spectroscopy to investigate the effects of anions such as Cl^-, P_i, and 2,3-DPG on the pK values of surface histidyl residues, in particular the β2His and β146His residues, in both deoxy and carbonmonoxy forms of Hb A. Our results clearly show that there are specific anion effects on the ionization properties of the histidyl residues in Hb A. Thus, the molecular basis of the anion Bohr effect of Hb depends on the experimental conditions.

2. Materials and methods

The pK values of the surface histidyl residues in deoxy-Hb A and HbCO A in ^2H_2O at 29°C

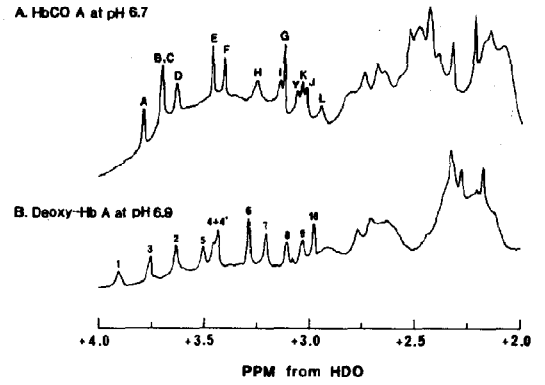

Fig. 1. 300 MHz ^{1}H-NMR spectra of 10% Hb A solutions in 0.1 M Hepes buffer at 29°C: (A) HbCO A at pH 6.7; (B) deoxy-Hb A at pH 6.9.

were determined using ^{1}H-NMR spectroscopy under a variety of buffer conditions chosen to illustrate the effects of chloride, inorganic phosphate, and 2,3-DPG [13,29,30]. 0.1 M Hepes buffer was chosen in order to provide an anion-free reference state, since Hepes is a zwitterionic buffer and does not require the addition of acids for adjustment of pH. The choice of 0.18 M NaCl was made to facilitate comparison with the results of Chu and Ackers [31] on the linkages between oxygen, protons, and chloride binding and dimer-tetramer assembly of Hb A. Labeling of the histidyl C2 (Cϵ1) resonances follows the original labeling scheme from this laboratory [12, 28], where resonances in deoxy-Hb A are numbered 1–10, and resonances in the carbonmonoxy form are given letters A–L and Y as is illustrated in fig. 1. The experimental results were taken from our laboratory [13,29,30]. The pK values were determined by a nonlinear least-squares fit of the ^{1}H chemical shift, δ, as a function of $[H^+]$ according to the following equation:

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

where δ^+ and δ^0 denote the chemical shifts in the protonated and unprotonated forms of the histidyl residue, respectively, and K is the proton dissociation equilibrium constant of the histidyl residue. The resulting values are presented in table 1. Table 2 presents the anion-induced pK changes (ΔpK) calculated from the pK data, using the pK

values obtained in 0.1 M Hepes as the anion-free reference state for a comparison involving chloride and phosphate, and pK values obtained in 0.1 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl) methane (Bis-Tris) as a reference for the 2,3-DPG study.

The original assignments of β2His to resonance 10 in the deoxy-Hb A and resonance G in the CO form were made in our laboratory [12], and were reconfirmed recently by Russu et al. [29] using Hb Deer Lodge (β2His → Arg).

The assignment of resonance 3 to β146His in deoxy-Hb A has been established for some time [11,12,28,32], but the identification of the corresponding resonance in the carbonmonoxy form has been the focus of some dispute and controversy [12,13,28,33–35]. It is beyond the scope and intention of this review to present evidence to resolve the dispute. In a separate paper, we shall report the assignment of the resonance arising from β146His of HbCO A by a careful comparison of spectra of Hb A, des(β146His)Hb, Hb York (β146His → Pro), and Hb Cowtown (β146His → Leu) (M.R. Busch, J.E. Mace, N.T. Ho and C. Ho, results to be published elsewhere). ^{1}H-NMR spectra of these Hb samples in the CO

Table 1

pK values of surface histidyl residues in deoxy-Hb A and HbCO A in ^2H_2O at 29 °C

Resonance	Site	0.1 M Hepes [a]	0.1 M Bis-Tris/Tris [b]	0.1 M Bis/Tris + 0.18 M NaCl [c]	0.1 M Bis-Tris + 2,3-DPG [d]	0.1 M phosphate [c]
Deoxy-Hb A						
1		8.03 ± 0.01	8.07 ± 0.04	7.83 ± 0.02	7.85 ± 0.02	7.89 ± 0.02
2		7.20 ± 0.02	7.28 ± 0.02	7.33 ± 0.01	7.17 ± 0.01	7.28 ± 0.02
3	β146	7.83 ± 0.02	7.98 ± 0.03	7.78 ± 0.02	7.99 ± 0.02	7.82 ± 0.01
4		7.11 ± 0.02	7.20 ± 0.03	7.27 ± 0.02	7.10 ± 0.01	7.24 ± 0.02
4′		7.32 ± 0.02	7.20 ± 0.03	7.34 ± 0.02	7.03 ± 0.02	7.24 ± 0.03
5		7.63 ± 0.03	7.76 ± 0.04	7.54 ± 0.03	7.65 ± 0.03	7.57 ± 0.02
6		6.95 ± 0.04	7.10 ± 0.03	7.07 ± 0.02	7.02 ± 0.01	7.23 ± 0.03
7		7.04 ± 0.03	7.07 ± 0.06	7.03 ± 0.03	7.12 ± 0.02	6.97 ± 0.03
8		6.59 ± 0.05	6.71 ± 0.06	6.70 ± 0.05	6.74 ± 0.04	6.76 ± 0.04
9		6.81 ± 0.03	6.82 ± 0.07	6.80 ± 0.04	6.86 ± 0.02	6.90 ± 0.04
10	β2	5.83 ± 0.03	6.35 ± 0.03	6.36 ± 0.02	7.25 ± 0.03	6.94 ± 0.02
HbCO A						
A		7.78 ± 0.01	7.87 ± 0.02	7.72 ± 0.01	7.87 ± 0.01	7.81 ± 0.01
B		7.25 ± 0.02	7.42 ± 0.02	7.30 ± 0.01	7.35 ± 0.02	7.40 ± 0.04
C		7.73 ± 0.02	7.85 ± 0.03	7.67 ± 0.01	7.82 ± 0.03	7.46 ± 0.04
D		7.48 ± 0.02	7.65 ± 0.02	7.52 ± 0.01	7.66 ± 0.01	7.58 ± 0.02
E		6.97 ± 0.03	7.15 ± 0.02	7.14 ± 0.02	7.13 ± 0.02	7.29 ± 0.03
F		6.82 ± 0.02	7.00 ± 0.03	7.01 ± 0.01	7.03 ± 0.01	7.14 ± 0.02
G	β2	6.24 ± 0.03	6.53 ± 0.02	6.53 ± 0.02	6.75 ± 0.01	6.81 ± 0.01
H		6.04 ± 0.01	6.20 ± 0.05	6.22 ± 0.03	6.02 ± 0.05	5.99 ± 0.03
I		6.76 ± 0.02	6.55 ± 0.03	6.69 ± 0.04	6.41 ± 0.05	6.48 ± 0.04
J		6.46 ± 0.04	6.81 ± 0.03	6.82 ± 0.04	6.95 ± 0.04	6.79 ± 0.03
K		6.73 ± 0.05	6.76 ± 0.06	6.62 ± 0.04	6.79 ± 0.03	6.69 ± 0.02
L		6.25 ± 0.06	6.25 ± 0.05	6.03 ± 0.01	broadens	broadens
Y	β146 [e]	7.38 ± 0.02	6.72 ± 0.03	NR [f]	6.82 ± 0.02	7.03 ± 0.04

[a] Russu et al., 1989 [29] and M.R. Busch, J.E. Mace, N.T. Ho and C. Ho (unpublished results).

[b] Russu et al., 1990 [30], pK values at 27 °C. The pK values for resonances L and Y are from M.R. Busch, J.E. Mace, N.T. Ho and C. Ho (unpublished results) and will require reevaluation of resonances I–K under these conditions.

[c] Russu et al., 1989 [29].

[d] Russu et al., 1990 [30], pK values at 27 °C, 1:1 ratio of 2,3-DPG to Hb A.

[e] Assignment from M.R. Busch, J.E. Mace, N.T. Ho and C. Ho (unpublished results).

[f] NR indicates resonance not sufficiently resolved under these conditions.

Table 2

Anion-induced pK changes of surface histidyl residues

Resonance	ΔpK in 0.18 M Cl^- [a]	ΔpK in 0.1 M phosphate [a]	ΔpK in 1:1 2,3-DPG [b]
Deoxy-Hb A			
1	−0.20	−0.14	−0.22
2	+0.13	+0.08	−0.11
3 (β146His)	−0.05	−0.01	+0.01
4	+0.16	+0.13	−0.10
4′	+0.02	−0.08	−0.17
5	−0.09	−0.06	−0.11
6	+0.12	+0.28	−0.08
7	−0.01	−0.07	+0.05
8	+0.11	+0.17	+0.03
9	−0.01	+0.09	+0.04
10 (β2His)	+0.53	+1.11	+0.90
HbCO A			
A	−0.06	+0.03	0.00
B	+0.05	+0.15	−0.07
C	−0.06	−0.27	−0.03
D	+0.04	+0.10	+0.01
E	+0.17	+0.32	−0.02
F	+0.19	+0.32	+0.03
G (β2His)	+0.29	+0.57	+0.22
H	+0.18	−0.05	−0.18
I	−0.07	−0.28	−0.14
J	+0.36	+0.33	+0.14
K	−0.11	−0.04	+0.03
L	−0.22	[c]	[c]
Y (β146His)	[c]	−0.35	+0.10

[a] Uses pK values from 0.1 M Hepes as reference state.
[b] Uses pK values from 0.1 M Bis-Tris/Tris as reference state.
[c] Indicates a resonance was lost to broadening or insufficient resolution.

form taken in 0.2 M phosphate near pH 7 clearly show the loss of a single peak in each of these three variant hemoglobins, and thus allow the assignment of this resonance to β146His. The variant Hbs in 0.1 M Hepes give spectra which show complex variations, but by close incremental variations of pH from 6.5 to 8.5, a single resonance seen in the spectrum of HbCO A is found to be missing in each of the three variants. Spectra acquired at incremental variations of the anion conditions between the 0.1 M Hepes and 0.2 M phosphate are necessary for the unambiguous identification of the β146His resonance in 0.1 M Hepes. This resonance is identified in table 1 and in fig. 1 as resonance Y, following the nomenclature of our laboratory [28].

3. Results and discussion

3.1. Effects of chloride and inorganic phosphate

The data given in tables 1 and 2 clearly show that β2His exhibits the largest anion-induced change in its pK values among all the surface histidyl residues detected. In the deoxy form, the pK value of β2His changes from 5.83 in 0.1 M Hepes to 6.36 in the presence of 0.18 M chloride, and to 6.94 in the presence of 0.1 M phosphate. In the CO form, the ΔpK is 0.29 pH units in the presence of 0.18 M chloride, and 0.57 pH units in the presence of 0.1 M phosphate. These results indicate the existence of an anion-binding site at or near β2His [29]. The greater increases in the pK values seen in the deoxy form suggest that the deoxy-Hb A binds anions at this site with a higher affinity than that in the CO form. These results are consistent with findings from X-ray crystallographic studies and electrostatic modeling [14,27,36]. X-ray diffraction studies reveal a site for inorganic anions at or near β2His [27]. Electrostatic modeling has suggested that multiple isoenergetic chloride-binding sites exist in the β-chain central cavity in deoxy-Hb, with one site between β82Lys and the α-amino group of β1Val, and the other involving residues β82Lys, β143His, and β2His [14,36].

In contrast to the striking changes in the pK values seen for β2His, β146His in deoxy-Hb does not show any substantial pK changes in the presence of chloride or phosphate. The pK of this residue remains very nearly constant at about 7.8. In the CO form, a decrease of 0.35 pH units is seen in the presence of phosphate, and a 0.66-pH unit decrease occurs in the 0.1 M Bis-Tris/Tris conditions where chloride varies from 5 to 60 mM. Resonance Y is not adequately resolved under the 0.1 M Bis-Tris + 0.18 M NaCl buffer conditions to allow the determination of its pK value [29]. Since the site-specific binding of an anion would be expected to raise the pK value of the residue at

which it is bound, the behavior of β146His under both deoxy and carbonmonoxy conditions would imply that it is not a site for anion binding, and the substantial changes in pK values seen for this residue in the CO form of Hb must originate from the intramolecular electrostatic effects of other ionizable groups.

Additional histidyl residues show variations in their pK values in the presence of anions which are less striking than those of β2His. In the deoxy form, resonances 2, 4, 6, and 8 each shows increases in its pK values both in the presence of chloride and also in the presence of phosphate, although the effect in chloride is weaker than that in phosphate for resonances 6 and 8, while the opposite is true for resonances 2 and 4. Resonances E, F, and J in the CO form show increases in their pK values for both conditions; however, resonance H increases its pK value in the 0.18 M chloride, but shows a slight decrease in the 0.1 M phosphate. Decreasing pK values in the presence of the anions can be seen for resonance 1 of the deoxy form under both anion conditions, for resonances C and I in the CO form in the presence of phosphate, and for resonance L in the CO form in the presence of chloride.

The physical basis for the anion-induced pK shifts is not yet fully understood. Experiments utilizing ^{35}Cl-NMR quadrupole relaxation studies show at least two classes of chloride-binding sites in Hb [37]. It seems possible that the low-affinity sites correspond at least in part to the histidyl residues having pK increases in the presence of anions.

Further interpretation may be possible using electrostatic calculations as carried out by Gurd and co-workers [14,36,38]. Modeling of the electrostatic interactions and the effects of the complete charge matrix of the protein molecule on single-site proton equilibria is described in detail by Matthew et al. [38]. The anion-induced perturbations of the electrostatic interactions could result in changes of the pK values of histidyl residues which vary in both magnitude and direction, as seen here in our ^{1}H-NMR data. These effects result from specific binding of anions, as well as the Coulombic effects of increasing ionic strength. Our understanding of these phenomena is likely to be aided by the efforts of Garcia-Moreno E. and Ackers [39] who have begun to model site-specific ion binding in Hb A.

The suggestion that electrostatic interactions within the Hb molecule give rise to the anion-induced changes in the individual histidyl titration curves is also consistent with the results from an analysis of titration coefficients when the data are fitted to the following equation:

$$\delta = \left(\delta^{+}[\mathrm{H}^{+}]^{n} + \delta^{0}K^{n}\right)/\left([\mathrm{H}^{+}]^{n} + K^{n}\right). \qquad (3)$$

Large deviations of the titration coefficient (n) from unity are usually interpreted as a sign of interaction of the histidyl residue with other ionizable groups titrating over the same pH range [12]. The generalized increase of the titration coefficient in the presence of chloride or phosphate is consistent with diminution of the electrostatic interactions of other groups as the Coulombic screening is increased at higher ionic strengths.

3.2. Effects of 2,3-DPG

Binding of the organic polyanion 2,3-DPG also induces pK changes in the surface histidyl residues of Hb A [30]. In examining tables 1 and 2, the largest pK change for deoxy-Hb A is seen for resonance 10, corresponding to β2His, where the pK increases by 0.90 pH units in the presence of 2,3-DPG (using 0.1 M Bis-Tris conditions as a reference). This change in the pK value reflects the interaction of this residue with the negatively charged 2,3-DPG molecule, which also results in a decrease of the titration coefficient calculated when the data are fitted to eq. 3, as is expected from an interaction with a charge group titrating over the same pH range [30]. The β2His behavior in the deoxy form is consistent with its involvement in the binding site of 2,3-DPG as determined by X-ray crystallographic analysis of deoxy-Hb A crystals with bound 2,3-DPG [27]. The binding site has been located in the central cavity of the Hb tetramer between the β-chains, and involves the α-amino groups of the β1Val, the imidazoles of β2His and β143His, and the ε-amino group of β82Lys. These results confirm the equivalence of the binding site as determined for crystal and solution forms of deoxy-Hb A.

In HbCO A, the resonance from β2His is resonance G, which increases its pK value by 0.22 pH units in the presence of 2,3-DPG, and supports the suggestion that in the ligated form of Hb, this residue remains involved in the binding site. The finding that resonance L broadens in the presence of 2,3-DPG gains significance when considered with the suggestion that it arises from β143His [40]. This would lend support to the contention that the binding of 2,3-DPG to HbCO A involves essentially the same residues as the binding to deoxy-Hb A. The results of Gupta et al. [41] using ^{31}P-NMR relaxation rate studies also support this suggestion.

Other histidyl resonances also show the effects of their interactions with 2,3-DPG. In the deoxy form, no other resonances show substantial increases in pK, but resonances labeled 1,2,4,4′ and 5 show decreases in their pK values [30]. Resonances J and Y increase their pK values, and resonances B, H, and I decrease their pK values in the presence of 2,3-DPG in the CO form. The crystal structures of Hb A show that these residues are likely to be distant from the 2,3-DPG-binding site, and the possibility that changes are due to a nonspecific ionic strength effect are ruled out by the differences seen for the binding of chloride and inorganic phosphate, as discussed above.

Electrostatic modeling of the binding of 2,3-DPG to deoxy-Hb A predicts changes of the pK values of histidyl residues located throughout the Hb molecule, and generally the pK values increase in the presence of 2,3-DPG [36,38,42]. The decreases in pK values that we observe are not predicted. Conformational changes of the Hb molecule could also affect the pK values of its surface histidyl residues. 2,3-DPG binding to deoxy-Hb A results in a number of alterations in the structure as determined from X-ray diffraction [27] and conformational changes in the heme pockets have been detected by ^{1}H-NMR spectroscopy from shifts in the hyperfine-shifted resonances in the deoxy form [43,44], and in the ring-current shifted resonances in the ligated form [45].

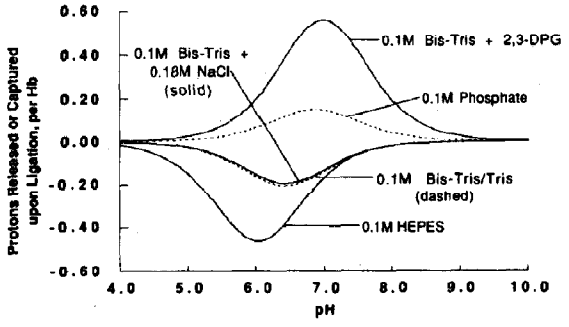

Fig. 2. Proton release or capture by β2His residues upon oxygenation of Hb A, from pK values as reported in table 1 for different buffer conditions. Curves representing different buffer conditions are identified within the figure.

3.3. Molecular basis of the Bohr effect in the presence of anions

The previous sections have presented evidence which demonstrates that the binding of anions, including Cl^-, P_i, and 2,3-DPG, substantially affects the pK values of the surface histidyl residues of Hb A. The effects can result from the direct binding of these effectors to specific histidyl residues, but must also involve longer-range interactions either by perturbations of electrostatic interactions, conformational alterations of the Hb molecule, or some combination of the two. Regardless of the precise mechanisms involved in these effects, the direct measurement of their pK values allows estimation of the role of specific histidyl residues in the Bohr effect of Hb A when the resonances can be assigned in the spectra of both the deoxy and CO forms of Hb A. For a particular residue, the number of Bohr protons as a function of pH is calculated from the difference in fractional proton occupancy at the site in the CO and deoxy forms of Hb A as determined from the pK values from table 1 [12,29,30].

As illustrated in fig. 2, the contributions of β2His to the Bohr effect as a function of pH have been calculated for the conditions studied. In 0.1 M Hepes, β2His captures about 0.46 protons per tetramer upon oxygenation at pH 6.0, and therefore contributes to the acid Bohr effect under these conditions. At pH 7.0, β2His still absorbs about 0.17 protons per tetramer, and thus, participates in the alkaline Bohr effect by making a

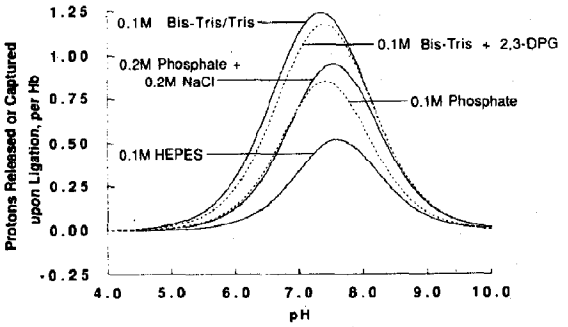

Fig. 3. Proton release by β146His residues upon oxygenation of Hb A, from pK values as reported in table 1 for different buffer conditions, and pK values for 0.2 M phosphate + 0.2 M NaCl conditions from Kilmartin et al. [32]. Curves representing different buffer conditions are identified within the figure.

negative contribution to the overall effect. As seen in fig. 2, the curves for the 0.1 M Bis-Tris/Tris and 0.1 M Bis-Tris + 0.18 M NaCl conditions nearly superimpose, with β2His still capturing up to 0.20 protons per tetramer upon ligation at about pH 6.5, thus, still opposing the macroscopically observed Bohr effect with a negative contribution. In the presence of 0.1 M phosphate, β2His releases up to 0.15 protons per tetramer, making a small, but positive contribution to the alkaline Bohr effect. The β2His residue releases up to 0.56 protons per tetramer upon ligation in 0.1 M Bis-Tris + 2,3-DPG, a significant contribution to the alkaline Bohr effect.

Fig. 3 similarly illustrates the contributions of β146His to the Bohr effect. The contributions of this residue to the Bohr effect remain positive under the conditions studied, but the magnitude of its contribution can be significantly altered by the differing conditions. Using pH 7.4 as a reference pH, the Bohr contribution of β146His in 0.1 M Hepes buffer is 0.49 protons per tetramer. The presence of 0.1 M phosphate increases the Bohr contribution of this residue to 0.85 protons per tetramer. In 0.2 M phosphate + 0.2 M NaCl, this residue contributes 0.93 protons per tetramer (pK data from Kilmartin et al. [32]), in 0.1 M Bis-Tris + 2,3-DPG β146His contributes 1.18 protons per tetramer to the Bohr effect, and 1.24 protons per tetramer are contributed by β146His in 0.1 M Bis-Tris.

The contribution of β146His to the Bohr effect has also been investigated by Shih and Perutz [46] by measurement and comparison of the alkaline Bohr effect and the individual Adair constants of Hb A and Hb Cowtown (β146His → Leu) under various solvent conditions. They have estimated that β146His contributes about 0.2 H^+/heme, independent of chloride concentration. Its contribution is estimated to be 94% of the total 0.84 H^+/tetramer alkaline Bohr effect measured in 0.1 M Hepes buffer (Cl^--free), 57% in 0.05 M Bis-Tris buffer (with minimal Cl^- concentration), and 50% in 0.1 M Bis-Tris with 0.1 M Cl^-. They have further concluded that the pK values of β146His in the T- and R-structures of the Hb molecule are largely chloride-independent. Measurement of the Bohr effect of Hb A in 0.1 M Hepes buffer (Cl^--free) in our laboratory (M.R. Busch, J.E. Mace, N.T. Ho and C. Ho, unpublished results) shows a maximum alkaline Bohr effect of 0.83 H^+/tetramer at pH 7.15, in good agreement with the results of Shih and Perutz [46]. However, our pK measurements by ^{1}H-NMR spectroscopy in 0.1 M Hepes buffer (Cl^--free) indicate the maximum contribution of β146His to the alkaline Bohr effect is 0.52 H^+/tetramer at pH 7.6, substantially less than the 0.8 H^+/tetramer contribution under the same conditions as estimated by Shih and Perutz [46]. Furthermore, in HbCO A we have found the pK values of β146His to be substantially affected by the presence of different anions in the solvent (see table 1), which results in significant changes in the magnitude of the contribution of β146His to the alkaline Bohr effect as shown in fig. 3. Our ^{1}H-NMR investigation of the CO form of Hb A, des(β146His)Hb, Hb York (β146His → Pro), and Hb Cowtown (β146His → Leu) in Cl^--free 0.1 M Hepes buffer (M.R. Busch, J.E. Mace, N.T. Ho and C. Ho, unpublished results) has revealed that the pK values of a number of histidyl residues are found to change in each of the variant hemoglobins when compared to Hb A. By a direct comparison of the functional properties of Hb Cowtown and Hb A for the determination of the participation of β146His in the alkaline Bohr effect, Shih and Perutz [46] have presumed that β146His is the only site in the Hb molecule responsible for the alteration of the proton-binding

behavior, i.e., that no other pK changes occur in the mutant protein. Our results show that this is not the case, and that further experimental verification is in order.

The examples of β2His and β146His clearly illustrate that the microscopic behavior of a specific group in Hb is determined by its site-specific chemical and electrostatic environments, defined both by the Hb structure and by the environment in which the Hb molecule resides. As a result, depending on solvent conditions, the individual behavior of a group could either contribute to the behavior seen macroscopically for the system, or oppose that behavior.

The example of β146His also provides an illustration of the significance of long-range electrostatic and/or conformational effects on the pK values. From these results, we have concluded that β146His is not a site for the binding of chloride or inorganic phosphate in either the deoxy or CO form, yet these anions can significantly increase the contribution of this residue to the Bohr effect. This supports the assertion that the detailed molecular mechanism for the alkaline Bohr effect is not unique, but is dependent on the nature of the solvent both by direct interactions and by longer-range electrostatic and/or conformational perturbations [11–13,28–30].

It is most fitting at this occasion to express our admiration as well as our friendship to Jeffries Wyman. His foresight and contributions to science, especially to hemoglobin, have formed the basis for much of our research on the molecular basis for the cooperative oxygenation and for the Bohr effect of hemoglobin. The phenomenological equation proposed by German and Wyman in 1937 [5] has provided the stimulus for our using ^{1}H-NMR spectroscopy to identify those histidyl residues that are involved in the Bohr effect.

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