A Proton Nuclear Magnetic Resonance Investigation of the Anion Bohr Effect of Human Normal Adult Hemoglobin†

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ABSTRACT: High-resolution proton nuclear magnetic resonance spectroscopy has been used to investigate the molecular mechanism of the Bohr effect of human normal adult hemoglobin in the presence of two allosteric effectors, i.e., chloride and inorganic phosphate ions. The individual hydrogen ion equilibria of 22–26 histidyl residues of hemoglobin have been measured in anion-free 0.1 M HEPES buffer and in the presence of 0.18 M chloride or 0.1 M inorganic phosphate ions in both deoxy and carbonmonoxy forms. The results indicate that the β2-histidyl residues are strong binding sites for chloride and inorganic phosphate ions in hemoglobin. The affinity of the β2-histidyl residues for these anions is larger in the deoxy than in the carbonmonoxy form. Nevertheless, the contribution of these histidyl residues to the anion Bohr effect is small due to their low pK value in deoxyhemoglobin in anion-free solvents. The interactions of chloride and inorganic phosphate ions with the hemoglobin molecule also result in lower pK values and/or changes in the shapes of the hydrogen ion binding curves for several other surface histidyl residues. These results suggest that long-range electrostatic interactions between individual ionizable sites in hemoglobin could play an important role in the molecular mechanism of the anion Bohr effect.

The oxygen-binding properties of the hemoglobin (Hb)[1] molecule are the result of homotropic interactions between its oxygen-binding sites as well as of heterotropic interactions between individual amino acid residues and solvent components. The latter include interactions with hydrogen ions, mono- and divalent anions (such as chloride and inorganic phosphate ions), and organic polyanions [such as 2,3-diphosphoglycerate (2,3-DPG)] (Benesch & Benesch, 1969; Antonini & Brunori, 1971). Thermodynamically, the effects of these solvent components upon the oxygen affinity of Hb imply that each of them interacts differentially with the unligated and ligated forms of the Hb molecule (Wyman, 1948, 1964). For instance, the decrease in the oxygen affinity of Hb upon lowering the pH toward 7 is a direct thermodynamic consequence of the higher affinity of deoxyhemoglobin (deoxy-Hb) for H^+ ions. This thermodynamic linkage is reflected by the release of H^+ ions upon oxygenation of Hb, a phenomenon known as the alkaline Bohr effect. Similarly, Hb in the deoxy form has a higher affinity for chloride and 2,3-DPG than Hb in the oxy form (Benesch & Benesch, 1969; Chiancone et al., 1972).

The heterotropic interactions of Hb with various solvent components are clearly not independent of each other. Various allosteric effectors can compete for the same binding sites on the Hb molecule, and/or a given solvent component can affect the affinity of an individual site for other allosteric effectors. This linkage has been first demonstrated for the Bohr effect of human normal adult hemoglobin (Hb A) by de Bruin and co-workers (de Bruin et al., 1974; Rollema et al., 1975; van Beek et al., 1979; van Beek & de Bruin, 1980). They have shown that the number of H^+ ions released upon oxygenation of Hb is strongly dependent upon the concentration of Cl^- ions in solution. An analysis of the normal and differential titration curves of oxy- and deoxy-Hb A has demonstrated that the release of about half of the Bohr protons is due to a difference in Cl^- binding to the oxy and deoxy forms (van Beek et al., 1979). In this model, the Cl^- ions bind to positively charged groups whose pK values are around neutral pH (such as histidyl residues and the NH_2 group of α1Val). The binding of Cl^- ions to an individual site should clearly result in an uptake of H^+ ions which, in turn, should depend upon the affinity of that site for the anion. Thus, the release of Cl^- ions upon oxygenation of Hb should be accompanied by a release of H^+ ions and an enhancement in the Bohr effect as observed experimentally (van Beek et al., 1979; van Beek & de Bruin, 1980; Fronticelli et al., 1988). The part of the Bohr effect which originates from the oxygen-linked interactions of the Hb molecule with solvent anions is generally known as the anion Bohr effect. This designation aims at distinguishing the changes in the H^+-binding equilibria which result solely from the oxygenation-induced conformational transitions of Hb from those produced by the differential affinity of the Hb molecule for anions. On the basis of proton nuclear magnetic resonance (NMR) measurements and electrostatic calculations, it has been suggested that the so-called intrinsic Bohr effect could also be modulated, in magnitude and in its mechanism, by the nature and the concentration of anions present in solution (Matthew et al., 1979a,b, 1982; Russu et al., 1980, 1982; Ho

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[1] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; Hb, hemoglobin; Hb A, human normal adult hemoglobin; HbCO, carbonmonoxyhemoglobin; HbO_2, oxyhemoglobin; deoxy-Hb, deoxyhemoglobin; met-Hb, methemoglobin; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DSS, 2,2-dimethyl-5-silapentane-5-sulfonate.

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& Russu, 1987). A unifying model for the anion Bohr effect has been proposed by Bucci and Fronticelli (1985).

The important role played by anions in the modulation of the oxygen affinity of Hb and in the Bohr effect has prompted extensive investigations aimed at identifying the oxygenation-linked anion-binding sites. Crystallographic analyses of Hb A and its carbamylated derivatives have located two oxygenation-linked Cl^--binding sites at α1Val, namely, one site between the α-amino group of α1Val and the β-hydroxyl group of α131Ser on the same chain and the other between the α-amino group of α1Val and the guanidinium group of α141Arg on the opposite chain (O'Donnell et al., 1979). 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 (Garner et al., 1975; O'Donnell et al., 1979; van Beek & de Bruin, 1980). An alternative approach for the identification of oxygenation-linked Cl^--binding sites in Hb has been used by Bonaventura and co-workers, who investigated the functional properties of a series of mutant and chemically modified Hbs (Bonaventura et al., 1975a,b, 1976; Bonaventura & Bonaventura, 1978). The extensive characterization of these Hbs containing single-site structural changes has revealed specific differences in the allosteric effects of anions and in the linkages between oxygen, H^+, and anion binding. These differences have pointed to a localization of the Cl^--binding sites within the cluster of positively charged amino acid residues in the central cavity between the two β chains. Among these residues, the most likely candidate for an oxygenation-linked Cl^--binding site is β82Lys. A similar conclusion has been reached by Nigen et al. (1980) on the basis of their studies of hybrids between carbamylated Hb A and Hb Providence I (β82Lys → Asn).

In the present work, we have used ^{1}H NMR spectroscopy to investigate the molecular basis of the anion Bohr effect. Previous work from this laboratory has demonstrated that ^{1}H NMR spectroscopy is ideally suited for the study of the structure–function relationship in Hb (Ho et al., 1978; Ho & Russu, 1981, 1987). This technique allows the observation of individual histidyl residues of the Hb molecule in the solution state and under experimental conditions relevant to the Hb function. For the elucidation of the molecular mechanism of the anion Bohr effect, the histidyl residues are of special interest since they are likely candidates for the Bohr groups and for the oxygenation-linked Cl^-- and phosphate-binding sites (van Beek et al., 1979; van Beek & de Bruin, 1980). We have previously shown that 22–26 surface histidyl residues of Hb A can be observed in the ^{1}H NMR spectra of the deoxy and CO forms (Russu et al., 1982; Russu & Ho, 1986). The individual titration curves and pK values of each of these histidyl residues have been determined by ^{1}H NMR in Hb solutions in 0.1 M Bis-Tris buffer with Cl^- ion concentrations ranging from 0.005 to 0.060 M. The results of this investigation have revealed the existence of a wide range of local conformations and electrostatic environments of the surface histidyl residues in Hb A under various ligation states. The H^+-binding equilibria of individual histidyl residues, and thus their corresponding contributions to the Bohr effect, have been found to depend upon solvent composition.

This finding has suggested that the molecular mechanism of the Bohr effect may not be unique and, in fact, depends upon the interactions of the Hb molecule with solvent components (Russu et al., 1980, 1982; Russu & Ho, 1986; Ho & Russu, 1987). We have now extended this work by investigating the effects of two anions, Cl^- and inorganic phosphate ions, upon the H^+-binding equilibria of each of the surface histidyl residues of Hb A in both deoxy and CO forms. To characterize these effects more rigorously, we have also obtained the individual titration curves of the histidyl residues of Hb A in 0.1 M HEPES buffer. This buffer, being a zwitterion, does not require addition of acids in order to adjust its pH. Thus, this solvent provides an anion-free "reference" state for the characterization of individual H^+-binding equilibria of the histidyl residues of Hb A.

Materials and Methods

Materials. Hb A was prepared by the procedure of Drabkin (1946) as described previously (Lindstrom & Ho, 1972). Hb Deer Lodge (β2His → Arg) was purified by anion-exchange chromatography (DEAE-Sephadex A-50) from a blood sample kindly provided by Dr. R. L. Nagel. The organic phosphates were removed by passing 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). All Hb samples were exchanged into D_2O either by dialysis or by pressure ultrafiltration as described previously (Russu et al., 1982). The Hb samples were deoxygenated by flushing the HbO_2 solutions with nitrogen in a rotary evaporator at 4 °C as described by Lindstrom and Ho (1972). The deoxygenation of the Hb samples was confirmed by the disappearance of the ring-current-shifted proton resonances between −6.0 and −7.0 ppm from HDO in the ^{1}H NMR spectra (Ho et al., 1978; Ho & Russu, 1981). The amount of methemoglobin (met-Hb) was measured by the optical absorbance at 631 nm [extinction coefficient 4.4 (mM aquo-met-Hb monomer)$^{-1}$ cm^{-1}; Antonini & Brunori, 1971] and was found to be less than 1% for all samples.

Hb A samples in 0.1 M HEPES buffer were obtained by adding to the Hb solutions an appropriate volume of concentrated buffer in D_2O. The buffer was titrated to the desired pH value using NaOD.

Hb A samples in 0.1 M Bis-Tris or Tris plus 0.18 M NaCl were prepared by dialyzing each sample against the buffer of the desired pH in D_2O. The Bis-Tris buffer was titrated over the pH range 6–8 using 2 N DCl. The amount of NaCl added to the buffer was adjusted at each pH value such that the total Cl^- concentration was constant throughout the titration. A similar adjustment of Cl^- concentration was made for the Tris buffer over the pH range 7.5–9.5. No differences in the NMR spectra of Hb samples in 0.1 M Bis-Tris and 0.1 M Tris buffer were detected over the pH range from 7.5 to 8.0. Our choice of using 0.18 M NaCl was made in order to be able to correlate, at a later stage, the results of the present investigation with the extensive data obtained by Chu and Ackers (1981) on the linkages between oxygen, H^+, and Cl^- binding and dimer–tetramer assembly. According to the results of de Bruin and co-workers (de Bruin et al., 1974; Rollema et al., 1975), at this Cl^- concentration and at the Hb concentration used in the NMR experiments (i.e., 1.5 mM on a tetramer basis), the anion Bohr effect is approximately half its maximum value.

Hb A samples in 0.1 M phosphate buffer were obtained by dialyzing each sample against the buffer of the desired pH value in D_2O. The pH values of the NMR samples of HbCO A were measured on a Beckman-3500 pH meter using a standard combination glass electrode. The change in pH upon deoxygenation was checked for several Hb samples by using a Radiometer microelectrode unit (E5021a) and was found to be negligible. The pH values are reported as direct pH meter readings since the deuterium isotope effect on the glass electrode [namely, pD = pH + 0.4 according to Glasoe and Long (1960)] is compensated by its effect on the pK value of

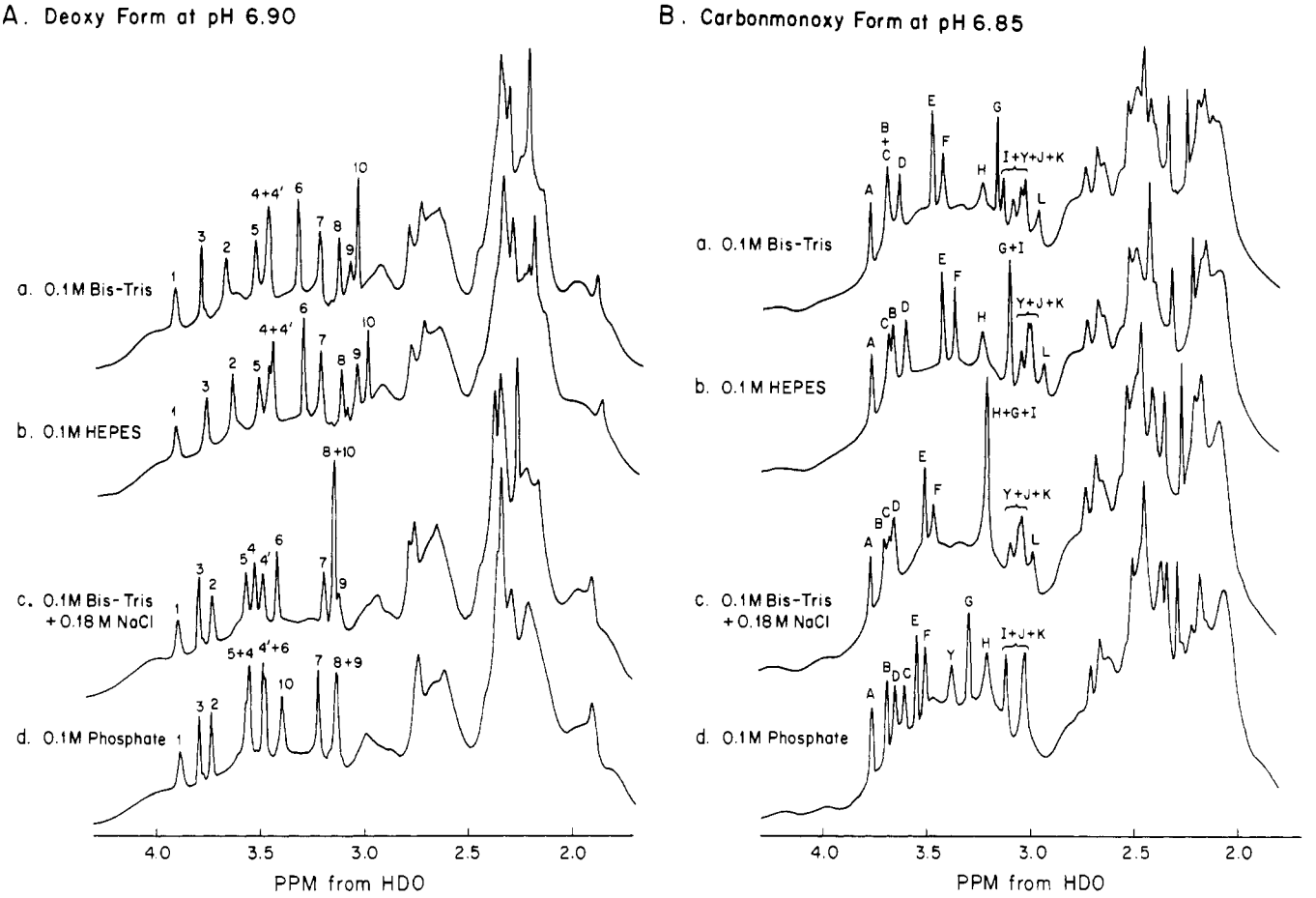

FIGURE 1: 300-MHz ^{1}H NMR spectra of 10% Hb A in D_2O and at 29 °C: (A) deoxy-Hb A at pH 6.9; (B) HbCO A at pH 6.85. In (A) and (B), (a) in 0.1 M Bis-Tris, (b) in 0.1 M HEPES, (c) in 0.1 M Bis-Tris plus 0.18 M NaCl, and (d) in 0.1 M phosphate.

imidazole (Tanokura et al., 1978). The pH measurements were carried out at room temperature (~25 °C).

Methods. High-resolution ^{1}H NMR spectra were obtained on a Bruker WH-300 or AM-300 spectrometer operating at 300 MHz and 29 °C. For each spectrum, 200–400 transients were accumulated with a repetition rate of 0.25 s^{-1}. The proton chemical shifts are expressed as parts per million (ppm) from the residual water (HDO) proton resonance, which is 4.73 ppm downfield from the proton resonance of 2,2-dimethyl-5-silapentane-5-sulfonate (DSS) at 29 °C. The chemical shift is defined as positive in the low-field direction with respect to HDO. The accuracy of the chemical shift measurements is ±0.01 ppm.

To obtain the individual titration curves of the histidyl residues, the chemical shift of each His C2-proton resonance was fitted as a function of pH to the equation (Markley, 1975):

$$\delta = \frac{\delta^+[H^+] + \delta^0 K}{[H^+] + K} \quad (1)$$

where $[H^+]$ is the concentration of H^+ ions and K is the H^+ dissociation equilibrium constant of the histidyl residue (i.e., $pK = -\log K$). δ^+ and δ^0 are the C2-proton chemical shifts in the protonated and unprotonated forms of the histidyl residue, respectively.

The chemical shifts of the His C2-proton resonances of the histidyl residue were also fitted as a function of pH to the equation (Markley, 1975):

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

where n is the titration coefficient[2] for the ^{1}H NMR titration of the histidyl residue and the rest of the symbols have the same meaning as those in eq 1. A nonlinear least-squares program was used for both fitting procedures.

Results

The aromatic proton resonances of Hb A under the experimental conditions investigated (namely, 0.1 M HEPES buffer, 0.1 M Bis-Tris buffer plus 0.18 M NaCl, and 0.1 M phosphate buffer) are illustrated in Figure 1A and Figure 1B for the deoxy and carbonmonoxy forms, respectively. These figures also include the corresponding spectra in 0.1 M Bis-Tris buffer (with Cl^- ion concentrations ranging from 0.005 to 0.060 M over the pH range of interest), which were the conditions of our previous ^{1}H NMR study of histidyl residues of Hb A (Russu et al., 1982). Under the experimental conditions investigated, the great majority of the His C2-proton resonances correspond closely to those previously observed in 0.1 M Bis-Tris buffer (Russu et al., 1982). Thus, we shall continue to use throughout the present work our original labeling of the His C2-proton resonances, namely, 1–10 in the deoxy form and A–L in the carbonmonoxy form. In the deoxy form, the resonance labeled 1 is assigned to the His C2-proton of β97, the resonance labeled 3 is assigned to the His C2-proton of β146, and the resonances labeled 7 and 8 are assigned to the

[2] The titration coefficient, n, as given in eq 2, 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 widespread use of this term in describing cooperativity in oxygen binding. For this reason, we have chosen to call the coefficient n in eq 2 the titration coefficient for the ^{1}H NMR titration of the histidyl residue.

Table I: pK Values of Surface Histidyl Residues in Deoxy- and Carbonmonoxy-Hb A in D_2O and at 29 °C, Based on Nonlinear Least-Squares Fitting of the Experimental Data to the ^1H NMR Titration Equation 1[a]

resonance	0.1 M HEPES	0.1 M Bis-Tris + 0.18 M NaCl	0.1 M phosphate
1 (β97)	8.03 ± 0.01	7.83 ± 0.02	7.89 ± 0.02
2	7.20 ± 0.02	7.33 ± 0.01	7.28 ± 0.02
3 (β146)	7.83 ± 0.02	7.78 ± 0.02	7.82 ± 0.01
4	7.11 ± 0.02	7.27 ± 0.02	7.24 ± 0.02
4′	7.32 ± 0.02	7.34 ± 0.02	7.24 ± 0.03
5	7.63 ± 0.03	7.54 ± 0.03	7.57 ± 0.02
6	6.95 ± 0.04	7.07 ± 0.02	7.23 ± 0.03
7 (β116 or β117)	7.04 ± 0.03	7.03 ± 0.03	6.97 ± 0.03
8 (β116 or β117)	6.59 ± 0.05	6.70 ± 0.05	6.76 ± 0.04
9	6.81 ± 0.03	6.80 ± 0.04	6.90 ± 0.04
10 (β2)	5.83 ± 0.03	6.36 ± 0.02	6.94 ± 0.02
A	7.78 ± 0.01	7.72 ± 0.01	7.81 ± 0.01
B	7.25 ± 0.02	7.30 ± 0.01	7.40 ± 0.04
C[b]	7.73 ± 0.02	7.67 ± 0.01	7.46 ± 0.04
D	7.48 ± 0.02	7.52 ± 0.01	7.58 ± 0.02
E	6.97 ± 0.03	7.14 ± 0.02	7.29 ± 0.03
F	6.82 ± 0.02	7.01 ± 0.01	7.14 ± 0.02
G (β2)	6.24 ± 0.03	6.53 ± 0.02	6.81 ± 0.01
I	6.76 ± 0.02	6.69 ± 0.04	6.48 ± 0.04
J (β116 or β117)	6.46 ± 0.04	6.82 ± 0.04	6.79 ± 0.03
K (β116 or β117)	6.73 ± 0.05	6.62 ± 0.04	6.69 ± 0.02
H	6.04 ± 0.01	6.22 ± 0.03	5.99 ± 0.03
L	6.25 ± 0.06	6.03 ± 0.01	[c]
Y (β146)[d]	[e]	[e]	7.03 ± 0.04

[a] Resonances labeled 1–10 correspond to the deoxy form, and those labeled A–Y correspond to the carbonmonoxy form. [b] Assignment of this resonance to the C2-proton of the β146His residue in a salt-bridge conformation is controversial. [c] Resonance L is broadened beyond detection under these conditions. [d] This resonance corresponds to the β146 His residue in a conformation in which the β146His–β94Asp salt bridge is broken. [e] Resonance Y is not resolved under these experimental conditions over the entire pH of interest.

Table II: pK Values of Surface Histidyl Residues in Deoxy- and Carbonmonoxy-Hb A in D_2O and at 29 °C, Based on Nonlinear Least-Squares Fitting of the Experimental Data to the ^1H NMR Titration Equation 2[a]

resonance	0.1 M HEPES	0.1 M Bis-Tris + 0.18 M NaCl	0.1 M phosphate
1 (β97)	8.02 ± 0.01	7.85 ± 0.02	7.81 ± 0.01
2	7.19 ± 0.01	7.34 ± 0.01	7.28 ± 0.02
3 (β146)	7.96 ± 0.02	7.80 ± 0.02	7.82 ± 0.03
4	7.09 ± 0.01	7.27 ± 0.02	7.25 ± 0.02
4′	7.32 ± 0.02	7.34 ± 0.03	7.23 ± 0.03
5	7.67 ± 0.03	7.56 ± 0.03	7.57 ± 0.03
6	6.84 ± 0.02	7.05 ± 0.02	7.23 ± 0.03
7 (β116 or β117)	6.99 ± 0.03	7.01 ± 0.03	6.91 ± 0.04
8 (β116 or β117)	6.10 ± 0.20	6.60 ± 0.10	6.64 ± 0.07
9	6.67 ± 0.04	6.69 ± 0.07	6.88 ± 0.05
10 (β2)	5.84 ± 0.08	6.13 ± 0.08	6.95 ± 0.02
A	7.86 ± 0.01	7.73 ± 0.01	7.82 ± 0.02
B	7.27 ± 0.01	7.30 ± 0.01	7.58 ± 0.09
C[b]	7.80 ± 0.02	7.71 ± 0.02	7.51 ± 0.07
D	7.56 ± 0.02	7.52 ± 0.02	7.59 ± 0.05
E	6.93 ± 0.02	7.13 ± 0.01	7.29 ± 0.03
F	6.76 ± 0.01	7.00 ± 0.01	7.13 ± 0.02
G (β2)	5.88 ± 0.08	6.46 ± 0.02	6.81 ± 0.02
I	6.68 ± 0.03	6.45 ± 0.08	6.30 ± 0.10
J (β116 or β117)	5.70 ± 0.20	6.71 ± 0.05	6.68 ± 0.03
K (β116 or β117)	6.20 ± 0.20	6.42 ± 0.07	6.59 ± 0.02
H	6.03 ± 0.03	6.27 ± 0.04	6.11 ± 0.06
L	[c]	5.8 ± 0.2	[d]
Y (β146)[e]	[f]	[f]	7.04 ± 0.03

[a] Resonances labeled 1–10 correspond to the deoxy form, and those labeled A–Y correspond to the carbonmonoxy form. [b] Assignment of this resonance to the C2-proton of the β146His residue in a salt-bridge conformation is controversial. [c] Not determined. [d] Resonance L is broadened beyond detection under these conditions. [e] This resonance corresponds to the β146His residue in a conformation in which the β146His–β94Asp salt bridge is broken. [f] Resonance Y is not resolved under these experimental conditions over the entire pH range of interest.

His C2-protons of β116 and β117 (Kilmartin et al., 1973; Russu et al., 1980, 1984; Ho & Russu, 1985; Perutz et al., 1985a). In the carbonmonoxy form, the resonances labeled J and K are assigned to the His C2-protons of β116 and β117 (Russu et al., 1984). The resonance labeled C has been previously assigned to the His C2-proton of β146 (Russu et al., 1980), but at present, this assignment is controversial (Perutz et al., 1985a,b; Russu & Ho, 1986; Shih et al., 1987; Ho & Russu, 1987). As we have previously shown, in HbCO A, in the presence of 0.1 M phosphate ions, an additional His C2-proton resonance (labeled Y in Figure 1B) can be observed in the NMR spectra (Russu et al., 1980; Russu & Ho, 1986). This resonance corresponds to the C2-proton of the β146His residue in a conformation in which the β146His–β94Asp salt bridge is broken (Kilmartin et al., 1973; Russu et al., 1980; Russu & Ho, 1986). As the concentration of inorganic phosphate ion is decreased, the resonance labeled Y gradually shifts upfield into the spectral region 2.75–3.0 ppm from HDO [see Figure 4 of Russu and Ho (1986)]. Under "stripped" conditions or in the presence of Cl^- ions, this resonance overlaps resonances I, J, K, and L and cannot be specifically resolved in the ^1H NMR spectrum. An additional effect of inorganic phosphate ions upon the ^1H NMR spectra of HbCO A consists of the broadening of resonance L beyond detection. A similar effect is observed in the presence of 2,3-DPG (I. M. Russu, S.-S. Wu, K. A. Bupp, N. T. Ho, and C. Ho, unpublished results). As a result of these effects, the ^1H NMR titration of resonance L cannot be monitored in the presence of inorganic or organic phosphate ions.

In the presence of 0.1 M Bis-Tris buffer, the resonance labeled 10 in deoxy-Hb A and that labeled G in HbCO A are assigned to the His C2-proton of β2 (Russu et al., 1982). As shown in Figure 1A,B, these two resonances are greatly affected in 0.1 M HEPES buffer or by the presence of Cl^- or phosphate ions. Due to these effects, new experiments for resonance assignments have been carried out. The His C2-proton resonance of β2 in 0.1 M HEPES buffer has now been assigned by comparing the ^1H NMR spectra of Hb A with those of Hb Deer Lodge (β2His → Arg) under the same experimental conditions. The results are illustrated in Figure 2A and Figure 2B for the deoxy and carbonmonoxy forms, respectively. The assignments of the β2His C2-proton resonances in the presence of 0.18 M Cl^- or 0.1 M phosphate ions have been obtained by monitoring the β2His resonances in 0.1 M Bis-Tris buffer, at constant pH, as a function of increasing concentrations of Cl^- or phosphate ions [results not shown; refer to Figure 4 in Russu and Ho (1986)].

The chemical shifts of each of the His C2-proton resonances in both deoxy and CO forms have been fitted as a function of pH to eq 1 which corresponds to a Henderson–Hasselbach type of equilibrium for the ionization of a single ionizable group. The results of this analysis[3] are presented in Table I. To characterize the deviations of the protonation equilibria of the surface histidyl residues from those predicted by the Henderson–Hasselbach equation, we have also fitted the experimental data to eq 2. The results of this analysis are given in Tables II and III for the apparent pK values and the titration coefficients of the ^1H NMR titration. The individual

[3] The pK values published recently from this laboratory (Ho & Russu, 1987) were based on a preliminary analysis of the present data, and some of them differ slightly from those reported in Tables I and II.

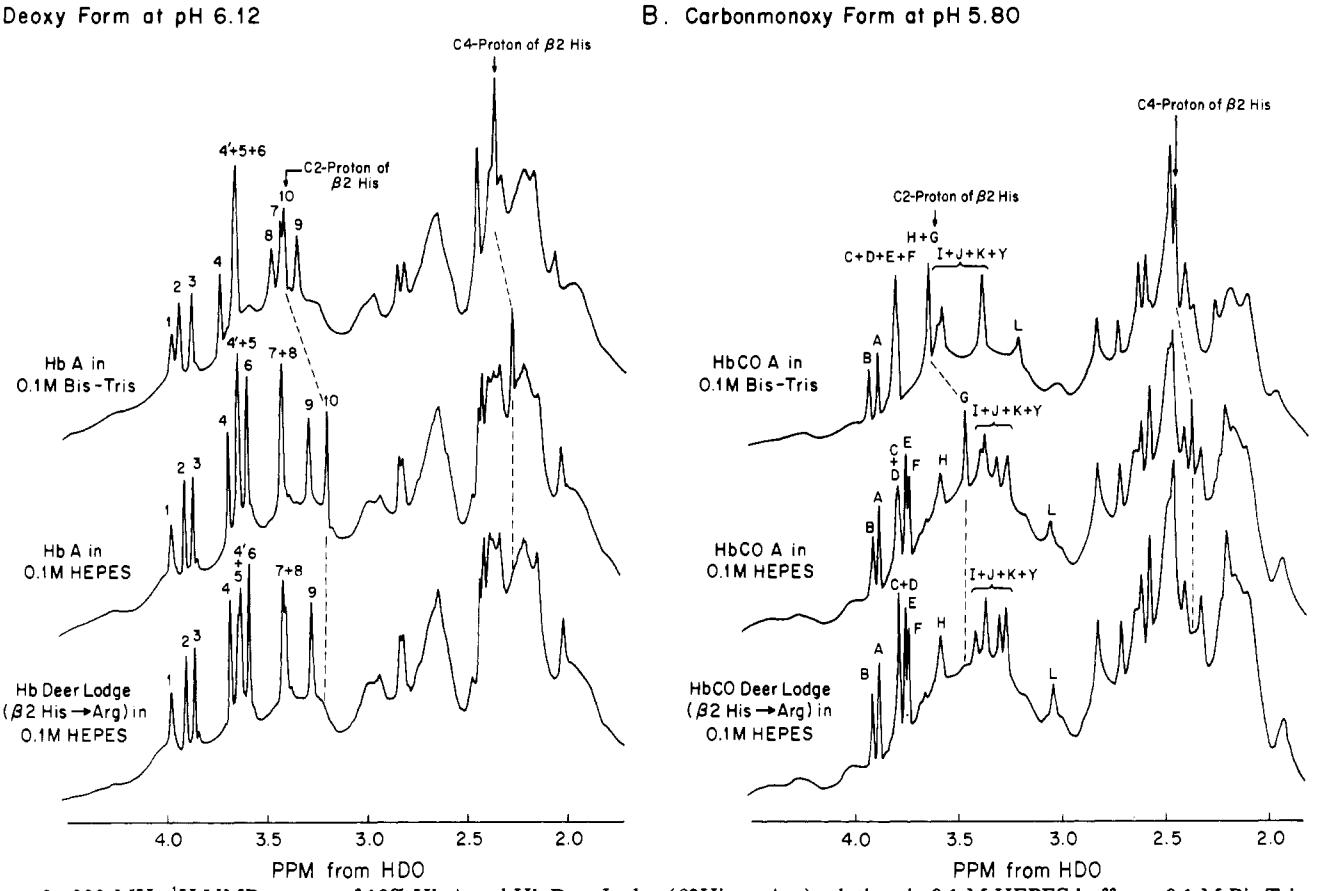

FIGURE 2: 300-MHz ^{1}H NMR spectra of 10% Hb A and Hb Deer Lodge (β2His → Arg) solutions in 0.1 M HEPES buffer or 0.1 M Bis-Tris buffer in D_2O at 29 °C: (A) deoxy form at pH 6.12; (B) carbonmonoxy form at pH 5.80.

NMR titration curves of histidyl residues are illustrated in Figure 3 for the β2His C2-proton, under the three sets of experimental conditions investigated.

Discussion

Inspection of the results presented in Table I reveals that, among the surface histidyl residues investigated, the β2His residues are affected to the largest extent by Cl^- and inorganic phosphate ions. In the deoxy form, the p*K* value of β2His increases by 0.53 pH unit in the presence of 0.18 M Cl^- and by 1.11 pH units in the presence of 0.1 M inorganic phosphate ions. Similarly, in the CO form, the p*K* value of the β2His increases by 0.29 pH unit in the presence of Cl^- ions and by 0.57 pH unit in the presence of inorganic phosphate ions. These findings indicate that both Cl^- and inorganic phosphate ions bind at a site at or near the β2His residues. The increases in the p*K* values of β2His induced by these anions also suggest that the affinity of this binding site for both Cl^- and phosphate ions is greater in the deoxy form than in the CO form. A binding site for inorganic anions at or near the β2His has been detected in deoxy-Hb A using X-ray diffraction techniques (Arnone, 1972). Our present results indicate that the same binding site also exists in the solution state. Electrostatic modeling of the Hb molecule has revealed the existence of multiple isoenergetic Cl^--binding sites within the β-chain central cavity in deoxy-Hb A. One of these is between β82Lys and the α-amino group of β1Val, and another is between β82Lys, β143His, and β2His (Matthew et al., 1981, 1982). Our present ^{1}H NMR results are consistent with these electrostatic calculations, but cannot distinguish between the two proposed Cl^--binding sites. An additional electrostatic contribution to the binding of inorganic anions at or near the β2His residues could originate from the electric field of the

Table III: Titration Coefficients, *n*, for the ^{1}H NMR Titration of Surface Histidyl Residues in Deoxy- and Carbonmonoxy-Hb A in D_2O and at 29 °C, Based on Nonlinear Least-Squares Fitting of the Experimental Data to the ^{1}H NMR Titration Equation 2[a]

resonance	0.1 M HEPES	0.1 M Bis-Tris + 0.18 M NaCl	0.1 M phosphate
1 (β97)	1.02 ± 0.02	0.94 ± 0.04	1.16 ± 0.02
2	0.79 ± 0.02	0.95 ± 0.02	1.01 ± 0.06
3 β146)	0.73 ± 0.02	0.92 ± 0.04	1.00 ± 0.04
4	0.74 ± 0.02	0.99 ± 0.04	0.94 ± 0.06
4′	1.06 ± 0.06	0.93 ± 0.05	1.16 ± 0.09
5	0.80 ± 0.05	0.86 ± 0.05	1.01 ± 0.08
6	0.61 ± 0.02	0.86 ± 0.03	1.18 ± 0.09
7 (β116 or β117)	0.64 ± 0.04	0.85 ± 0.05	0.76 ± 0.06
8 (β116 or β117)	0.48 ± 0.06	0.70 ± 0.10	0.74 ± 0.07
9	0.65 ± 0.04	0.73 ± 0.08	0.90 ± 0.10
10 (β2)	1.02 ± 0.08	0.79 ± 0.06	1.05 ± 0.07
A	0.81 ± 0.02	0.96 ± 0.03	0.99 ± 0.03
B	0.79 ± 0.01	1.05 ± 0.02	0.68 ± 0.08
C[b]	0.82 ± 0.02	0.83 ± 0.04	0.90 ± 0.10
D	0.69 ± 0.02	0.99 ± 0.04	0.97 ± 0.08
E	0.66 ± 0.02	0.90 ± 0.03	1.04 ± 0.08
F	0.73 ± 0.02	0.98 ± 0.03	1.17 ± 0.06
G (β2)	0.67 ± 0.04	0.85 ± 0.03	1.01 ± 0.06
I	0.72 ± 0.04	0.61 ± 0.04	0.80 ± 0.10
J (β116 or β117)	0.48 ± 0.04	0.72 ± 0.06	0.69 ± 0.03
K (β116 or β117)	0.41 ± 0.05	0.68 ± 0.05	0.76 ± 0.02
H	0.98 ± 0.03	1.12 ± 0.08	1.14 ± 0.08
L	*c*	0.8 ± 0.1	*d*
Y (β146)[e]	*f*	*f*	1.21 ± 0.09

[a] Resonances labeled 1–10 correspond to the deoxy form, and those labeled A–Y correspond to the carbonmonoxy form. [b] The assignment of this resonance to the C2-proton of the β146His residue in a salt-bridge conformation is controversial. [c] Not determined. [d] Resonance L is broadened beyond detection under these conditions. [e] This resonance corresponds to the β146His residue in a conformation in which the β146His–β94Asp salt bridge is broken. [f] Resonance Y is not resolved under these conditions over the entire pH range of interest.

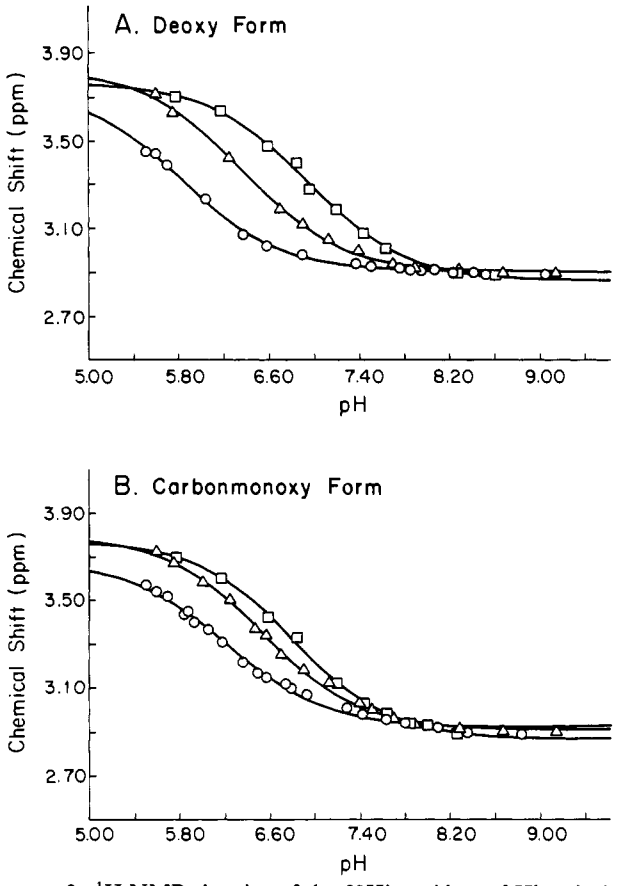

FIGURE 3: ^{1}H NMR titration of the β2His residues of Hb A in 0.1 M HEPES buffer (○), 0.1 M Bis-Tris buffer plus 0.18 M NaCl (△), and 0.1 M phosphate buffer (□) in D_2O at 29 °C: (A) deoxy-Hb A; (B) HbCO A. The titration curves were obtained by fitting the experimental data to eq 1.

A helix dipole at its N-terminal end where β2His is located. As shown by Hol et al. (1978), phosphate ions often bind at the N-terminal of the α-helices in proteins as a result of the interaction between the helix dipole and the charged anion.

The effects of the anions investigated upon the remaining surface histidyl residues of Hb A are subtle and complex. Several histidyl residues have pK values which are higher in the presence of Cl^- and/or phosphate ions than the corresponding ones in 0.1 M HEPES [for example, histidyl residues whose C2-proton resonances are labeled 2, 4, 6, and 8 (β116- or β117His) in the deoxy form and E, F, J (β116- or β117His), and H in the CO form]. On the other hand, several other histidyl residues exhibit decreases in their pK values in the presence of Cl^- and/or inorganic phosphate ions. Among those, the most representative ones are the histidyl residues whose C2-proton resonances are labeled 1 (β97His) and 5 in the deoxy form, and C, I, and L in the CO form. It is interesting to note that, among these histidyl residues, some are not affected by phosphate or chloride ions in the same manner. Several histidyl residues show variations in their pK values when only one of the anions is present (e.g., resonances labeled 5 and 8 in the deoxy form, and resonances labeled I and H in the CO form).

The physical origin of the anion-induced effects observed in the present work is not fully understood at present. In the first approximation, the small increases in the pK values of several histidyl residues induced by Cl^- and/or phosphate ions could be interpreted as being due to the weak binding of the anions at or near these residues. Experimental evidence obtained by ^{35}Cl NMR quadrupole relaxation indicates the existence of at least two classes of chloride-binding sites in Hb (Chiancone et al., 1972). The low-affinity sites may include the histidyl residues observed in the present work to increase their pK values in the presence of anions.

An alternative interpretation of the observed effects is suggested by our finding that the interactions of solvent anions with Hb can also result in lowering of the pK values of certain histidyl residues. As suggested by Gurd and co-workers, the individual H^+-binding equilibrium of an ionizable group in Hb is influenced by its electrostatic interactions with the rest of the charged amino acid residues of the molecule (Matthew et al., 1979a,b, 1981, 1982). The magnitude of these effects clearly depends upon the ionization states of the interacting amino acid residues and upon the ionic strength of the solvent. The binding of anions to well-defined sites on the Hb molecule also affects the pairwise interactions between the histidyl residue of interest and other charges on the Hb molecule (i.e., other ionizable groups and bound anions). Furthermore, increasing the ionic strength of the solvent could also modulate the Coulombic effects of other charged sites upon the histidyl residue of interest and its H^+ affinity. The direction and the magnitude of these anion-induced electrostatic effects on histidyl ionization would clearly depend upon the exact location of the histidyl residue in the three-dimensional structure of the Hb molecule, i.e., relative to other charged amino acid residues and relative to the anion-binding sites.

Our suggestion that the anion-induced changes in the individual histidyl titration curves originate from variations in the electrostatic interactions within the Hb molecule is further supported by the results of the analysis of the shape of the histidyl titration curves presented in Table III. In 0.1 M HEPES buffer, in the absence of Cl^- and phosphate ions, a large number of His C2-proton resonances have titration coefficients less than 1. Large deviations from unity of the n value in eq 2 are usually interpreted as an indication of interactions of the histidyl residue with other ionizable groups (Markley, 1975; Russu et al., 1982). In the present case, these other ionizable groups include amino acid residues of Hb which titrate over the same pH range as the histidyl residue of interest and bound titratable anions (i.e., phosphate ions). In the presence of Cl^- or phosphate ions, the titration coefficients of all the surface histidyl residues increase toward unity. These changes are very likely to originate from decreases in the electrostatic interactions of the histidyl residues with other ionizable groups at higher ionic strengths. Most illustrative of this effect are the β116His and β117His residues whose mutual electrostatic interaction is clearly reflected in the deviation of the corresponding titration coefficients from unity. These deviations are diminished at higher ionic strength where the Coulombic screening is increased. In addition to this nonspecific ionic strength effect, the pK values shown in Tables I and II suggest that β116His and/or β117His could also be binding sites for inorganic anions as previously inferred from electrostatic calculations (Matthew et al., 1981, 1982; Flanagan et al., 1981).

In summary, our present NMR results indicate that small anions such as Cl^- and inorganic phosphate ions affect the surface histidyl residues of Hb through direct binding to these sites and/or by perturbing the network of electrostatic interactions which determines the H^+- and anion-binding equilibria at an individual site. A quantitative analysis of the latter effects requires extensive computer modeling and is beyond the scope of the present work. Regardless of the molecular mechanisms involved, our present NMR data provide direct measurements of the H^+-binding equilibria of the histidyl residues and thus can be used to estimate the role of

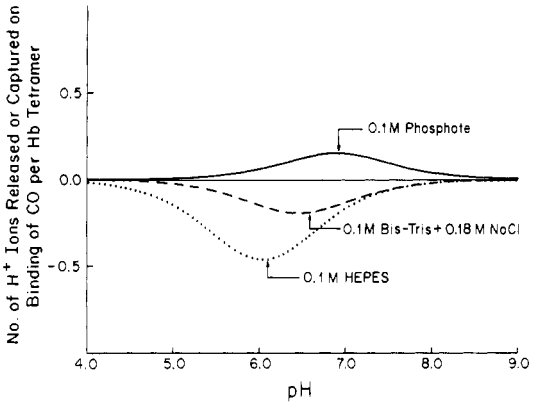

FIGURE 4: Number of H^+ ions released or captured by the β2His residues upon ligand binding to the Hb tetramer. The calculations are based on the pK values reported in Table I.

these amino acid residues in the anion Bohr effect of Hb A.

The contribution of the β2His residues to the Bohr effect under the solvent conditions investigated is shown in Figure 4. The number of H^+ ions released by these residues upon Hb oxygenation has been calculated on the basis of the pK values given in Table I as described previously (Russu et al., 1982). In 0.1 M HEPES buffer, the β2His captures up to 0.5 H^+ ion/Hb tetramer upon ligand binding to Hb A. According to the measurements of the total Bohr effect carried out by Bucci and Fronticelli (1985), this result suggests that, in 0.1 M HEPES buffer, β2His residues make a significant contribution to the acid Bohr effect. In the presence of 0.18 M chloride ions, the number of H^+ ions captured by the β2His residues upon Hb ligation is small. Under these conditions, the pH range in which these residues change their ionization state when ligand binds to Hb corresponds to that of the alkaline Bohr effect (de Bruin et al., 1974; Rollema et al., 1975). Thus, we can conclude that, under these experimental conditions, the β2His makes a negative contribution to the alkaline Bohr effect. In the presence of 0.1 M phosphate ions, the change in the pK values of β2His upon ligation corresponds to a positive contribution to the alkaline Bohr effect of up to 0.2 H^+ ion/Hb tetramer. The protonation properties of the β2His residues and their linkage to anion binding provide an excellent illustration of the variable role that a given amino acid residue can play in the Bohr effect of Hb A. Depending upon solvent conditions, an individual ionizable group could contribute to the macroscopic behavior of the whole molecule or it could oppose it. Hence, the detailed molecular mechanism of the Bohr effect is clearly dependent upon the nature and the concentrations of the allosteric effectors present in the solvent as pointed out previously (Russu et al., 1980, 1982; Russu & Ho, 1986; Ho & Russu, 1987).

The thermodynamic analysis of the anion Bohr effect carried out by de Bruin and co-workers (van Beek et al., 1979; van Beek & de Bruin, 1980) and by Bucci and Fronticelli (1985) indicates that, in addition to the α1Val, the histidyl residues of Hb are the most likely candidates for a contribution to the anion Bohr effect. This role has also been inferred from the ability of the histidyl residues to vary their affinity for H^+ ions upon anion binding over the pH range relevant to the Bohr effect. The number of H^+ ions released (or captured) by a given histidyl residue upon anion binding to Hb can be calculated on the basis of the corresponding change in the pK value in going from 0.1 M HEPES buffer to the buffer containing the anion of interest (Table I). Several representative examples of these calculations are given in Figure 5 for β2His and β97His in the deoxy form and for β116His (or β117His)

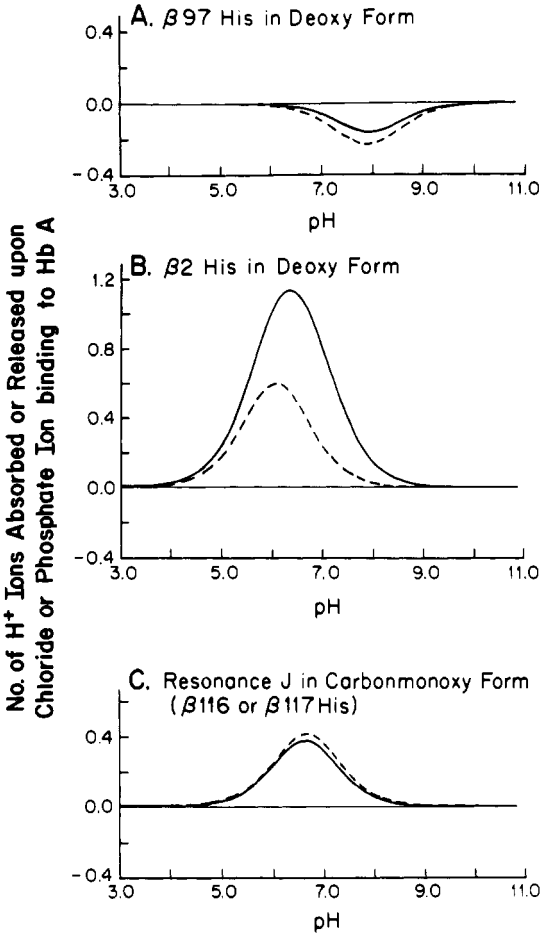

FIGURE 5: Number of H^+ ions released or captured upon chloride or phosphate ion binding to Hb A: (A) β97His in deoxy-Hb A; (B) β2His in deoxy-Hb A; (C) resonance labeled J (β116His or β117His) in HbCO A. Dashed line represents 0.1 M Bis-Tris (or Tris) buffer plus 0.18 M NaCl in D_2O, and solid line represents 0.1 M phosphate buffer in D_2O. The calculations were based on eq 1 and on the pK values of the corresponding histidyl residues given in Table I.

in the CO form. The total number of H^+ ions captured (or released) by the histidyl residues upon Cl^- or inorganic phosphate ion binding to deoxy or ligated Hb A is shown in Figure 6. It should be noted that these calculations include only the histidyl residues which are observable by ^1H NMR under all experimental conditions of interest, namely, 11 histidyl residues per $\alpha\beta$ dimer in both deoxy and CO forms (the histidyl residues whose C2-proton resonances are labeled L and Y in HbCO A have not been included since they cannot be resolved in the ^1H NMR spectra under all solvent conditions investigated). In the presence of 0.18 M Cl^- ions, the 22 histidyl residues of Hb A monitored in the present work bind less H^+ ions in the deoxy form than in the CO form. Assuming that the *same* histidyl residues are observable by ^1H NMR in both deoxy and CO forms of Hb A, this result suggests that, at the Cl^- concentration used here, other amino acid residues of Hb A (such as α1Val) make the dominant contribution to the anion Bohr effect. On the other hand, in the presence of 0.1 M phosphate ions, the total number of H^+ ions bound by the histidyl residues in the deoxy form is enhanced relative to that in the CO form. This fact suggests that the 22 histidyl residues investigated could make a significant contribution to the anion Bohr effect induced by inorganic phosphate ions. An additional contribution to the anion Bohr effect under these conditions would clearly result from the change in the protonation equilibria of the inorganic phosphate ions upon binding to Hb.

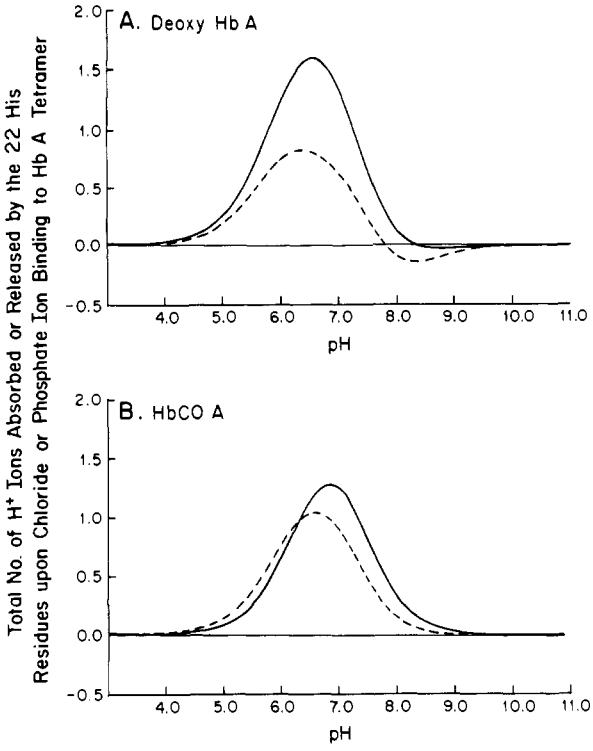

FIGURE 6: Total number of H^+ ions released or captured by the 22 histidyl residues investigated upon chloride or phosphate ion binding to the Hb A tetramer: (A) deoxy-Hb A; (B) HbCO A. Dashed line represents 0.1 M Bis-Tris (or Tris) buffer plus 0.18 M NaCl in D_2O, and solid line represents 0.1 M phosphate buffer in D_2O. The calculations were based on eq 1 and on the pK values given in Table I.

In conclusion, our present ^1H NMR results identify the β2His residue as one of the strongest binding sites for Cl^- and inorganic phosphate ions in Hb A. Although the binding of ions to this histidyl residue appears to be stronger in the deoxy form than in the CO form, the β2His residues do not make a large contribution to the anion Bohr effect. Moreover, our present ^1H NMR data clearly indicate that the molecular mechanism of the anion Bohr effect also involves long-range interactions between ionizable individual sites.

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Amino Acid Sequence of *Salmonella typhimurium* Branched-Chain Amino Acid Aminotransferase†

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ABSTRACT: The complete amino acid sequence of the subunit of branched-chain amino acid aminotransferase (transaminase B, EC 2.6.1.42) of *Salmonella typhimurium* was determined. An *Escherichia coli* recombinant containing the *ilvGEDAY* gene cluster of *Salmonella* was used as the source of the hexameric enzyme. The peptide fragments used for sequencing were generated by treatment with trypsin, *Staphylococcus aureus* V8 protease, endoproteinase Lys-C, and cyanogen bromide. The enzyme subunit contains 308 residues and has a molecular weight of 33 920. To determine the coenzyme-binding site, the pyridoxal 5-phosphate containing enzyme was treated with tritiated sodium borohydride prior to trypsin digestion. Peptide map comparisons with an apoenzyme tryptic digest and monitoring radioactivity incorporation allowed identification of the pyridoxylated peptide, which was then isolated and sequenced. The coenzyme-binding site is the lysyl residue at position 159. The amino acid sequence of *Salmonella* transaminase B is 97.4% identical with that of *Escherichia coli*, differing in only eight amino acid positions. Sequence comparisons of transaminase B to other known aminotransferase sequences revealed limited sequence similarity (24–33%) when conserved amino acid substitutions are allowed and alignments were forced to occur on the coenzyme-binding site.

The branched-chain amino acid aminotransferase [EC 2.6.1.42 (transaminase B, TmB[1])] of *Salmonella typhimurium* catalyzes the final step in the biosynthesis of isoleucine, leucine, and valine. The enzyme is essential only for isoleucine synthesis since valine and leucine can be synthesized by alternate aminotransferases. *Salmonella* TmB was purified, crystallized, and partially characterized by Coleman and Armstrong (1971). Lipscomb et al. (1974) identified the enzyme as a hexameric protein of 183 kDa composed of identical 31.5-kDa subunits. On the basis of this subunit size, amino acid analysis gave an estimated 288 amino acid residues per subunit. No sulfhydryls are required for activity, and the intact hexamer contains no disulfide bridges. One mole of pyridoxal 5-phosphate is bound per subunit. Serine was identified as the C-terminal residue. Randall et al. (1979) and Randall (1982) identified the first 20 residues of the amino terminus, with the N-terminal residue being threonine.

The present study on the *S. typhimurium* enzyme was undertaken to verify, by direct methods, the structure of TmB of *Escherichia coli* K-12, which was deduced from nucleotide sequence analysis (Kuramitsu et al., 1985). The study also identifies the coenzyme-binding site and reports a new procedure for the purification and crystallization of large quantities of the *Salmonella* TmB.

Materials and Methods

Transaminase B, the *ilvE* gene product of *S. typhimurium*, was isolated and crystallized from a recombinant organism, *E. coli* JA199 (pDU 11), a gift from the late Dr. R. O. Burns. The organism contains a deletion mutation of the *ilvGEDAYC* gene cluster, as well as a deletion mutation of the *trpE5* gene and a mutation in the *leuB6* gene. It harbors a multicopy plasmid, pDU11, a pBR322 derivative containing the *ilvGEDAY* gene cluster of *S. typhimurium*, and a gene for tetracycline resistance (*tet*[r]). When grown under derepressing conditions (limiting isoleucine), the organism produces up to 12% of its soluble protein as TmB.

For enzyme purification, the cells were grown in a modified Davis and Mingioli medium (1950) and contained 86 mM K_2HPO_4, 47 mM KH_2PO_4, 16 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 70 mM D-glucose, 0.85 mM L-valine, 0.76 mM L-leucine, 0.49 mM L-tryptophan, 0.076 mM L-isoleucine, and tetracycline hydrochloride at a concentration of 20 μg/mL and were incubated on a rotary shaker for 18 h at 37 °C. Cells were harvested by centrifugation; the resulting pellets were resuspended with 0.1 M potassium phosphate buffer, pH 7.4, and recentrifuged.

For the purification protocol, all steps were performed at 4 °C, and all buffers used were potassium phosphate. Cells were suspended in 0.1 M buffer, pH 6.5, and disrupted by

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[1] Abbreviations: TmB, transaminase B; AAT, aspartate:α-ketoglutarate aminotransferase; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; EDTA·4Na, ethylenediaminetetraacetate tetrasodium salt; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; CM, carboxymethyl; PE, pyridylethyl; UWGCG, University of Wisconsin Genetics Computer Group.

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