Roles of the β146 Histidyl Residue in the Molecular Basis of the Bohr Effect of Hemoglobin: A Proton Nuclear Magnetic Resonance Study†

Mark R. Busch, James E. Mace,‡ Nancy T. Ho, and Chien Ho*
Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213
Received July 30, 1990; Revised Manuscript Received October 9, 1990

ABSTRACT: Assessment of the roles of the carboxyl-terminal β146 histidyl residues in the alkaline Bohr effect in human normal adult hemoglobin by high-resolution proton nuclear magnetic resonance spectroscopy requires assignment of the resonances corresponding to these residues. Previous resonance assignments in low ionic strength buffers for the β146 histidyl residue in the carbonmonoxy form of hemoglobin have been controversial [see Ho and Russu (1987) *Biochemistry 26*, 6299–6305; and references therein]. By a careful spectroscopic study of human normal adult hemoglobin, enzymatically prepared des(His146β)-hemoglobin, and the mutant hemoglobins Cowtown (β146His → Leu) and York (β146His → Pro), we have resolved some of these conflicting results. By a close incremental variation of pH over a wide range in chloride-free 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer, a single resonance has been found to be consistently missing in the proton nuclear magnetic resonance spectra of these hemoglobin variants. The spectra of each of these variants show additional perturbations; therefore, the assignment has been confirmed by an incremental titration of buffer conditions to benchmark conditions, i.e., 0.2 M phosphate, where the assignment of this resonance is unambiguous. The strategy of incremental titration of buffer conditions also allows extension of this resonance assignment to spectra taken in 0.1 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane buffer. Participation of the β146 histidyl residues in the Bohr effect has been calculated from the p*K* values determined for the assigned resonances in chloride-free 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer. Our results indicate that the contribution of the β146 histidyl residues is 0.52 H^+/hemoglobin tetramer at pH 7.6, markedly less than the 0.8 H^+/hemoglobin tetramer estimated by study of the mutant hemoglobin Cowtown (β146His → Leu) by Shih and Perutz [(1987) *J. Mol. Biol. 195*, 419–422]. We have found that at least two histidyl residues in the carbonmonoxy form of this mutant have p*K* values that are perturbed, and we suggest that these p*K* differences may in part account for this discrepancy. Furthermore, summation of the positive contribution of the β146 histidyl residues and the negative contribution of the β2 histidyl residues to the maximum Bohr effect measured in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer suggests that additional sites in the hemoglobin molecule account for proton release upon ligation greater than the contribution of the β146 histidyl residues. Our results show that the p*K* values of β146 histidyl residues in the carbonmonoxy form of hemoglobin are substantially affected by the presence of chloride and other anions in the solvent, and thus, the contribution of this amino acid residue to the alkaline Bohr effect can be shown to vary widely in magnitude, depending on the solvent composition. These results demonstrate that the detailed molecular mechanisms of the alkaline Bohr effect are not unique but are affected both by the hemoglobin structure and by the interactions with the solvent components in which the hemoglobin molecule resides. The implication of these results is that one needs to consider both the macroscopic and microscopic properties of a protein molecule before one can fully and accurately understand the molecular basis of its biological function.

The oxygen-binding properties of the hemoglobin (Hb)[1] molecule are mediated both by interactions between its oxygen-binding sites (homotropic interactions) and by interactions of individual amino acid residues with solvent components (heterotropic interactions). Heterotropic effectors include hydrogen ion, chloride, inorganic phosphate, and organic polyanions such as 2,3-diphosphoglycerate (2,3-DPG) (Benesch & Benesch, 1969; Antonini & Brunori, 1971). The functional consequences of these heterotropic effectors imply differential interactions of each effector with the ligated and unligated forms of the Hb molecule (Wyman, 1948, 1964). The higher affinity of deoxyhemoglobin (deoxy-Hb) for H^+ ions as pH increases above 6.5 leads to the thermodynamic consequence of increasing oxygen affinity, with oxygenation of Hb resulting

† Preliminary results were presented at the 10th International Biophysics Congress, July 28–Aug 3, 1990, Vancouver, BC, Canada, and at the 4th Symposium of the Protein Society, Aug 11–15, 1990, San Diego, CA. This work was supported by a research grant from the National Institutes of Health (HL-24525). M.R.B. was supported in part by a postdoctoral fellowship from the American Heart Association, Western Pennsylvania Chapter; and J.E.M. was supported in part by a grant on Research Experience for Undergraduates in Biological Sciences awarded to Carnegie Mellon University by the National Science Foundation (BBS-8803975) during the summer of 1988.

* Address correspondence to this author.
‡ Present address: Department of Biochemistry and Biophysics, School of Medicine, University of California at San Francisco, San Francisco, CA 94143.

[1] Abbreviations: Hb, hemoglobin; deoxy-Hb, deoxyhemoglobin; HbO_2, oxy-Hb; HbCO, carbonmonoxyhemoglobin; met-Hb, methemoglobin; Hb A, human normal adult hemoglobin; T, deoxy-type quaternary structure of Hb; R, oxy-type quaternary structure of Hb; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; TAPS, *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; 2,3-DPG, 2,3-diphosphoglycerate; des(His146β)Hb, Hb A with the β146 histidyl residue enzymatically removed.

0006-2960/91/0430-1865$02.50/0

in a release of H^+ ions by the Hb molecule, known as the alkaline Bohr effect (Bohr et al., 1904). This physiologically relevant effect 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^+ ions, oxygen affinity increases with decreasing pH, and the Hb molecule absorbs H^+ ions upon oxygenation, known as the acid Bohr effect (Antonini & Brunori, 1971).

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 that occur upon changing the ligation state of the Hb molecule. Any group that changes its ionization constant between the deoxy and CO (or oxy)[2] forms of the Hb molecule participates in the Bohr effect. It is inappropriate to assume that all sites have pK changes that result in positive contributions to the macroscopically observed effect (Busch & Ho, 1990). 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 [for example, see Russu et al. (1982, 1989, 1990, and references therein)].

Intensive experimental and theoretical studies during the past two decades have been devoted to the identification of those amino acid residues whose pK values change in going from the unligated to ligated forms of the Hb molecule and thereby participate in the Bohr effect. It is beyond the scope or intent of this paper to chronicle the entirety of these investigations [for a recent review of the Bohr effect, see Ho and Russu (1987)], but insofar as this paper seeks to clarify some of the conflicting results regarding the amino acid residues involved in the Bohr effect, it is necessary to briefly summarize a number of the more important investigations in this area.

Perutz (1970) proposed the carboxyl-terminal histidine residues of the β-chains [β146His(HC3)] as potential contributors to the alkaline Bohr effect of Hb. X-ray diffraction results using deoxy-Hb A have shown an intrachain salt bridge between the imidazole of β146His and the carboxyl group of β94Asp(FG1) (Perutz, 1970; Fermi, 1975). The electrostatic interactions resulting from this salt bridge would increase the pK value of the β146His residue. Upon oxygenation, this salt bridge is broken, the β146His residue moves away from the β94Asp(FG1), and its pK should revert to a lower value (Perutz, 1970; Baldwin, 1980; Shaanan, 1983).

Functional studies of Hb molecules in which the β146His residue has been altered by mutation or by enzymatic modification are consistent with the participation of this amino acid residue in the Bohr effect, in that each example shows a reduction in the magnitude of the Bohr effect relative to Hb A. Hb Hiroshima (β146His → Asp), Hb York (β146His → Pro), and Hb Cowtown (β146His → Leu) each show a reduction of the alkaline Bohr effect of about 40%–60% (Imai, 1968; Bare et al., 1976; Shih et al., 1984). Hb Cochin-Port Royal (β146His → Arg) and Hb Barcelona (β94Asp → His) (mutation of the salt-bridge partner of β146His) each show a reduction of the Bohr effect of about 20%–30% (Wajcman et al., 1975, 1982; Phillips et al., 1983). Removal of the β146His residues by digestion with carboxypeptidase B to form des(His146β)Hb results in a reduction of the alkaline Bohr effect, which varies from 40% to 60% depending on the concentration of chloride in the solvent (Kilmartin & Wootton, 1970; Kilmartin et al., 1980). These results must be interpreted carefully, however, since the use of mutant or modified Hbs to identify amino acid residues involved in the Bohr effect requires a complete analysis of the pH dependence of the H^+ release upon ligation (Saroff, 1972) and, in the case of des(His146β)Hb for example, the reduction of the Bohr effect cannot be accounted for simply by the deletion of a Bohr group (Saroff, 1972; Matsukawa et al., 1984).

Proton nuclear magnetic resonance (NMR) spectroscopy is particularly suited to studying the involvement of histidyl residues in the Bohr effect, in that the technique allows the observation of individual C2 ($C_{\epsilon 1}$) and C4 ($C_{\delta 2}$) hydrogens of histidyl residues in the solution state and under experimental conditions relevant to Hb function. This laboratory has previously shown that 22–26 C2 ($C_{\epsilon 1}$) protons of the surface histidyl residues can be resolved and titrated in the ^1H NMR spectra of the deoxy and CO forms of Hb A (Russu et al., 1980, 1982; Russu & Ho, 1986; Ho & Russu, 1987). Detailed information regarding the molecular basis of the Bohr effect requires an assignment of these resonances to the corresponding histidyl residues, and considerable effort has been given to the assignment of the resonance arising from β146His.

The C2 ($C_{\epsilon 1}$) proton resonance arising from β146His was first identified by a comparison of ^1H NMR spectra of des(His146β)Hb and Hb A in 0.2 M phosphate + 0.2 M chloride in our laboratories (Kilmartin et al., 1973). Under these conditions, the pK value at 30 °C of β146His decreases from 8.0 in the deoxy-Hb A to 7.1 in HbCO A, thus contributing about 50% of the alkaline Bohr effect. In 0.1 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-Tris) and/or 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffers with chloride ranging from 5 to 60 mM, a comparison of the ^1H NMR spectra of des(His146β)Hb and Hb A led to the assignment of resonance 3 in deoxy-Hb A and resonance C in HbCO A to β146His (Russu et al., 1980, 1982; Russu & Ho, 1986).[3] The assignment of resonance 3 in deoxy-Hb A to β146His has been well established. The pK values of β146His in 0.1 M Bis-Tris/Tris at 27 °C were found to be 8.0 and 7.85 in the deoxy and CO forms of Hb A, respectively, leading to the conclusion that under these conditions the contribution of β146His to the alkaline Bohr effect was less than 5%. Since the total Bohr effect in 0.1 M Bis-Tris is substantially the same as that in 0.2 M phosphate + 0.2 M chloride, it has been suggested that other amino acid residues also contribute to the Bohr effect and that the detailed molecular mechanism of the Bohr effect is dependent on the solvent composition.

In addition to questioning this conclusion on the basis of biochemical and structural studies of mutant and modified Hbs, the assignment for β146His in HbCO A made in 0.1 M Bis-Tris/Tris buffers was reexamined by Perutz and coworkers (Perutz et al., 1980, 1985a,b; Kilmartin et al., 1980) by a comparison of ^1H NMR spectra of HbCO A with those of mutants HbCO Cowtown (β146His → Leu), HbCO Wood (β97His → Leu), HbCO Malmö (β97His → Gln), des(His146β)HbCO, HbCO Abruzzo (β143His → Arg), HbCO

[2] HbCO is used in place of HbO_2 due to the increased stability of HbCO to acid and alkali denaturation and to oxidation to met-Hb. It has been demonstrated that the H^+-binding properties of HbCO are equivalent to those of HbO_2 (Antonini et al., 1963).

[3] Resonance nomenclature follows that of Russu et al. (1982); i.e., resonances A–L and Y represent the C2 ($C_{\epsilon 1}$) proton resonances of histidyl residues in HbCO A, and resonances 1–10 represent those in deoxy-Hb A.

Barcelona (β94Asp → His), and HbCO Fort de France (α45His → Arg) in 0.2 M *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES) or 0.1 M Bis-Tris buffer. They proposed that resonance C originates from β97His and that β146His gave rise to resonance H. The resulting Δp*K* of 1.8 pH units implied that β146His should contribute 75% of the alkaline Bohr effect.

Russu and Ho (1986) demonstrated that resonance H cannot originate from β146His by an examination of ^{1}H NMR spectra of HbCO Cowtown (β146His → Leu), des(His146β)HbCO, and HbCO A in 0.1 M Bis-Tris and 0.2 M HEPES buffers. The sensitivity of resonance H to O_2 and/or methemoglobin (met-Hb) in the Hb sample has been documented and has been suggested as a possible reason for the absence of this resonance in the spectra of Perutz and co-workers (Perutz et al., 1985a,b). The conclusion that resonance H does not correspond to β146His was accepted by Perutz and co-workers, but its loss is attributed to the method used for exchange of Hb into deuterated solvent (Shih et al., 1987).

The identity of resonance C has remained in question, as the resonance is present in the spectrum of Hb Cowtown (β146His → Leu) (Perutz et al., 1985a,b; Russu & Ho, 1986; Shih et al., 1987), suggesting that it cannot be assigned to β146His. Its assignment to β97His by Perutz et al. (1985a,b) was also questioned due to the multiple spectral alterations seen for HbCO Wood (β97His → Leu) and HbCO Malmö (β97His → Gln) in comparison with HbCO A, as well as the loss of this resonance in HbCO Barcelona (β94Asp → His) and HbCO Abruzzo (β143His → Arg) (Russo & Ho, 1986; Ho & Russo, 1987).

Thus, the roles of β146His in the Bohr effect remain an open question. Shih and Perutz (1987) have investigated the contribution of β146His 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. They have further concluded that the p*K* values of β146His in the deoxy-type (T) and oxy-type (R) structures of the Hb molecule are largely chloride independent.

In the present work, in order to resolve some of the conflicting results regarding the ^{1}H NMR assignments of β146His in HbCO A and the roles of this amino acid residue in the Bohr effect, we have prepared des(His146β)Hb and obtained Hb Cowtown (β146His → Leu) and Hb York (β146His → Pro) in order to assign the C2 ($C_{\epsilon 1}$) proton of β146His in HbCO A in 0.1 M HEPES buffer. We have determined the p*K* of this residue under these conditions and also extended the assignment to the 0.1 M Bis-Tris conditions. With these p*K* values and oxygen equilibrium data, also taken in 0.1 M HEPES buffer, we have calculated the contribution of β146His to the alkaline Bohr effect. The p*K* value of β146His in HbCO A, and, concomitantly, its contribution to the Bohr effect, varies significantly with the solvent composition. It should be noted that the p*K* value of β146His in deoxy-Hb A is relatively insensitive to the solvent composition (Russu et al., 1989, 1990; Busch & Ho, 1990). Furthermore, our results have demonstrated that the use of mutant or modified Hbs to study the detailed molecular mechanisms of the Bohr effect in Hb A has limitations, which arise from the conformational and/or electrostatic perturbations that can accompany the mutation or modification of the Hb molecule.

Materials and Methods

Materials. Carboxypeptidase B was obtained from Sigma and used without further purification. [1,3-^{13}C(99%)]Glycerol and D_2O were obtained from Cambridge Isotope Labortories, and stock solutions were prepared in D_2O. All other chemicals used were reagent grade and were obtained from commercial suppliers.

Hb A was prepared and purified by standard procedures used in our laboratory (Lindstrom & Ho, 1972). Hb Cowtown (β146His → Leu) and Hb York (β146His → Pro) were purified according to Shih et al. (1984), using a CM-cellulose column (Whatman CM-52) in 20 mM phosphate and with a pH gradient from 6.6 to 7.8. Purity of both mutants was checked with isoelectric focusing.

The preparation of des(His146β)Hb followed the methods of Kilmartin et al. (1981), with minor modifications. The carboxypeptidase B digestion of isolated β-chains of Hb A was carried out in 0.1 M *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer to allow analysis of the amino acids released without the interference of Tris in the detection of histidine. Following removal of the enzyme on a DE-52 column, the des(His146β) chains were isolated by using a CM-52 column equilibrated in 0.01 M sodium acetate buffer at pH 6.0, with a gradient to 0.1 M sodium acetate. Constant CO saturation of the buffers used in this column was necessary to avoid loss of product due to oxidation. Removal of the β146His residue was confirmed by amino acid analysis of the isolated product prior to recombination with isolated α-chains and subsquent purification by ion-exchange chromatography. The purity of the des(His146β)Hb was checked by using isoelectric focusing. Functional properties of the product were comparable with previously studied samples.

Organic phosphates were removed by passing the Hb solutions through a Sephadex G-25 column equilibrated with 0.01 M Tris buffer + 0.1 M NaCl at pH 7.6 (Berman et al., 1971). Hb samples were exchanged into D_2O buffers by repeated centrifugation in Centricon concentrators (Amicon), with occasional use of stirred ultrafiltration cells and/or dialysis into multiple changes of buffer.[4] Deoxygenation of samples was accomplished by conversion of HbCO to HbO_2 in a rotary evaporator at 4 °C, followed by deoxygenation under nitrogen as previously described (Lindstrom & Ho, 1972). Deoxygenation was confirmed by the disappearance of the ring-current-shifted proton resonances that occur between −6.0 and −7.0 ppm upfield from HDO in the ^{1}H NMR spectrum. Samples were examined for met-Hb before and after ^{1}H NMR spectroscopy by examining the optical absorbance at 631 nm; those samples showing increased absorbance indicating met-Hb formation were discarded.

Hb samples in 0.1 M HEPES buffer were 10 μM in EDTA to reduce met-Hb formation, and the pH was adjusted with NaOD in D_2O. The pH values of Hb samples in 0.2 M phosphate + 0.2 M NaCl and in 0.1 M Bis-Tris were measured with a combination electrode (Radiometer Type GK2322C) and a Radiometer pHM64 pH meter at ~25 °C. The pH values are reported as direct readings, since the deuterium effect on the glass electrode [i.e., pD = pH + 0.4 (Glascoe & Long, 1960)] is compensated by its effects on the p*K* value of the imidazole (Tanokura et al., 1978). Determination of pH values in the HEPES buffer is described under Methods.

Methods. The NMR spectra were obtained at 29 °C on Bruker WM-300 and AM-300 spectrometers operating at 300.13 MHz for ^{1}H NMR and 75.468 MHz for ^{13}C NMR.

[4] The ^{1}H NMR spectra that we have observed are unaffected by the method of solvent exchange, provided that no met-Hb is allowed to form and that CO saturation is maintained.

^{1}H NMR spectra were obtained with 256–400 transients at a repetition rate of 0.33 s^{-1}, and the free induction decay was processed with slight resolution enhancement (parameters: line broadening = −4 Hz; Gaussian broadening = 0.15) to resolve closely spaced peaks. The effect of this resolution enhancement on peak intensities is expected to be minimal; nonetheless, peak intensities were not used for resonance assignment purposes. Proton chemical shifts are expressed as parts per million from the residual proton (HDO) resonance in D_2O media, which is 4.73 ppm downfield from the proton resonance of a standard, 2,2,-dimethyl-2-silapentane-5-sulfonate (DSS), at 29 °C. The proton chemical shift is defined as positive in the low-field direction with respect to HDO.

The pK values of the histidyl residues were determined by a nonlinear least-squares fit of the chemical shift of each C2 ($C_{\epsilon 1}$) proton resonance, δ, as a function of pH according to the equation:

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

where δ^+ and δ^0 are the chemical shifts in the protonated and unprotonated forms of the histidyl residue, respectively, and K is the H^+ dissociation equilibrium constant of the histidyl residue (Markley, 1975a). The chemical shifts of the His C2 ($C_{\epsilon 1}$) proton resonances were also fit as a function of pH to the equation:

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

where n is the titration coefficient for the ^{1}H NMR titration of the histidyl residue[5] and the other symbols are defined as above.

^{13}C NMR spectra were obtained with 64–128 transients at a repetition rate of 0.33 s^{-1}, with broad-band proton decoupling centered at the HDO frequency. Chemical shifts in the ^{13}C NMR spectra were measured relative to an internal [1,3-^{13}C(99%)]glycerol standard (~1 mM final concentration) at 63.865 ppm downfield relative to tetramethylsilane (TMS) at 29 °C. By use of a broad-band probe in the NMR spectrometer, ^{13}C and ^{1}H NMR spectra could be acquired consecutively on the same sample under the same conditions.

The pH values of Hb samples in chloride-free 0.1 M HEPES buffer were determined from the ^{13}C NMR chemical shifts of the buffer carbons relative to the internal [1,3-^{13}C-(99%)]glycerol standard in order to avoid the chloride efflux from the pH electrode.[6] In a separate titration experiment, the ^{13}C NMR chemical shifts of each of the six buffer carbons were determined as a function of pH by titration of 0.1 M HEPES buffer in D_2O with NaOD and by subsequent reverse titration with DCl. The pH values were determined with a combination electrode and a Radiometer pHM64 pH meter, calibrated with NBS standard borate, phthalate, and phosphate buffers thermostated at 29 °C (Bates, 1973). The chemical shifts observed in the NaOD and subsequent reverse DCl titrations were superimposable, and the data were then fit to eq 1, with the resulting pK and chemical shift limits then used in the calculation of pH from the ^{13}C NMR chemical shifts of each sample. The two peaks with the largest chemical shift dispersion ($\delta^0 - \delta^+$ = 2.98 and 2.25 ppm) were used for pH calculations. The accuracy of the pH determinations is estimated to be ±0.02 pH unit.

Oxygen dissociation curves of Hb solutions in 0.1 M HEPES buffer in D_2O were measured with an Aminco Hem-O-Scan oxygen dissociation analyzer, with Hb concentrations of ~10%. The pH of each sample was determined as described above for NMR samples.

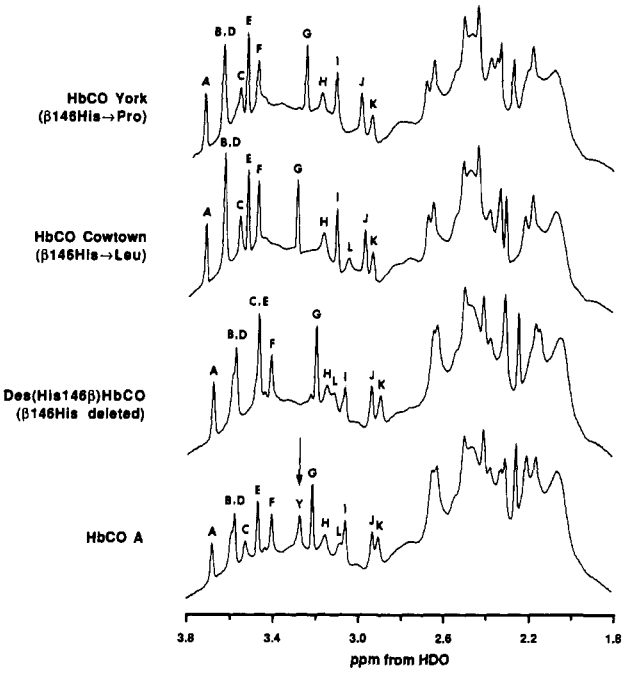

FIGURE 1: 300-MHz ^{1}H NMR spectra of 8%–10% solutions of HbCO York (β146His → Pro), HbCO Cowtown (β146His → Leu), des-(His146β)HbCO, and HbCO A in D_2O in 0.2 M phosphate + 0.2 M NaCl at pH 7.1 and 29 °C. Resonances from C2 ($C_{\epsilon 1}$) protons of histidyl residues are seen from 2.8 to 3.8 ppm; the region from 1.8 to 2.8 ppm consists of resonances from C4 ($C_{\delta 2}$) histidyl protons as well as resonances from residues of phenylalanine, tyrosine, and tryptophan.

RESULTS

Assignment of the C2 ($C_{\epsilon 1}$) Resonance of β146His in HbCO A in Chloride-Free 0.1 M HEPES. The aromatic proton resonances of HbCO A, des(His146β)HbCO, HbCO Cowtown (β146His → Leu), and HbCO York (β146His → Pro) in 0.2 M phosphate + 0.2 M NaCl in D_2O at pH 7.1 are shown in Figure 1. In this paper, we are primarily concerned with the C2 ($C_{\epsilon 1}$) proton resonances of the histidyl residues that resonate over the region from 2.8 to 3.8 ppm downfield of HDO. The proton resonances over the region from 1.8 to 2.8 ppm downfield from HDO arise from the C4 ($C_{\delta 2}$) protons of histidyl residues, as well as protons of aromatic amino acid residues of tyrosine, phenylalanine, and tryptophan in Hb.

The resonance labeled Y is missing from the ^{1}H NMR spectra of each of the three carboxyl-terminal variants and is, therefore, assigned to the C2 ($C_{\epsilon 1}$) proton of β146His under these conditions. In the spectrum of des(His146β)HbCO, peak C has shifted to an upfield position where it cannot be resolved from resonance E but returns to its unperturbed position in the spectra of HbCO Cowtown and HbCO York. HbCO Cowtown and HbCO York also show shifts in peaks G and I, and HbCO York has lost peak L due to broadening. This assignment of resonance Y is consistent with that made by this laboratory (Kilmartin et al., 1973; Russu et al., 1980, 1982; Russu & Ho, 1986) under these high-salt conditions. The loss

[5] 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. To avoid possible confusion with the use of this term, Hill coefficient, to describe the cooperativity of oxygen binding in Hb, we have chosen to call n of eq 2 the titration coefficient.

[6] Given manufacturer's data on the rate of efflux of saturated KCl (3.86 M) from the reference electrode of a combination electrode and the small size of the NMR samples (0.3–0.5 mL), the chloride accumulation from a pH electrode was estimated to be as high as 1 mM/min of exposure.

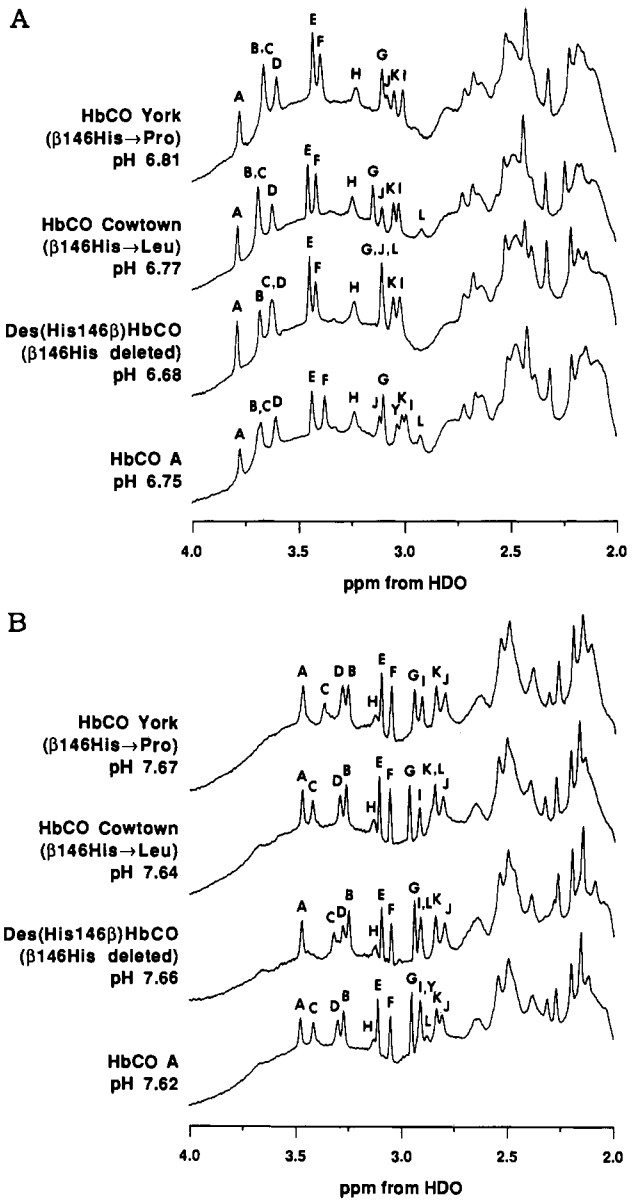

FIGURE 2: 300-MHz ^{1}H NMR spectra of 8%–10% solutions of HbCO York (β146His → Pro), HbCO Cowtown (β146His → Leu), des-(His146β)HbCO, and HbCO A in D_2O in 0.1 M HEPES at 29 °C: (A) pH = ~6.8 and (B) pH = ~7.6.

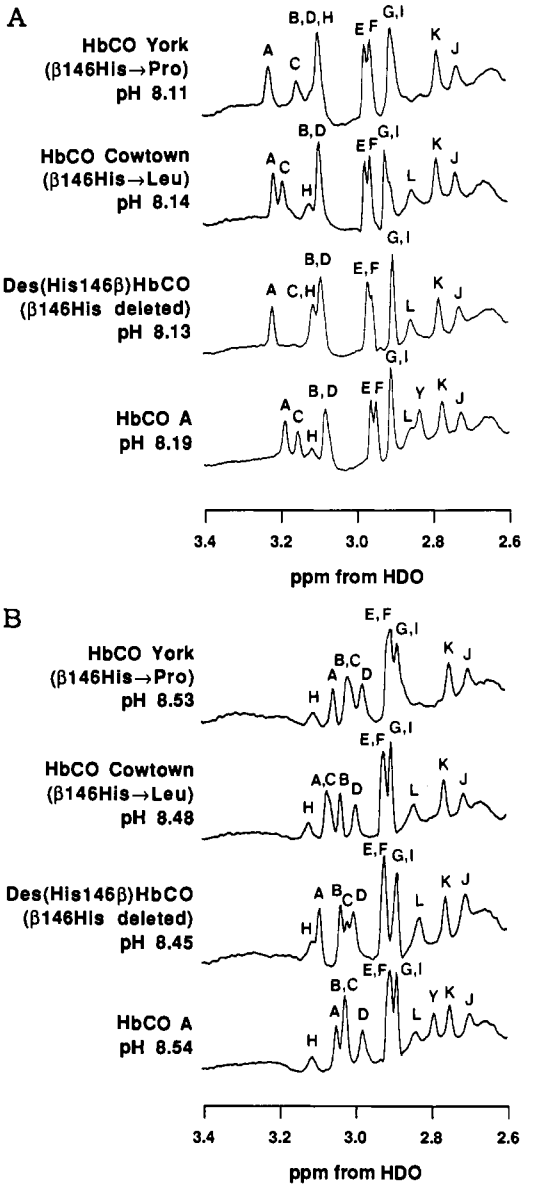

FIGURE 3: 300-MHz ^{1}H NMR spectra of 8%–10% solutions of HbCO York (β146His → Pro), HbCO Cowtown (β146His → Leu), des-(His146β)HbCO, and HbCO A in D_2O in 0.1 M HEPES at 29 °C: (A) pH = ~8.1 and (B) pH = ~8.5.

of resonance Y is consistent at other pH values in phosphate buffers, and therefore, this assignment will serve as a benchmark to confirm resonance assignments made in other buffer conditions.

Examination of ^{1}H NMR spectra taken in chloride-free 0.1 M HEPES buffer at pH 6.8 (Figure 2A) shows that, in the case of des(His146β)HbCO, resonance C is shifted upfield, similar to the situation in the phosphate buffer shown in Figure 1, and cannot be resolved from resonance D, but in the spectra of HbCO Cowtown and HbCO York, it has returned to its unperturbed position as seen in HbCO A. At pH 7.6 in 0.1 M HEPES (Figure 2B), resonance C can be clearly resolved from resonances B and D in des(His146β)HbCO, as well as in HbCO Cowtown and HbCO York. However, resonance C in des(His146β)HbCO is shifted upfield compared to that in HbCO A, HbCO Cowtown, and HbCO York.

The resonances upfield of resonance H (2.8–3.2 ppm) can be clearly resolved as six separate resonances in HbCO A at pH 6.8 (Figure 2A). In the case of des(His146β)HbCO, only three peaks are seen in this same upfield region, but by following these peaks throughout the pH range, it can be demonstrated that five overlapping resonances are, in fact, present and are seen as these three peaks. In HbCO Cowtown, five peaks representing five titratable resonances are seen at this pH, and in HbCO York, only four titratable resonances are seen in this region due to the loss of resonance L in HbCO York. The spectra at this pH value do not show the direct 1:1 correspondence that would simplify resonance assignments.

As pH increases to 7.6 in the chloride-free 0.1 M HEPES buffer (Figure 2B), the arrangement of these six resonances is such that the loss of a resonance is difficult to ascertain in the des(His146β)HbCO, HbCO Cowtown, and HbCO York and can only be determined by following the complete range of pH titration in these conditions.

As seen in Figure 3A in 0.1 M HEPES at pH 8.1, the region of the spectra from 2.7 to 2.9 ppm more clearly shows the loss of resonance Y in the cases of des(His146β)HbCO and HbCO Cowtown and the loss of resonances Y and L in HbCO York. As seen in the spectra at lower pH values, resonance C is still shifted upfield in des(His146β)HbCO. In Figure 3B, spectra at pH 8.5 in 0.1 M HEPES still clearly show the loss of peak

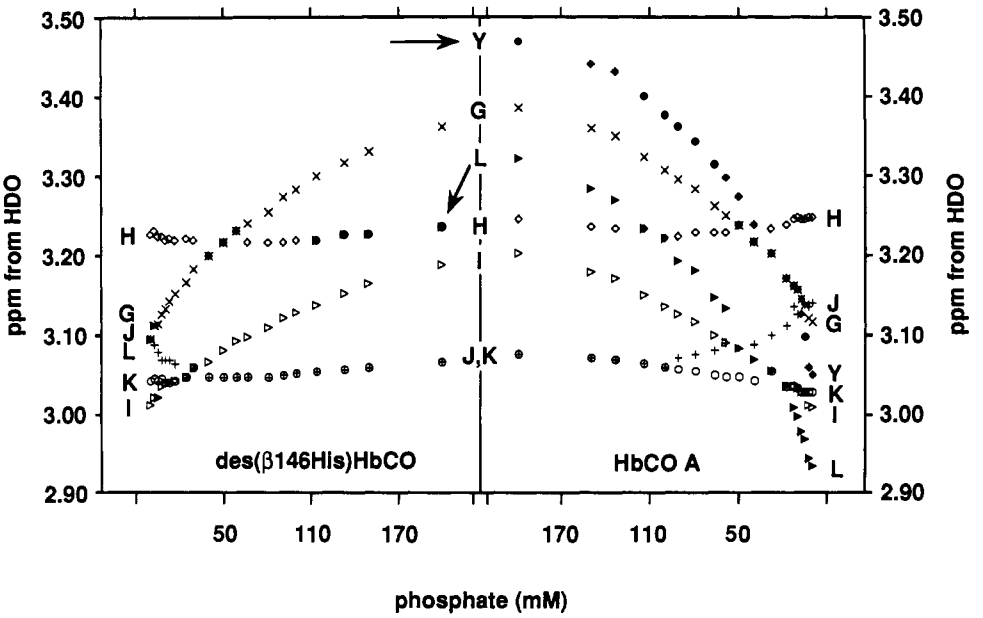

FIGURE 4: Phosphate dependence of resonances G–L and Y in HbCO A and G–L in des(His146β)HbCO in 0.1 M HEPES at pH 6.7 and phosphate concentrations from 0 to 200 mM. The horizontal axis, representing concentration of phosphate, is reflected about the center of the plot to allow representation of both HbCO A and des(His146β)HbCO, and the vertical axis represents the ^{1}H chemical shifts of the peaks of interest as a function of the increasing phosphate concentration toward the center. Symbols: resonance G (×), resonance H (◇), resonance I (△), resonance J (+), resonance K (○), resonance L (▲), and resonance Y (◆).

Y at ~2.8 ppm, but resonances A–D and H in des(His146β)HbCO, HbCO Cowtown, and HbCO York are showing some perturbations relative to HbCO A as well.

With the careful tracking of resonances as a function of pH in chloride-free 0.1 M HEPES buffer, and very close increments of pH (44 pH values between 6.3 and 9.0 in the case of HbCO A), we have been able to identify the shift of resonance C in the spectra of des(His146β)HbCO and to discover the loss of single resonance (resonance Y) in the spectra of des(His146β)HbCO and HbCO Cowtown; in HbCO York, resonance Y is also missing, along with the loss of resonance L in this mutant. Resonance L is found to shift significantly in the case of des(His146β)HbCO in 0.1 M HEPES buffer.

With a limited pH range where the 1:1 correspondence of spectra allows for a clear identification and location of the missing resonance Y, we believe that it is necessary to further verify the location of this resonance at the lower pH values in 0.1 M HEPES buffer, where the overlapping resonances and the concomitant shift of resonance L in des(His146β)-HbCO have hindered the assignment of this resonance. Thus, we have sought to bridge the 0.1 M HEPES conditions at pH 6.8, where the resonances in the spectrum of HbCO A are best resolved in the region of interest (see Figure 2A), to conditions where the resonance assignments are unambiguous due to better 1:1 correspondence of spectra. Spectra in 0.2 M phosphate at pH 6.7 are nearly as well resolved as in Figure 1, and we have, therefore, incrementally titrated the buffer conditions between 0.1 M HEPES and 0.2 M phosphate + 0.1 M HEPES at pH 6.7 and collected the ^{1}H NMR spectra at each interval. The results of this incremental variation of buffer content for both HbCO A and des(His146β)HbCO are presented in Figure 4. The horizontal axis, representing the concentration of phosphate, is reflected about the center of the plot to allow representation of both HbCO A and des(His146β)HbCO, and the vertical axis represents the ^{1}H chemical shifts of the peaks of interest as a function of the increasing phosphate concentration toward the center. The ^{1}H NMR spectra corresponding to the conditions at the center of the plot are shown in Figure 5, depicting both HbCO A and

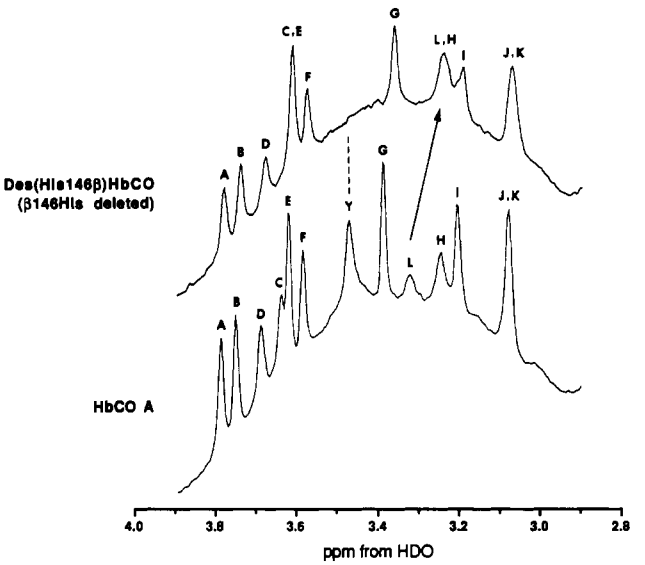

FIGURE 5: 300-MHz ^{1}H NMR spectra of 8%–10% solutions of des(His146β)HbCO and HbCO A in D_2O in 0.1 M HEPES + 0.2 M phosphate at pH 6.7 and 29 °C, corresponding to the conditions seen at the center of Figure 4.

des(His146β)HbCO in 0.2 M phosphate + 0.1 M HEPES at pH 6.7, and clearly show the loss of resonance Y and a slight shift in the position of resonance L. This bridging of buffer conditions has confirmed our identification of peak Y in chloride-free 0.1 M HEPES and its assignment to β146His.

The loss of resonance Y in the ^{1}H NMR spectra of des(His146β)HbCO can also be seen by comparison of the spectra of HbCO A and des(His146β)HbCO in 0.1 M HEPES + 640 mM NaCl at pH 6.95, as shown in Figure 6. The loss of resonance Y in the spectrum of des(His146β)HbCO at 3.34 ppm, just downfield of resonance G, is illustrated in Figure 6B. Analogous to the incremental titration of buffer conditions from 0.1 M HEPES to 0.2 M phosphate, Figure 6 illustrates the significant effects of increasing concentrations of chloride on the spectra of both HbCO A and des(His146β)HbCO in 0.1 M HEPES at pH 6.95 with chloride concentrations ranging

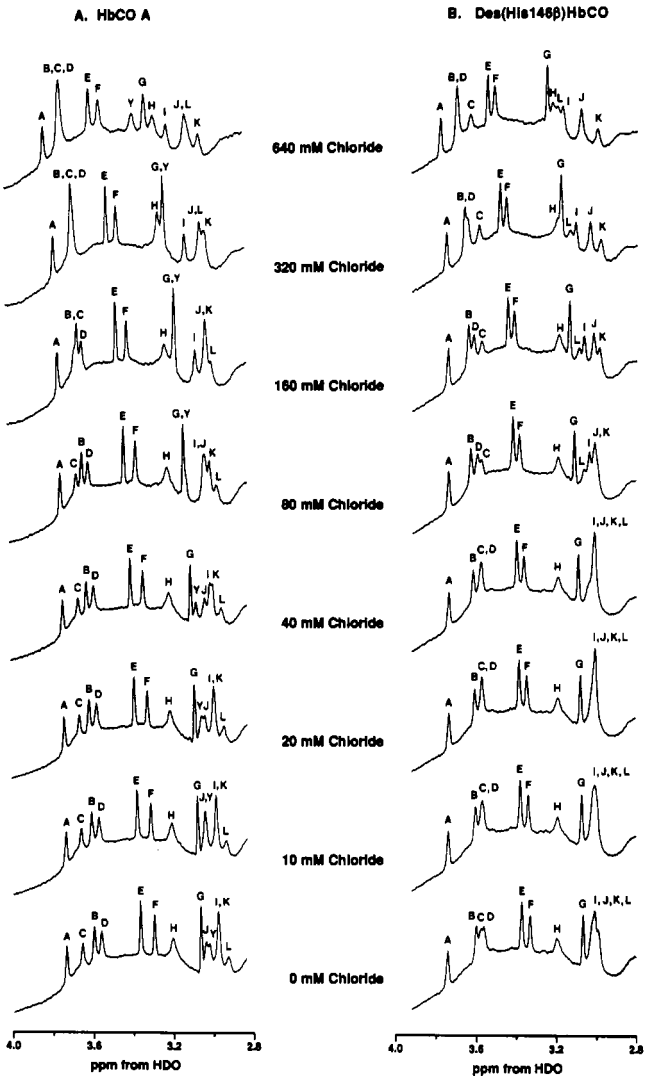

FIGURE 6: 300-MHz ^{1}H NMR spectra of 8%–10% solutions of HbCO A and des(His146β)HbCO in D_2O in 0.1 M HEPES and 0–640 mM chloride at pH 6.95 and 29 °C: (A) HbCO A and (B) des-(His146β)HbCO.

from 0 to 640 mM. A similar experiment performed at pH 7.70 is not shown. The resonances in the region from 2.9 to 3.1 ppm (G–L, Y) show significant sensitivity to chloride. As an example (Figure 6A), resonances J and Y can be resolved in the chloride-free conditions, but the two resonances overlap at 10 mM chloride. At 20 mM chloride, the resonances are once again resolved, but their relative positions have switched. Resonance J shows little sensitivity to the increasing concentration of chloride, but resonance Y is substantially affected.

Assignment of the C2 ($C_{\epsilon 1}$) Resonance of β146His in HbCO A in 0.1 M Bis-Tris. The assignment of resonance Y to β146His in chloride-free 0.1 M HEPES has prompted us to reexamine our earlier work (Russu et al., 1980, 1982) in 0.1 M Bis-Tris/Tris buffers to determine the position of resonance C in des(His146β)HbCO and resonance Y in HbCO A under these conditions. As seen in Figure 7, resonance C is clearly resolved at 3.71 ppm in the spectrum of HbCO A in 0.1 M Bis-Tris at pH 6.8, but in des(His146β)HbCO, resonance C is essentially overlapped by resonance D. The upfield shift of resonance C seen in des(His146β)HbCO in 0.1 M Bis-Tris/Tris conditions, similar to that seen in 0.1 M HEPES, explains the report by Russu et al. (1980, 1982) of the loss of this resonance. In addition, the studies of Russu et al. (1980, 1982) were performed at a lower temperature (27 °C) with a spectrometer frequency of 250 MHz using the technique of

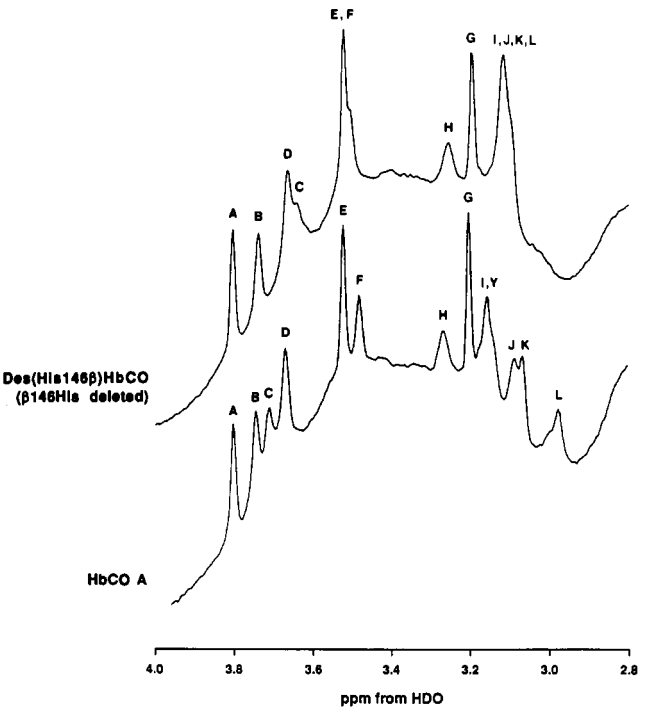

FIGURE 7: 300-MHz ^{1}H NMR spectra of 8%–10% solutions of des(His146β)HbCO and HbCO A in D_2O in 0.1 M Bis-Tris at pH 6.8 and 29 °C.

NMR correlation spectroscopy to improve the signal-to-noise ratio (Dadok & Sprecher, 1974). Resolution enhancement by use of an exponential multiplication function in the time domain was also applied, which can distort the intensity of individual resonances. With additional samples, we have compared the ^{1}H NMR spectra of HbCO A and des-(His146β)HbCO over a wider range of pH and buffer conditions and have been able to resolve resonances C and D in the spectra of des(His146β)HbCO. Again analogous to the 0.1 M HEPES situation, resonance L has shifted downfield in des(His146β)HbCO in 0.1 M Bis-Tris, relative to its position in HbCO A, and again this has complicated the resonance assignments by elimination of the 1:1 peak correspondence.

Two independent approaches to the location of resonance Y in the spectra of HbCO A in 0.1 M Bis-Tris have been taken, both employing the strategy of incremental variation of buffer conditions to conditions where the resonance assignments are known. Starting with the HbCO A spectrum in 0.1 M HEPES at pH 6.8 (see Figure 2A), buffer conditions have been incrementally varied to reach 0.1 M Bis-Tris at the same pH. This approach has narrowed the location of resonance Y to a peak at 3.16 ppm (see Figure 7), which was shown to represent two overlapping resonances, I and Y, but this approach alone could not conclusively determine which peak was due to resonance Y. The second approach took advantage of the clear loss of resonance Y in the spectra of des(His146β)HbCO in 0.2 M phosphate buffer at pH 6.35 (spectra not shown); incremental titration of samples of HbCO A and des(His146β)HbCO from 0.1 M Bis-Tris to 0.2 M phosphate at pH 6.35, analogous to the titration illustrated in Figure 4, has allowed clear assignment of resonance Y in 0.1 M Bis-Tris at pH 6.36, as is illustrated in Figure 8. Following the pH titration in 0.1 M Bis-Tris, therefore, has allowed the choice of the correct resonance at pH 6.7.

pK Values of Surface Histidyl Residues in Chloride-Free 0.1 M HEPES. The chemical shifts of each C2 ($C_{\epsilon 1}$) proton resonance have been fit to eq 1 by using a nonlinear least-squares algorithm, and the results are presented in Table I.

Table I: pK Values of Histidyl Residues in Deoxy-Hb A and HbCO A in Chloride-Free 0.1 M HEPES in D_2O at 29 °C from a Nonlinear Least-Squares Fit of Experimental Data to Equation 1

resonance	site	pK	$\delta^+ - \delta^0$	δ^+	δ^0	$V \times 10^4$
deoxy-Hb A						
1		8.08 ± 0.01	1.033 ± 0.003	3.986 ± 0.001	2.953 ± 0.003	0.3
2		7.21 ± 0.02	0.921 ± 0.017	3.956 ± 0.014	3.034 ± 0.008	1.5
3	β146	7.84 ± 0.02	0.806 ± 0.012	3.852 ± 0.004	3.047 ± 0.012	0.7
4		7.19 ± 0.02	0.752 ± 0.014	3.714 ± 0.013	2.962 ± 0.007	1.1
4′		7.40 ± 0.02	0.891 ± 0.013	3.696 ± 0.009	2.805 ± 0.009	1.2
5		7.80 ± 0.02	0.755 ± 0.015	3.606 ± 0.006	2.852 ± 0.014	1.2
6		7.12 ± 0.03	0.750 ± 0.021	3.597 ± 0.019	2.846 ± 0.008	1.9
7	β116 or β117	7.25 ± 0.02	0.743 ± 0.011	3.471 ± 0.009	2.729 ± 0.006	0.8
8	β116 or β117	7.10 ± 0.04	0.470 ± 0.017	3.317 ± 0.015	2.847 ± 0.006	1.1
9		6.99 ± 0.02	0.766 ± 0.014	3.400 ± 0.013	2.634 ± 0.004	0.5
10	β2	–[a]	–	–	–	–
HbCO A						
A		7.77 ± 0.01	0.946 ± 0.004	3.863 ± 0.002	2.917 ± 0.004	0.7
B		7.25 ± 0.08	0.917 ± 0.005	3.904 ± 0.004	2.987 ± 0.003	1.2
C		7.75 ± 0.01	0.858 ± 0.004	3.775 ± 0.002	2.917 ± 0.004	0.7
D		7.53 ± 0.01	0.821 ± 0.006	3.740 ± 0.004	2.920 ± 0.005	1.9
E		7.16 ± 0.02	0.783 ± 0.009	3.676 ± 0.007	2.893 ± 0.005	3.0
F		6.91 ± 0.02	0.810 ± 0.010	3.718 ± 0.009	2.908 ± 0.003	1.9
G	β2	–[a]	–	–	–	–
H		–[a]	–	–	–	–
I		–[a]	–	–	–	–
J	β116 or β117	6.95 ± 0.01	0.719 ± 0.001	3.404 ± 0.001	2.685 ± 0.001	0.8
K	β116 or β117	7.06 ± 0.02	0.428 ± 0.005	3.170 ± 0.004	2.742 ± 0.002	0.7
L		7.23 ± 0.03	0.137 ± 0.002	2.977 ± 0.002	2.840 ± 0.001	0.3
Y	β146	7.38 ± 0.02	0.341 ± 0.005	3.122 ± 0.003	2.782 ± 0.003	1.1

[a] Without addition of other acids, Hb samples in chloride-free 0.1 M HEPES buffers are limited to pH ≥6.3. As a result, the δ^+ values of resonances with low pK values are poorly defined, and the calculated pK values also reflect an increased uncertainty. These values are not reported here and are not used in further comparisons or calculations.

Table II: pK Values of Histidyl Residues in Deoxy-Hb A and HbCO A in Chloride-Free 0.1 M HEPES in D_2O at 29 °C from a Nonlinear Least-Squares Fit of Experimental Data to Equation 2

resonance	site	pK	$\delta^+ - \delta^0$	δ^+	δ^0	n	$V \times 10^4$
deoxy-Hb A							
1		8.16 ± 0.04	1.153 ± 0.049	4.000 ± 0.006	2.848 ± 0.049	0.90 ± 0.04	0.3
2		7.10 ± 0.05	1.163 ± 0.060	4.129 ± 0.057	2.966 ± 0.020	0.76 ± 0.05	0.8
3	β146	8.00 ± 0.04	1.044 ± 0.049	3.909 ± 0.012	2.864 ± 0.048	0.74 ± 0.04	0.3
4		7.01 ± 0.05	1.046 ± 0.052	3.933 ± 0.050	2.887 ± 0.015	0.69 ± 0.04	0.3
4′		7.40 ± 0.02	0.877 ± 0.029	3.687 ± 0.023	2.811 ± 0.018	1.02 ± 0.06	1.3
5		8.08 ± 0.08	1.177 ± 0.089	3.710 ± 0.022	2.533 ± 0.086	0.61 ± 0.05	0.3
6		–[a]	–	–	–	–	–
7	β116 or β117	7.17 ± 0.03	0.898 ± 0.035	3.578 ± 0.032	2.680 ± 0.014	0.79 ± 0.04	0.4
8	β116 or β117	–[a]	–	–	–	–	–
9		6.93 ± 0.05	0.851 ± 0.047	3.470 ± 0.046	2.618 ± 0.009	0.90 ± 0.05	0.5
10	β2	–[a]	–	–	–	–	–
HbCO A							
A		7.81 ± 0.01	1.023 ± 0.001	3.888 ± 0.001	2.865 ± 0.001	0.88 ± 0.01	0.2
B		7.20 ± 0.01	1.028 ± 0.013	3.980 ± 0.012	2.952 ± 0.006	0.84 ± 0.04	0.7
C		7.78 ± 0.01	0.929 ± 0.006	3.799 ± 0.003	2.870 ± 0.006	0.88 ± 0.04	0.3
D		7.53 ± 0.01	0.965 ± 0.002	3.811 ± 0.001	2.846 ± 0.001	0.77 ± 0.02	0.7
E		6.84 ± 0.05	1.193 ± 0.048	3.997 ± 0.047	2.805 ± 0.010	0.60 ± 0.03	0.8
F		–[a]	–	–	–	–	–
G	β2	–[a]	–	–	–	–	–
H		–[a]	–	–	–	–	–
I		–[a]	–	–	–	–	–
J	β116 or β117	6.85 ± 0.03	0.810 ± 0.023	3.482 ± 0.023	2.671 ± 0.004	0.87 ± 0.03	0.7
K	β116 or β117	6.87 ± 0.05	0.551 ± 0.023	3.271 ± 0.023	2.720 ± 0.005	0.74 ± 0.02	0.4
L		6.74 ± 0.2	0.254 ± 0.034	3.068 ± 0.033	2.814 ± 0.007	0.48 ± 0.07	0.1
Y	β146	7.19 ± 0.05	0.560 ± 0.028	3.261 ± 0.025	2.701 ± 0.012	0.50 ± 0.04	0.3

[a] Without addition of other acids, Hb samples in chloride-free 0.1 M HEPES buffers are limited to pH ≥6.3. As a result, the δ^+ values of resonances with low pK values are poorly defined, and the calculated pK values also reflect an increased uncertainty. These values are not reported here and are not used in further comparisons or calculations.

Since the pH of Hb samples in chloride-free 0.1 M HEPES is limited to pH ≥6.3 without addition of other acids, for several resonances having low pK values, the δ^+ (the chemical shift of the protonated form) is not well-defined, and this indicates that experimental results were insufficient to accurately define the titration curve. This is reflected in increased uncertainties for the calculated δ^+ and pK values, and therefore, these values are not reported in the tables (see, for example, resonances 10, G, H, and I in Table I).

Deviations from the protonation equilibrium as predicted by the Henderson–Hasselbalch equation were determined by the fit of the data to eq 2, which includes the titration coefficient, n. The results of this analysis are given in Table II, with incomplete titrations listed as in Table I.

Since the NMR spectra of the carboxyl-terminal variants in chloride-free 0.1 M HEPES have been collected at a large

Table III: pK Values of Histidyl Residues in HbCO A, des(His146β)HbCO, HbCO Cowtown (β146His → Leu), and HbCO York (B146His → Pro) in Chloride-Free 0.1 M HEPES in D_2O at 29 °C and in HbCO A in 0.1 M Bis-Tris in D_2O at 29 °C

		0.1 M HEPES				0.1 M Bis-Tris
resonance	site	HbCO A (N = 44)[a]	des(His146β)HbCO (N = 34)	HbCO Cowtown (N = 27)	HbCO York (N = 21)	HbCO A (N = 20)
A		7.77 ± 0.01	7.80 ± 0.01	7.78 ± 0.01	7.80 ± 0.02	7.84 ± 0.02
B		7.25 ± 0.09	7.27 ± 0.01	7.25 ± 0.01	7.30 ± 0.02	7.30 ± 0.01
C		7.75 ± 0.01	7.66 ± 0.01	7.78 ± 0.01	7.74 ± 0.02	7.80 ± 0.02
D		7.53 ± 0.01	7.53 ± 0.01	7.54 ± 0.02	7.56 ± 0.02	7.53 ± 0.02
E		7.16 ± 0.02	7.18 ± 0.02	7.12 ± 0.03	7.22 ± 0.02	7.09 ± 0.02
F		6.91 ± 0.02	6.99 ± 0.01	6.88 ± 0.02	7.03 ± 0.01	6.94 ± 0.01
G	β2	–[b]	–	–	–	6.49 ± 0.01
H		–[b]	–	–	–	6.11 ± 0.01
I		–[b]	–	–	–	6.24 ± 0.02
J	β116 or β117	6.95 ± 0.01	6.97 ± 0.01	6.89 ± 0.01	7.00 ± 0.03	6.86 ± 0.03
K	β116 or β117	7.06 ± 0.02	7.10 ± 0.01	6.93 ± 0.03	7.12 ± 0.03	6.64 ± 0.04
L		7.23 ± 0.03	6.76 ± 0.04	6.46 ± 0.08	broadens[c]	6.25 ± 0.05
Y	β146	7.38 ± 0.02	–	–	–	6.63 ± 0.03

[a] N represents the number of pH values at which chemical shift data were taken. [b] Values are not reported for those resonances where calculated δ^+ values and pK values have large uncertainties. These values are not used in this comparison. See footnote a in Table I for details. [c] This resonance broadens beyond detection in HbCO York in 0.1 M HEPES.

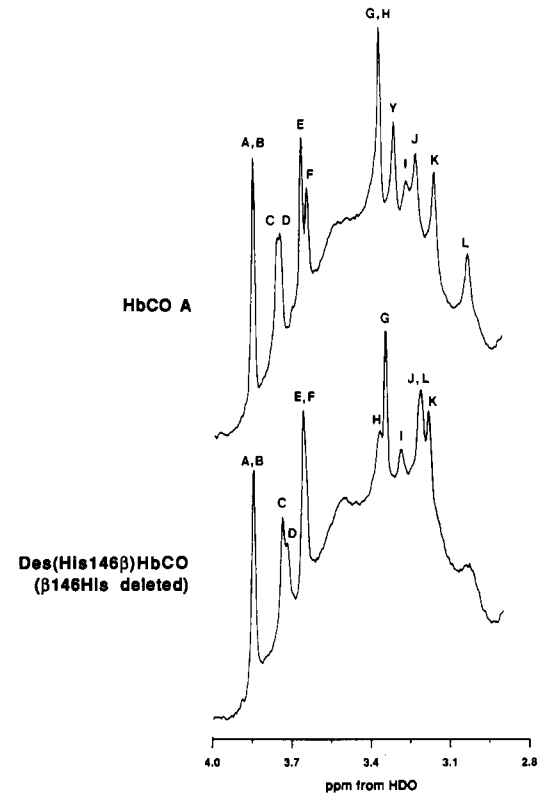

FIGURE 8: 300-MHz ^1H NMR spectra of 8%–10% solutions of HbCO A and des(His146β)HbCO in D_2O in 0.1 M Bis-Tris at pH 6.36 and 29 °C.

number of pH values in order to conclusively assign the proton resonance of β146His in the CO form of Hb, it is also possible to calculate the pK values of the observed histidyl residues in des(His146β)HbCO, HbCO Cowtown, and HbCO York. Table III summarizes these pK values, obtained by fitting of the data to eq 1. Also included in Table III are the pK results obtained for HbCO A in 0.1 M Bis-Tris, with resonance assignments revised from those previously published (Russu et al., 1980, 1982, 1990; Ho & Russu, 1987) by the assignment of resonance Y to β146His as described above. A preliminary set of pK values in 0.1 M Bis-Tris has been published by Busch and Ho (1990) and is updated here.

Oxygen Dissociation and the Bohr Effect of Hb A in Chloride-Free 0.1 M HEPES. Oxygen dissociation data, given

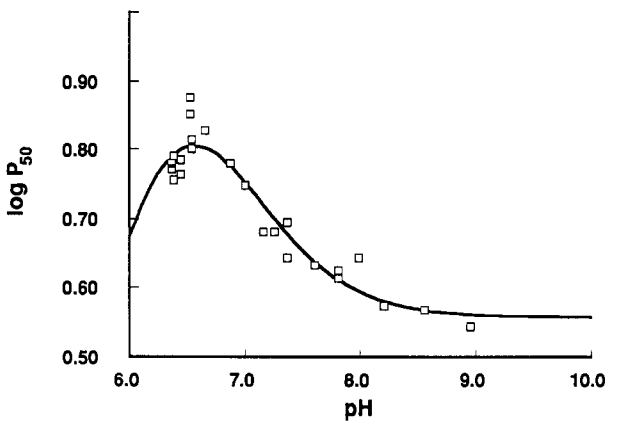

FIGURE 9: Oxygen dissociation data for 8%–10% Hb A in D_2O in 0.1 M HEPES at 29 °C presented as log P_{50} as a function of pH. The solid line represents the nonlinear least-squares fit of the data to eq 3.

as log P_{50}, are shown in Figure 9 as a function of pH. The log P_{50} data have been fit to the linkage equation as given by Wyman (1948):

$$\log P_{50} = \text{constant} + \log \frac{([H^+] + K_1')([H^+] + K_2')}{([H^+] + K_1'')([H^+] + K_2'')} \quad (3)$$

where K_1 and K_2 are the ionization constants of two hypothetical oxygen-linked groups and the single prime refers to deoxy-Hb and the double prime to HbCO. The log P_{50} curve represents the nonlinear least-squares fit of the data to eq 3 (resulting in pK_1' = 7.15, pK_1'' = 6.40, pK_2' = 4.20, and pK_2'' = 6.37). Cooperativity values calculated from the individual oxygen dissociation curves range from ~1.7 to 3.1 over the entire pH range studied, indicating that the cooperativity of the oxygenation of Hb A is maintained under these conditions.

By use of the ionization constants from the linkage equation (eq 3), the Bohr effect can be calculated as the number of protons released upon oxygenation, ΔH^+, as a function of pH from the equation:

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

The Bohr effect of Hb A in chloride-free 0.1 M HEPES at

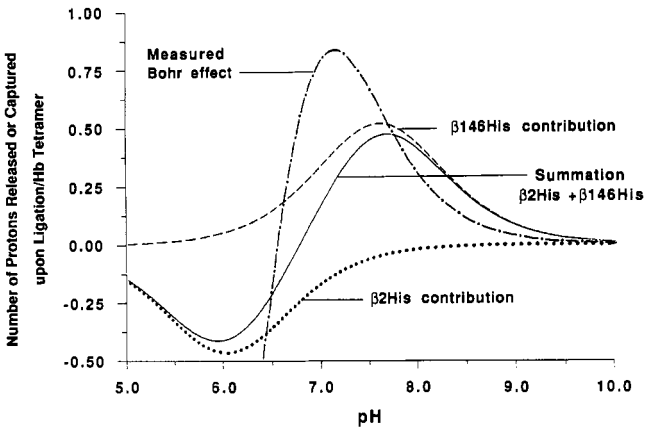

FIGURE 10: Alkaline Bohr effect of Hb A in D_2O in 0.1 M HEPES at 29 °C, reconstructed from eq 4 by substitution of the parameters resulting from the nonlinear least-squares fit of the oxygen dissociation data to eq 3, and the contributions of β146His and β2His as calculated from the pK values determined by ^{1}H NMR spectroscopy. The summation of the β146His and β2His contributions is also shown. The pK values for β2His are taken from Russu et al. (1989). Curve symbols: estimated Bohr effect (–·–), β146His contribution (– – –), summation of β146His and β2His (—), and β2His contribution (···).

29 °C as estimated by this method is shown in Figure 10.

Discussion

Assignment of the Histidyl C2 ($C_{\epsilon 1}$) Proton Resonance Y in HbCO A. The experimental evidence upon which our assignment of resonance Y to β146His is based begins with the benchmark assignment of resonance Y in 0.2 M phosphate conditions, as illustrated in Figures 1 and 5. Consistent with this assignment, the ^{1}H NMR spectrum of des(His146β)-HbCO relative to that of HbCO A in 0.1 M HEPES + 640 mM Cl^- at pH 6.95 also shows the loss of resonance Y, seen immediately downfield of resonance G (Figure 6). The incremental addition of chloride to samples of both HbCO A and des(His146β)HbCO in 0.1 M HEPES at pH 6.95 (Figure 6) and 7.70 (results not shown) demonstrated the sensitivity of many resonances in the spectra to the presence of chloride and validated our concern regarding careful control and exclusion of chloride ions in the Hb samples in 0.1 M HEPES. In contrast to the report by Perutz et al. (1985a) that "...very similar spectra were obtained on addition of 0.1 M NaCl to our HEPES buffers, or on substitution of Bis-Tris for HEPES...", examination of Figure 6 clearly shows that chloride concentrations as low as 10 mM are sufficient to perturb resonances in the spectra, and by 80 mM Cl^-, significant perturbations of the spectra have occurred (see the spectral region around 3.0 ppm in Figure 6), particularly at pH 6.95. Furthermore, although spectra taken in HEPES and in Bis-Tris are generally similar, that part of the spectrum in the aromatic region upfield of resonance H shows substantial differences that need to be taken into account in making spectral assignments (Figures 2 and 7).

Comparison of the spectra of HbCO A and des-(His146β)HbCO at close pH increments (44 and 34 pH values, respectively) over a pH range of 6.3–9.0 is also crucial to our assignment of resonance Y in 0.1 M HEPES. In addition, HbCO York and HbCO Cowtown were studied at 21 and 27 pH values, respectively, in order to accurately dissect multiple overlapping and shifting resonances. To illustrate the importance of this point, Russu and Ho (1986) and Shih et al. (1987) concluded that all resonances present in the spectrum of HbCO A are present in the spectrum of HbCO Cowtown in 0.1 M Bis-Tris or 0.2 M HEPES buffer, so-called "stripped" conditions. In Figure 1 of Shih et al. (1987), spectra of HbCO A and HbCO Cowtown in 0.2 M HEPES at pH 6.75 are compared, and the authors refer to four closely spaced resonances upfield of resonance H. Our results demonstrate that, in the case of HbCO A, six overlapping resonances exist in this region of the spectrum and only five resonances are present in the spectrum of HbCO Cowtown, the difference resulting from the loss of resonance Y (see, for example, Figure 2A). In the ^{1}H NMR spectra of each of the carboxyl-terminal Hb variants in 0.1 M HEPES, the loss of resonance Y is consistent over the pH range studied but is more clearly seen at higher pH values (Figure 3) and, therefore, demonstrates the need to examine spectra over a wide range of conditions for conclusive resonance assignments.

The resonance assignments in 0.1 M HEPES have been further confirmed by the incremental titration of buffer conditions between 0.1 M HEPES and 0.2 M phosphate + 0.1 M HEPES at pH 6.7, as illustrated in Figure 4 for HbCO A and des(His146β)HbCO. Incremental addition of chloride to samples of des(His146β)HbCO and HbCO A in 0.1 M HEPES at pH 6.95 and 7.70, with the resulting concentration of chloride ranging from 0 to 640 mM, also confirmed the loss of resonance Y in the spectra of des(His146β)HbCO (Figure 6B). The use of incremental titration of buffer conditions has demonstrated the identity of peaks in differing buffer conditions and has allowed a clear chain of evidence to relate complex spectra (e.g., in 0.1 M HEPES at pH 6.8, Figure 2A) to those where the 1:1 correspondence of peaks between the native and variant Hbs simplifies the resonance assignments (e.g., in 0.2 M phosphate, Figures 1 and 5).

The availability of three distinct carboxyl-terminal Hb variants for the resonance assignment of β146His has also proven to be significant, in that each variant has specific spectral alterations in addition to the loss of resonance Y, which could be misleading. ^{1}H NMR spectra of HbCO Cowtown show a clear downfield shift of resonance G and minor shifts of resonances K and J (see Figures 1 and 2). The spectra of HbCO York, in addition to the loss of resonance Y, have also lost resonance L due to broadening in nearly all conditions in which this mutant has been examined; resonance C has also shifted slightly in the spectra of HbCO York (see Figures 2B and 3), with both δ^+ and δ^0 showing downfield shifts.

Alterations in the spectra of des(His146β)HbCO are clearly seen for resonances C and L. The shift of resonance L results from a change in pK value of this residue (see Table III) as well as an alteration of the δ^+ for this residue. The resulting shift displaces the perturbed resonance into the region of overlapping resonances where resonance Y has been lost, and this circumstance complicates assignments in this region, particularly at low pH values, and has resulted in our use of incremental titration of buffers to assign this region.

The shift of resonance C in des(His146β)HbCO also results from a change in pK value (see Table III) and an altered δ^+, but the resulting upfield shift in the spectrum places the resonance coincident with resonance D from pH 6.3 to pH 7.3 in 0.1 M HEPES. This shift of resonance C in des-(His146β)HbCO also occurs in 0.1 M Bis-Tris (see Figure 7) and was misinterpreted as a loss of this resonance, which led to its assignment to β146His by Russu et al. (1980, 1982). Russu and Ho (1986) subsequently reported the finding of resonance C in the spectra of HbCO Cowtown, in concurrence with the observation of Perutz et al. (1985a,b), but the interpretation of these findings has remained in dispute. The experimental evidence presented here clearly indicates that resonance C does not represent β146His. Perutz and co-

workers (Perutz et al., 1985a,b; Shih et al., 1987) maintain that resonance C arises from β97His on the basis of their study of Hb Wood (β97His → Leu) and Hb Malmö (β97His → Gln). This assignment has been questioned due to the multiple alterations seen in the aromatic proton resonances, as well as alterations demonstrable in the α- and β-heme pockets of both these mutants in the CO form as monitored by ring-current-shifted proton resonances (Weichelman et al., 1976; Ho & Russu, 1985; Russu & Ho, 1986). Thus, the origin of resonance C is in need of further experimental verification.

pK Values of β146His and Its Contribution to the Alkaline Bohr Effect. The pK value determined for β146His by the fit of chemical shift data for resonance Y to eq 1 is 7.38 in HbCO A, and from resonance 3 the pK value is 7.84 in deoxy-Hb A, both in 0.1 M HEPES at 29 °C. When fit to eq 2, where the titration parameter n is used, the pK is 7.19 (n = 0.50) in the CO form and 8.00 (n = 0.74) in the deoxy form. The low value of n (0.50) seen for the fit of the data from the CO form to eq 2 strongly suggests that significant interactions with another ionizable group titrating over this pH range are taking place (Markley, 1975a; Russu et al., 1982, 1989, 1990).

It has not escaped our attention that the chemical shift dispersion value ($\delta^+ - \delta^0$) for resonance Y is smaller compared to other histidyl C2 ($C_{\epsilon 1}$) proton resonances in Hb (0.34 vs typical 0.9 ppm). However, the observed protonation shifts of resonances K and 8 are similar to that of resonance Y. Resonances K and J in HbCO A and resonances 7 and 8 in deoxy-Hb A have been identified as the pair of resonances corresponding to β116His and β117His (Russu et al., 1984). The close proximity of the imidazole side chains of these residues can be observed from the appropriate crystal structures (Baldwin, 1980; Fermi et al., 1984). The mutual electrostatic interactions of the two adjacent histidyl residues are reflected in the values of the titration coefficient, n; a diminution of the electrostatic interaction of these amino acid residues due to increased Coulombic screening at higher ionic strength was reported by Russu et al. (1989). The close proximity of β146His (resonance Y) and β143His can similarly be shown, suggesting that a similar interaction is possible. The close proximity of a tyrosyl residue has resulted in shielding effects of −0.45 ppm for the observed protonation shift of 48His of bovine pancreatic ribonuclease (Markley, 1975b; Markley & Ibanez, 1978). The possible shielding effect of the aromatic amino acid residues β118Phe and β145Tyr in the Hb molecule may also be reflected in the observed protonation shifts.

The contribution of β146His to the Bohr effect as a function of pH can be calculated from the difference in proton occupancy of this His residue in the CO and deoxy forms of Hb by using the pK values determined for resonance Y in HbCO A and resonance 3 in deoxy-Hb A and is shown in Figure 10 for 0.1 M HEPES. By use of pK values from the fit of the ^{1}H chemical shift data to eq 1, the maximum contribution of β146His to the Bohr effect is seen to be 0.52 H^+/Hb tetramer at pH 7.6. With pK values generated by the fit of the data to eq 2, the maximum contribution of β146His to the Bohr effect is seen at pH 7.24, where 0.60 H^+/Hb tetramer is released upon oxygenation.

The contribution of β146His to the Bohr effect has also been investigated by Shih and Perutz (1987) 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 to the alkaline Bohr effect is estimated to be 94% of the total 0.84 H^+/Hb tetramer measured in 0.1 M HEPES buffer (Cl^- free) between pH 7 and 8. They have further concluded that "In the absence of chloride, HisHC3(146)β is the only residue contributing significantly to the alkaline Bohr effect."

Although our estimate of the Bohr effect is dependent on the accuracy of our results from the Hem-O-Scan apparatus, our experimental results (Figure 10) show a maximum alkaline Bohr effect of 0.83 H^+/Hb tetramer at pH 7.15 in 0.1 M HEPES, in good agreement with the value of 0.84 H^+/Hb tetramer obtained by Shih and Perutz (1987). However, our pK measurements by ^{1}H NMR spectroscopy in chloride-free 0.1 M HEPES buffer indicate that the maximum contribution of β146His to the alkaline Bohr effect is 0.52 H^+/Hb tetramer at pH 7.6 (0.60 H^+/Hb tetramer if the pK values from eq 2 are used), substantially less than the 0.8 H^+/Hb tetramer contribution of β146His in the same conditions as estimated by Shih and Perutz (1987). At pH 7.15, where we estimate the Bohr effect of Hb A in 0.1 M HEPES reaches its maximum of 0.83 H^+/Hb tetramer, β146His contributes 0.40 H^+/Hb tetramer.

In a separate study (Russu et al., 1989), the C2 ($C_{\epsilon 1}$) proton resonances of β2His of Hb A in 0.1 M HEPES buffer have been identified, and the pK values have been determined in both HbCO A (pK = 6.24) and deoxy-Hb A (pK = 5.83). With these pK values, the participation of β2His in the Bohr effect as a function of pH can similarly be calculated, and the result of this calculation is also shown in Figure 10, along with the summation of the contributions of β146His, which releases protons upon ligation of Hb, and β2His, which captures protons upon ligation of Hb. At pH 7.15, where the estimated Bohr effect is at its maximum value of 0.83 H^+/Hb tetramer, the summation of the contributions of β146His and β2His is 0.27 H^+/Hb tetramer, suggesting that a minimum of 0.56 H^+/Hb tetramer is contributed to the Bohr effect at this pH by other sites in the Hb molecule.

By a direct comparison of the functional properties of Hb Cowtown and Hb A for the assessment of the contribution of β146His to the alkaline Bohr effect, Shih and Perutz (1987) have attributed to β146His the entirety of the observed alteration of the H^+-binding behavior; that is, no other pK changes occur in the mutant protein. Examination of the pK values determined for HbCO Cowtown (Table III) reveals that the pK values of histidyl residues corresponding to resonances K and L are found to change in HbCO Cowtown when compared to HbCO A. Further experimental verification is in order to examine the possibility that these pK changes may account in part for the difference. The results of the summation of the contributions of β146His and β2His, as measured by ^{1}H NMR spectroscopy, are inconsistent with the attribution of all of the macroscopically observed Bohr effect in the absence of chloride to β146His by Shih and Perutz (1987). These results clearly demonstrate that additional sites in the Hb molecule must participate in the Bohr effect in the absence of chloride.

Derewenda et al. (1990) have compared the crystal structure of HbCO A and HbCO Cowtown and concluded that no significant differences exist between the structures with the exception of the carboxyl-terminal residues. Perutz et al. (1984) have previously reported the same conclusion regarding the crystal structures of deoxy-Hb A and deoxy-Hb Cowtown. The structural comparisons are cited as evidence to indicate that the reduced alkaline Bohr effect of Hb Cowtown can be attributed exclusively to the loss of the imidazole group of β146His. Our experimental evidence indicates that there is an alteration of at least two histidyl pK values in the CO form

of Hb Cowtown, specifically those of resonances K and L (Table III). In addition, the downfield shift seen for resonance G (β2His) in HbCO Cowtown (see Figures 1 and 2) suggests that the pK value and/or the chemical environment of this amino acid residue have (has) been perturbed relative to HbCO A. Thus, we must conclude that the substitution of leucine for histidine in Hb Cowtown can result in perturbations of pK values at other sites in the Hb molecule, without having significant effects on the conformation of the Hb crystals as determined by X-ray crystallography.

The physical basis for these observations may involve the alteration of the electrostatic charge matrix of the protein molecule, as described by Matthew et al. (1985), or alteration of other properties of the Hb molecule. As an example, substitution studies at the amino-terminal residue of myoglobin have demonstrated a 0.3 pH unit perturbation of the pK value of the terminal α-amino group upon substitution of alanine for glycine at the amino terminus, without detectable conformational alteration of the protein molecule (Busch et al., 1985). The change of pK was attributed to an alteration of hydration in the vicinity of the amino acid residue, detected by a pH-dependent study of the dynamics of the amino-terminal residue. The deletion of β146His, or its substitution by leucine (Hb Cowtown) or proline (Hb York), could similarly perturb the hydration behavior or the electrostatic interactions in the vicinity of the change and result in perturbations of pK values in the variant Hbs.

Table III summarizes the pK values of the observed histidyl residues for HbCO A and each of the three carboxyl-terminal variants in the CO form. The obvious change in the pK value for resonance C from 7.75 in HbCO A to 7.66 in des-(His146β)HbCO results in the upfield shift of this resonance in the spectra and does not occur in either HbCO Cowtown or HbCO York and, therefore, allows us to examine the possible origins of the observed behavior. The results suggest that the loss of the imidazole side chain of β146His, as in the cases of HbCO Cowtown and HbCO York, is secondary to the influence of displacement of the terminal-carboxyl group from the removal of β146His, as occurs in the des-(His146β)Hb, in affecting the behavior of resonance C. The displacement of the terminal-carboxyl group to β145Tyr would disrupt the salt bridge between the terminal-carboxyl group of β146His and the ϵ-amino group of α40 Lys that is observed in the crystal structure of deoxy-Hb (Perutz, 1970; Baldwin, 1976). This salt bridge is not seen in the ligated (R) structure, since the α40Lys move 13 Å further away in this structure (Kilmartin et al., 1980). We do not yet have sufficient data to predict the consequences of deletion or variation of β146His in the deoxy form. Nevertheless, our results strongly support the position that alteration of the β146His residue, either by deletion or by substitution, results in demonstrable pK changes at other sites in the Hb molecule and that the use of carboxyl-terminal variants to quantitatively assess the contributions of β146His of Hb A to the Bohr effect requires careful evaluation.

The assignment of the resonances of β146His in different buffer conditions has allowed the determination of pK values as shown in Table IV, where it is clear that the pK values of β146His in HbCO A are found to be substantially affected by the presence of different anions in the solvent (Russu et al., 1989, 1990; Busch & Ho, 1990), which results in significant changes in the magnitude of the contribution of β146His to the alkaline Bohr effect (Figure 11). These experimental results do not support the prediction that the pK values of β146His in the R-state structure are largely chloride independent (Shih & Perutz, 1987).

Table IV: pK Values of β146 Histidyl Residues in Deoxy-Hb A and HbCO A at 29 °C in Different Buffer Conditions

conditions	deoxy-Hb A	HbCO A
0.1 M HEPES	7.84	7.38
0.1 M Bis-Tris[a]	7.98	6.63
0.1 M phosphate[b]	7.82	7.03
0.1 M Bis-Tris + 2,3-DPG[c]	7.99	6.82
0.2 M phosphate + 0.2 M NaCl[d]	8.0	7.1

[a] Value from deoxy-Hb A at 27 °C from Russu et al. (1990); value for HbCO A from this study. Chloride concentration varies from 5 to 60 mM under these conditions. [b] Values from Russu et al. (1989). [c] Values at 27 °C from Russu et al. (1990), 1:1 molar ratio of 2,3-DPG to Hb A. [d] Values at 30 °C from Kilmartin et al. (1973).

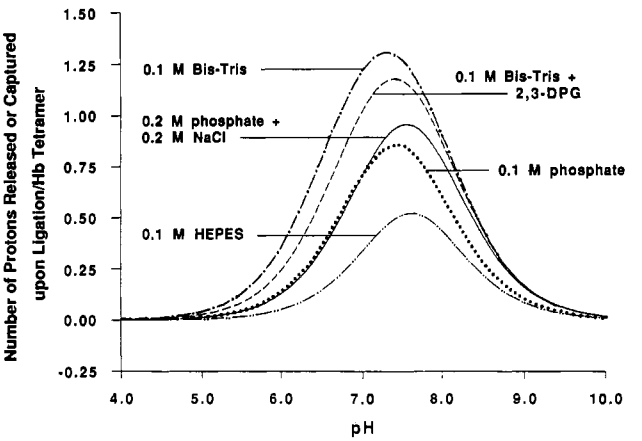

FIGURE 11: Contribution of β146His to the alkaline Bohr effect upon oxygenation of Hb A, from pK values reported in Table IV for different buffer conditions. Curve symbols: 0.1 M Bis-Tris (-·-), 0.1 M Bis-Tris + 2,3-DPG (---), 0.2 M phosphate + 0.2 M NaCl (—), 0.1 M phosphate (···), and 0.1 M HEPES (-··-).

Identification of resonance Y in the spectra of HbCO A in 0.1 M Bis-Tris conditions and determination of its pK value (Table III) allow calculation of the contribution of β146His to the Bohr effect under these conditions [the pK value of resonance 3 of deoxy-Hb A is 7.98, from Russu et al. (1990)]. At the 1.84 H^+/Hb tetramer maximum Bohr effect of Hb in 0.1 M Bis-Tris seen at pH 6.72 [results from Russu et al. (1980)], we have calculated a contribution of 1.0 H^+/Hb tetramer for β146His, slightly higher that the 0.88 estimated for these conditions by Shih and Perutz (1987). The maximum contribution of β146His in 0.1 M Bis-Tris is 1.30 H^+/Hb tetramer at pH 7.30, which exceeds the total Bohr effect seen at that pH. This serves as a reminder that the total Bohr effect as measured is the summation of all pK changes throughout the Hb molecule and that the participation of individual sites may contribute to the macroscopically observed effect but could also oppose the macroscopic behavior seen for the molecule. The variable role of a single amino acid residue has been examined for β2His in Hb A by Russu et al. (1989, 1990) and by Busch and Ho (1990). This histidyl residue has been shown to respond to experimental conditions by variation of its participation in the Bohr effect in both direction and magnitude and serves as a excellent example of the alterations in the microscopic behavior of an individual site that can result from the interactions with the solvent environment.

Conclusions

The assessment of the role of β146His in the molecular basis of the macroscopically observed alkaline Bohr effect by ^{1}H NMR spectroscopy clearly illustrates that the microscopic behavior of a specific group in Hb, which results from the

site-specific chemical and electrostatic environment of the group, is determined both by the Hb structure and by the environment in which the Hb molecule resides. The variable role of a given residue under different solvent conditions has been demonstrated for β146His, and in previous studies for β2His (Russu et al., 1989, 1990; Busch & Ho, 1990), and implies that the detailed molecular mechanisms of the Bohr effect are not unique but are adaptive to solvent conditions. These findings offer new insights in our understanding of the roles that various amino acid residues of a protein molecule may play in their interactions with substrates, ions, lipids, proteins, or nucleic acids that are necessary in carrying out its biological function.

ACKNOWLEDGMENTS

We are grateful to Dr. Irina M. Russu for extensive discussions, Dr. Shing-Shing Wu for discussions in regard to preliminary experimental results on the effect of anions on the ^{1}H NMR spectra of histidyl residues, Mr. Virgil Simplaceanu for assisting us in our NMR measurements, and Dr. William E. Brown for carrying out the amino acid analyses of our Hb samples. We thank Dr. Toshio Asakura for sending us a blood sample containing Hb York and Dr. Daniel T.-b. Shih for a blood sample containing Hb Cowtown. This paper was written during our stay (M.R.B., N.T.H., and C.H.) at the Institute of Molecular Biology, Academia Sinica, Taiwan, R.O.C. We extend our appreciation to the staff of the Institute of Molecular Biology for their hospitality as well as for the stimulating environment that they provided us during our visit.

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