Contribution of Surface Histidyl Residues in the α-Chain to the Bohr Effect of Human Normal Adult Hemoglobin: Roles of Global Electrostatic Effects†

Dazhen Philip Sun, Ming Zou, Nancy T. Ho, and Chien Ho*

Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213

Received December 19, 1996; Revised Manuscript Received April 7, 1997⊗

ABSTRACT: We have applied site-directed mutagenesis to our *Escherichia coli* hemoglobin expression plasmid and constructed five recombinant mutant hemoglobins (r Hbs): r Hb(α20His→Gln or α:H20Q); r Hb(α:H50Q); r Hb(α:H72Q); r Hb(α:H89Q); and r Hb(α:H112Q). We have constructed these r Hbs to help us assess the contribution of the surface histidyl residues in the α-chain to the alkaline Bohr effect of human normal adult hemoglobin (Hb A). In our laboratory, we have monitored the variation of proton nuclear magnetic resonances arising from the C2 protons of the histidyl residues of Hb A as a function of pH and buffer conditions. Several of these resonances have been assigned to the individual histidyl residues on the surface of the hemoglobin molecule using naturally occurring mutant hemoglobins and chemically modified hemoglobins. In the present work, we have identified the C2 proton resonances of five surface histidyl residues of the α-chain, α20, α50, α72, α89, and α112, in both the carbonmonoxy and deoxy forms, by comparing the proton nuclear magnetic resonance spectra of Hb A with those of the r Hbs. For the assignment of the C2 proton resonances of α20His and α112His, we have used combinations of mutations to compensate for the spectral perturbations resulting from the single mutations, which obscure the resonance assignment. On the basis of the new findings, in solvent containing 0.1 M chloride, the overall contributions from surface histidyl residues of both the α- and β-chain and from other previously identified alkaline Bohr groups account for approximately 75% of the observed Bohr effect at pH 7.3 (the maximum Bohr effect under the prescribed solvent conditions). Our results show that some histidyl residues contribute to the Bohr effect and some oppose the net Bohr effect. In some cases, the addition of anions can diminish or reverse the contributions of specific histidyl residues to the overall Bohr effect. Thus, the Bohr effect, a heterotropic effect, depends on the intricate arrangement and interactions of all hydrogen and anion binding sites in the hemoglobin molecule. It is an excellent example of global electrostatic effects in proteins.

In human normal adult hemoglobin (Hb A),[1] the binding of oxygen to the hemes is mediated in part by electrostatic interactions between individual amino acid residues and the various components of the solvent (i.e., heterotropic interactions). These heterotropic effectors include hydrogen ion, chloride, inorganic phosphate, and 2,3-diphosphoglycerate (2,3-DPG) (Benesch & Benesch, 1969; Antonini & Brunori, 1971). The effect of hydrogen ions on the oxygen affinity of Hb A is manifested in the Bohr effect (Bohr *et al.*, 1904). At pH values above 6.5, the oxygen affinity increases as the H^+ concentration decreases (the alkaline Bohr effect); at pH values below 6.5, the oxygen affinity increases as the H^+ concentration increases (the acid Bohr effect) (Wyman, 1948, 1964). The Bohr effect is physiologically important in facilitating the binding of oxygen to Hb A in the lung tissue and the release of oxygen into the tissues where aerobic activities produce an acidic environment. In this paper, we report the results of our recent investigation of the alkaline Bohr effect of Hb A at pH values from 6 to 8. At pH values below 6, i.e., the region for the acid Bohr effect, the Hb molecule becomes unstable, making it difficult to obtain meaningful experimental data.

The alkaline Bohr effect and other heterotropic effects can be quantitatively described using Wyman's linkage model (Wyman, 1948, 1964). In this model, H^+ bindings at individual H^+ binding sites are thermodynamically linked to the oxygen binding at the hemes through electrostatic interactions and conformational switches of the Hb molecule. At the molecular level, the local electrostatic and chemical environments of a particular proton binding site can be altered by the global conformational change induced by oxygen binding to the hemes. This can take place either by changing the local chemical environment (i.e., changes in the stereochemistry, the formation and breakage of salt bridges and hydrogen bonds, *etc.*) or by the rearrangement of charge distribution in the Hb molecule; both can take place during conformational transitions (Matthew *et al.*, 1985). As a result, upon oxygenation, differences can be observed in the H^+ dissociation constants of amino acid residues, of which the local electrostatic environments have been altered significantly.

† Preliminary results were presented at the 1996 meeting of the Biophysical Society. This work is supported by a research grant from the National Institutes of Health (HL-24525).

* Address all correspondence to Dr. Chien Ho, Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213. Telephone: (412) 268-3395. FAX: (412) 268-7083. E-mail: chienho@andrew.cmu.edu.

⊗ Abstract published in *Advance ACS Abstracts,* May 15, 1997.

[1] Abbreviations: Hb A, human normal adult hemoglobin; r Hb, recombinant hemoglobin; 2,3-DPG, 2,3-diphosphoglycerate; FPLC, fast protein liquid chromatography; NMR, nuclear magnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

S0006-2960(96)03121-2 CCC: $14.00

Table 1: Summary of Historical Assignment of C2 Proton Resonances Using Naturally Occurring Mutant Human Hbs[a]

α20	deoxy	6 ?	Craescu *et al.* (1986)	Hb Le Lamentin (α:H20Q)
	CO	E	Craescu *et al.* (1986)	Hb Le Lamentin (α:H20Q)
α72	deoxy	2	Craescu *et al.* (1986)	Hb Daneshgah−Tehran (α:H72R)
	CO	B	Craescu *et al.* (1986)	Hb Daneshgah−Tehran (α:H72R)
*β*2	deoxy	10	Russu *et al.* (1982)	Hb Deer Lodge (*β*:H2R)
	CO	G	Russu *et al.* (1982)	Hb Deer Lodge (*β*:H2R)
*β*77	deoxy	4′	Craescu *et al.* (1986)	Hb J Iran (*β*:H77D)
	CO	A	Craescu *et al.* (1986)	Hb J Iran (*β*:H77D)
*β*97	deoxy	1	Craescu *et al.* (1986)	Hb Malmö (*β*:H97Q)
	CO	C?	Perutz *et al.* (1985b)	Hb Malmö (*β*:H97Q)
			Craescu *et al.* (1986), Russu & Ho (1986)	Hb Wood (*β*:H97L)
*β*116/117	deoxy	7/8	Russu *et al.* (1984)	Hb A_2 ($\alpha_2\delta_2$)
	CO	J/K	Russu *et al.* (1984)	Hb A_2 ($\alpha_2\delta_2$)
*β*143	deoxy	9 ?	Russu *et al.* (1982)	Hb Little Rock (*β*:H143Q)
	CO	C or L ?	Russu & Ho (1986)	Hb Abruzzo (*β*:H143R)
*β*146	deoxy	3	Russu *et al.* (1980)	des-His(*β*146)-Hb
	CO	Y	Russu & Ho (1986)	des-His(*β*146)-Hb
			Busch *et al.* (1991)	Hb York (*β*:H146P)
				Hb Cowtown (*β*:H146L)
				des-His(*β*146)-Hb

[a] A question mark (?) indicates ambiguous assignments needing confirmation.

On the basis of the crystal structures of oxy- and deoxy-Hb A, Perutz and co-workers (Perutz, 1970; Perutz *et al.*, 1980, 1985a,b; Shih & Perutz, 1987; Shih *et al.*, 1993) believe that only a few amino acid residues contribute to the Bohr effect. These amino acid residues all have significant local stereochemical changes upon the global conformational changes of the Hb molecule. The suggestion that amino acid residues other than a number of "special" Bohr groups contribute to or significantly oppose the Bohr effect has been a topic of scientific debate for quite some time. Careful analysis of the titration properties of various H^+ binding sites on the Hb molecule has led to a more complete picture of the molecular basis of the Bohr effect (Busch *et al.*, 1991, and the references cited therein). It appears that the correct mechanism of the Bohr effect is the change in the H^+ affinity between the oxy and deoxy conformations of Hb A. Amino acid residues that have p*K* changes when the Hb molecule undergoes conformational transitions upon oxygenation will contribute to the total Bohr effect. Some amino acid residues that have differential affinities for anions between the deoxy-Hb and oxy-Hb states are also potential Bohr groups. Proton nuclear magnetic resonance (NMR) experiments on the C2 proton resonances of histidyl residues carried out in our laboratory have provided strong evidence for this model of differential H^+ affinity (Russu *et al.*, 1980, 1982, 1989, 1990; Russu & Ho, 1986; Ho & Russu, 1987; Busch & Ho, 1990; Busch *et al.*, 1991). In addition, efforts using electrostatic calculations of the charge matrix of the Hb molecule based on binding equilibria of individual H^+ binding sites have suggested that the Bohr effect is a thermodynamic consequence of the unique charge distributions in the two quaternary structures of the Hb molecule (Matthew *et al.*, 1981, 1982, 1985). The present paper presents additional evidence that supports this view.

The side-chain imidazoles of histidyl residues have intrinsic p*K* values in the physiological pH range. These and other groups (such as the α-amino groups of the amino-terminal residues, see below) are the likely sites for H^+ binding (or release) when the Hb molecule undergoes conformational switching at pH above 6. Extensive ^{1}H-NMR spectroscopic studies on resonances arising from the C2 protons on the imidazoles of histidyl residues have been conducted in our laboratory (Russu *et al.*, 1980, 1982, 1984, 1989, 1990; Russu & Ho, 1986; Busch & Ho, 1990; Busch *et al.*, 1991). These resonances have been labeled with numbers 1, 2, ..., 10 in the deoxy form and with the letters A, B, ..., L, and Y in the CO form (see Figures 2 and 5). The H^+ dissociation constants of these resonances under various conditions have been determined to a high degree of accuracy. There are 10 histidyl residues in the α-chain (Dickerson & Geis, 1983). Among them, five are on the surface of the Hb molecule, i.e., α20His, α50His, α72His, α89His, and α112His. Similarly, there are nine histidyl residues in the *β*-chain (Dickerson & Geis, 1983). Among them, *β*2His, *β*77His, *β*97His, *β*116His, and *β*117His are on the surface of the Hb molecule. *β*143His is partially exposed to solvent in the central cavity of the Hb molecule. *β*146His participates in salt-bridge formations in the deoxy form of Hb A and is partially exposed to solvent upon oxygenation (Perutz, 1970; Baldwin, 1980).

In the past, several assignments of the C2 proton resonances of the histidyl residues of Hb A have been made using naturally occurring mutant Hbs and chemically modified Hbs (Table 1). Craescu *et al.* (1986) assigned α20His, α72His, *β*77His, and *β*97His. However, their assignments were not definitive for two reasons. First, Craescu *et al.* (1986) investigated the C2 proton resonances of the histidyl residues of the oxy form of Hb A, while using notations which had been used to label the C2 proton resonances of the carbon-monoxy form of Hb A. Although the ^{1}H-NMR spectra of HbCO A are similar to those of HbO_2 A under identical experimental conditions, the spectra are not always identical. Russu and Ho (1986) carried out ^{1}H-NMR studies to investigate surface conformational differences between HbCO A and HbO_2 A. They found that a number of resonances exhibit changes as the fraction of HbO_2 A increases in a sample containing both HbO_2 A and HbCO A. Second, as some of our data have suggested, single amino acid substitutions of the surface histidyl residues often can introduce nonlocalized perturbations in the C2 proton resonances of the histidyl residues of Hb A (Ho & Russu, 1985). These perturbations make the tracking of individual resonances ambiguous for spectra taken at isolated pH values. Craescu *et al.* (1986) investigated the resonances of naturally occurring mutant Hbs at only a few pH values. Thus, the

assignments of Craescu *et al.* (1986) of α20 and α72 are not conclusive. In this present study, we have confirmed their assignments. There have also been controversies surrounding the assignment of resonance C. It has been assigned to β97His and β143His by different researchers (Perutz *et al.*, 1985b; Craescu *et al.*, 1986; for a discussion of this topic, see Russu & Ho, 1986, and the discussion below).

In this paper, we attempt to complete and confirm the resonance assignments of the surface histidyl residues in the α-chain of Hb A. We have used molecular biology techniques to construct recombinant mutant Hbs, each of which has a single histidine to glutamine substitution on the surface of the α-chain of Hb A. We have applied site-directed mutagenesis to our *Escherichia coli* expression plasmid (pHE2) (Shen *et al.*, 1993) and have produced recombinant (r) Hb (α20His→Gln or α:H20Q), r Hb (α:H50Q), r Hb (α:H72Q), r Hb (α:H89Q), and r Hb (α:H112Q). By comparing the C2 proton resonances of the histidyl residues of the r Hbs with those of Hb A, we have identified the C2 resonances of these five histidyl residues of Hb A. The ^{1}H-NMR spectra of r Hb(α:H20Q) and r Hb-(α:H112Q) alone are not sufficient to assign the C2 resonances of α20His and α112His of deoxy-Hb A. Double mutants, r Hb (α:H20Q/E23L) and r Hb (α:E23L/H112Q), have been constructed to compensate for the spectral perturbations resulting from the single histidine to glutamine substitution at the α20His and α112His sites. The additional data provided by these double mutants have assisted us in assigning the C2 proton resonances of α20His and α112His of Hb A.

MATERIALS AND METHODS

The *E. coli* Hb expression plasmid, pHE2, was constructed in our laboratory (Shen *et al.*, 1993). Site-directed *in vitro* mutagenesis was performed on this plasmid using a Muta-Gene kit from BIORAD (catalog no. 170-3576). The growth of *E. coli* cells and purification of r Hbs are described in Shen *et al.* (1993) and Kim *et al.* (1994, 1995, 1996).

In the last step of the chromatography purification procedure, the fast protein liquid chromatography (FPLC) Mono-S column (Pharmacia Cation Exchanger, HR16/10) yielded multiple fractions of purified r Hbs. Following the procedure developed in our laboratory (Shen *et al.*, 1993), purified Hbs were oxidized from the CO form to the ferric form (or met form) by mixing HbCO samples with a 3-fold excess concentration of potassium ferricyanide (Fisher) at room temperature, with stirring for >1 h. Ferricyanide was prepared in concentrated stock solution before being added to the HbCO samples. The ferric Hbs were separated from the excess ferricyanide using a gel filtration column (Pharmacia Sephadex G-25) equilibrated with 0.1 M sodium phosphate at pH 6.5. The purified ferric Hbs were left at room temperature overnight before they were reduced back to the CO form. The reduction of ferric Hbs back to the CO form was achieved by mixing the samples with about a 3-fold excess concentration of sodium dithionite (Fluka Chemie AG, Switzerland) with stirring and with CO blowing across the surface of the sample, i.e., in an oxygen-free environment. Within 5 min, each mixture was again applied to the G-25 gel filtration column equilibrated with 0.1 M sodium phosphate at pH 7.0. The buffer was presaturated with CO to ensure a complete conversion of Hb to HbCO.

The electrospray ionization mass spectrometric analyses were performed on a VG Quattro-BQ (Fissons Instruments, VG Biotech, Altrincham, U.K.) as described in Shen *et al.* (1993). Automated cycles of Edman degradation were performed on an Applied Biosystems gas/liquid-phase sequencer (Model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (Model 120A).

The preparation of 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES, Sigma) in D_2O buffer at different pH values (chloride free) is described in Busch *et al.* (1991). HbCO solution was prepared by blowing CO across the surface of a Hb solution. Using a Centricon centrifugal concentrator with a membrane cutoff of 30 000 Da (Centricon-30, Amicon, Inc.), the HbCO sample was then exchanged into the deuterated HEPES buffer. Deoxy-Hb solution was prepared by first blowing oxygen across the surface of the HbCO solution in a rotary evaporator, under a Sylvania 150-W flood light, and in an ice bath, for about 1 h. Then, pure nitrogen was blown across the surface of the solution for about 90 min to complete the deoxygenation process.

The NMR experiments were performed at 29 °C on a Bruker AM-300 NMR spectrometer operating at 300.15 MHz for ^{1}H. The ^{1}H-NMR spectra of Hbs in H_2O were obtained using a jump-and-return pulse sequence (Plateau & Guéron, 1982) with a delay time of 1.0 s. Those of Hb solutions in D_2O were obtained using a single 90° pulse and a delay time of 1.0 s (delay times of up to 3 s were tested; they show no marked enhancement in resolution or intensity). A total of 256−1024 transients were accumulated for each spectrum. For the study of the C2 proton resonances of the histidyl residues of Hb A, the free induction decay was processed with a −4-Hz line broadening and 0.2 Gaussian broadening to enhance resolution. Gaussian-enhanced spectra were checked with unbroadened spectra to identify spectral artifacts. Proton chemical shifts were referenced to the residual water signal (HDO) in D_2O samples and the water signal (H_2O) in H_2O samples, which occur at 4.76 ppm downfield from that of the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 29 °C.

RESULTS

Electrospray Ionization Mass Spectrometry and Edman Degradation Analyses on Purified Hbs. Electrospray ionization mass spectrometry on purified Hbs shows efficient cleavage of the amino-terminal methionine residues of the fractions of purified recombinant Hbs selected to study in this paper. The molecular masses of the selected fractions of r Hbs as determined by the mass spectrometry are consistent with the calculated masses of these r Hbs with the desired mutations and without the N-terminal methionine. The amino-terminal sequences of the selected fractions of purified r Hbs have also been checked with Edman degradation. At least 95% of either the α- or the β-chain of any of the selected fractions of r Hbs has valine as the N-terminal residue. Only a small percentage (5% at most) of any of those fractions retains the methionine on the N-terminal.

^{1}H-NMR Studies of the Structure of Recombinant Hbs. ^{1}H-NMR is an excellent tool for monitoring changes in the tertiary and quaternary structures of Hb A and its variants (Ho, 1992) and of recombinant Hbs (Shen *et al.*, 1993; Kim

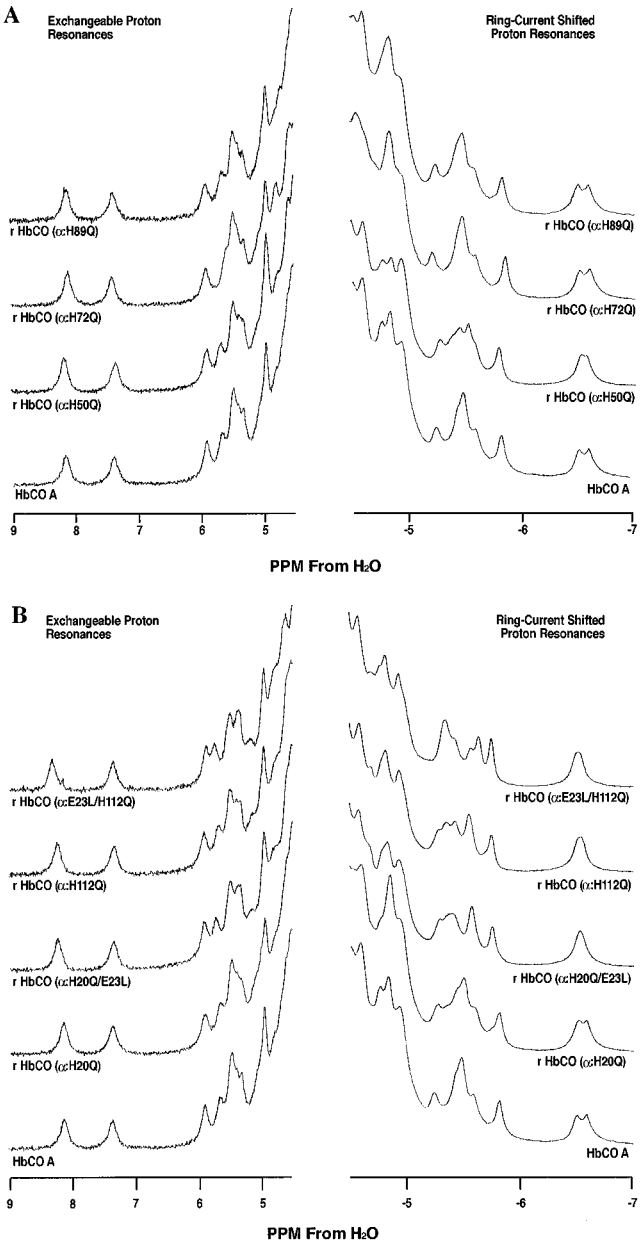

FIGURE 1: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of recombinant Hbs and Hb A in the CO form in H_2O in 0.1 M sodium phosphate at pH 7.0 and 29 °C: (A) HbCO(α:H50Q), r HbCO(α:H72Q), r HbCO(α:H89Q), and HbCO A; and (B) HbCO(α:H20Q), r HbCO(α:H20Q/E23L), r HbCO(α:H112Q), r HbCO(α:E23L/H112Q), and HbCO A.

et al., 1994, 1995, 1996; Ho *et al.*, 1996; Barrick *et al.*, 1997). Figure 1A,B show the exchangeable proton resonances and the ring-current-shifted proton resonances of the seven recombinant mutants in the CO form compared to those of HbCO A. In Figure 1A, r HbCO (α:H50Q), r HbCO (α:H72Q), and r HbCO (α:H89Q) are compared with HbCO A. The exchangeable proton resonances of the recombinant mutants that arise from the exchangeable protons in the subunit interface have no marked difference compared to those of Hb A. The subunit interfaces of these mutants are, therefore, not affected by the mutation on the surface of the α-chain of Hb A. The ring-current-shifted resonances are sensitive to the orientation or the conformation of the heme groups relative to the amino acid residues in the heme pockets, i.e., tertiary structures (Ho, 1992). The ring-current-shifted resonances of r HbCO (α:H50Q), r HbCO (α:H72Q), and r HbCO (α:H89Q) differ only slightly from those of HbCO A. Our experience has been that minor differences in the intensity and positions of ring-current-shifted resonances are common features in many recombinant Hb mutants that we have studied (Shen *et al.*, 1993, Kim *et al.*, 1994, 1995, 1996; Ho *et al.*, 1996). In other words, these changes reflect slight adjustments of the conformation of the hemes and/or the amino acid residues in the heme pockets as a result of the mutation.

Separately, in Figure 1B, we compare the exchangeable proton resonances and the ring-current-shifted proton resonances of r HbCO (α:H20Q), r HbCO (α:H20Q/E23L), r HbCO (α:H112Q), r HbCO (α:E23L/H112Q), and HbCO A. The exchangeable proton resonances of the two single mutants are identical to those of Hb A. The double mutants have slight differences in the positions of the resonances around 5.7 ppm from H_2O and in the intensity of the resonances around 5.4 ppm. The small spike occurring at 8.2 ppm in the exchangeable proton resonance region of r HbCO (α:E23L/H112Q) is a spectral artifact. The chemical shift values of the exchangeable proton resonances depend in part on the electrostatic interactions between the donor and acceptor groups. The differences observed might be a reflection of the change in the charge matrices of the double mutants compared to Hb A. The integrity of the exchangeable proton resonances (9−4 ppm from H_2O) suggests that the subunit interfaces of these mutants have no significant deviation from those of Hb A in the CO form. Except for r HbCO (α:H20Q), the ring-current-shifted resonances of the mutants in Figure 1B have some variant features compared to those of Hb A. The two resonances at around −6.5 ppm are unresolved in r HbCO (α:H20Q/E23L), r HbCO (α:H112Q), and r HbCO (α:E23L/H112Q). These two resonances come from the methyl protons of E11Val (α- and β-chain) and are particularly sensitive to the relative conformation of the heme groups (Ho, 1992). ^{1}H-NMR studies of Hb A at different pH values have shown that these two resonances are also sensitive to pH changes of the solvent (Lindstrom & Ho, 1973). The fact that they are not resolved under this buffer condition indicates that either there are slight adjustments in the relative position of the hemes or there are some changes in the electrostatic environment around the E11Val residues or both. The amino acid residue α23Glu is located on the exterior surface of the α-chain between α20His and α112His (X-ray crystal structure from the Protein Data Bank). Although not in the close vicinity of the hemes, the substitution of Leu for α23Glu has led to small adjustments in the heme pocket. Deviations in other ring-current-shifted resonances of r HbCO (α:H20Q/E23L), r HbCO (α:H112Q), and r HbCO (α:E23L/H112Q) are also observed. These changes in the ring-current-shifted resonances are common in many recombinant mutants studied in our laboratory (Shen *et al.*, 1993; Kim *et al.*, 1994, 1995, 1996; Ho *et al.*, 1996). They are more an indication of the sensitivity of the resonances to the heme pocket conformation than a signal of prominent structural deviations in the heme pocket.

Assignment of the C2 Resonances of α20His, α50His, α72His, α112His, and α89His in HbCO. The C2 proton resonances of the histidyl residues of r HbCO (α:H20Q), r HbCO (α:H50Q), r HbCO (α:H72Q), r HbCO (α:H112Q), and HbCO A in 0.1 M HEPES (chloride-free) in D_2O buffer at 29 °C are shown in Figure 2. Resonances B, D, E, and

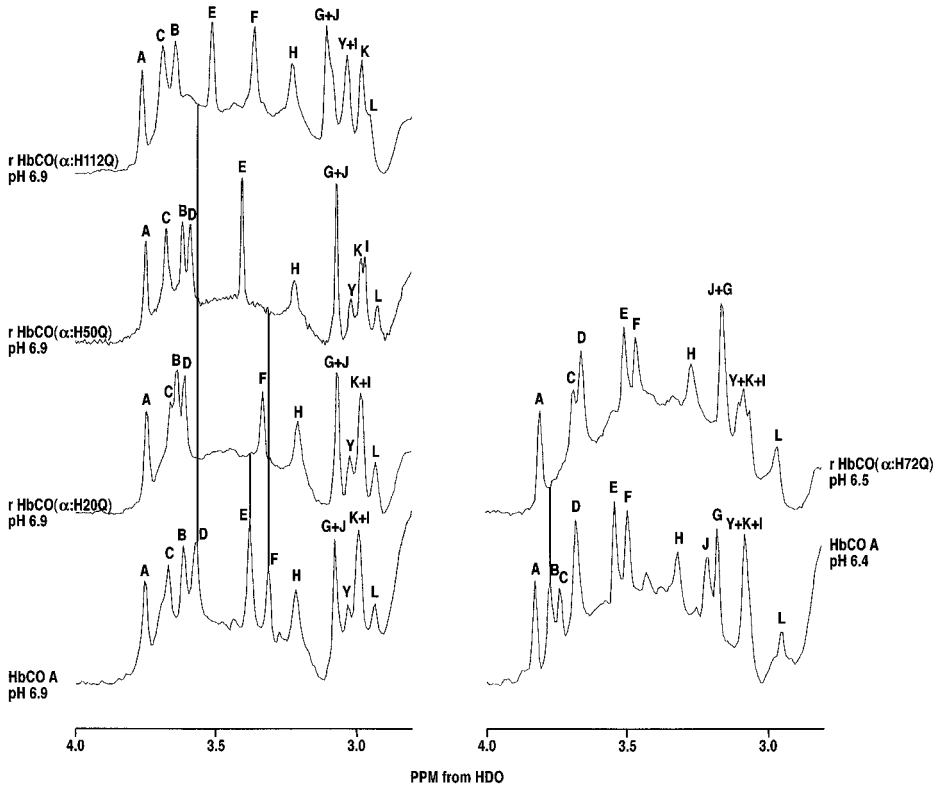

FIGURE 2: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of r HbCO(α:H20Q), r HbCO(α:H50Q), r HbCO(α:H72Q), r HbCO(α:H112Q), and HbCO A in D_2O in 0.1 M HEPES (chloride-free) and 29 °C.

F are missing in the spectra of r HbCO (α:H72Q), HbCO (α:H112Q), r HbCO (α:H20Q), and r HbCO (α:H50Q), respectively. These lead to the assignment of resonance B to α72His, D to α112His, E to α20His, and F to α50His. In Figure 2, the ^{1}H-NMR spectra are shown only at selected pH values for clarity. We have confirmed these assignments in a range of pH values from 6.5 to 8.0 (results not shown).

It is difficult to resolve resonances G, I, J, K, and Y in 0.1 M HEPES buffer. However, the chemical shift positions of resonances G, I, L, and Y are sensitive to varying concentrations of sodium chloride and sodium phosphate (Busch *et al.*, 1991). Utilizing this fact, we have studied the C2 resonances of the histidyl residues of r HbCO (α:H89Q) in HEPES buffer at varying sodium chloride and sodium phosphate concentrations. Figure 3 shows, in buffer containing various amounts of NaCl, resonances I and L of Hb A are well separated, and resonance I is definitely missing in the spectra of r HbCO (α:H89Q). In buffer containing various concentrations of sodium phosphate, resonances I and L of Hb A overlap each other. The mutation at α89 reduces the intensity of the degenerate I + L resonance (Figure 4). This gives additional evidence that the missing resonance is either I or L. These results lead to the assignment of resonance I to α89His in HbCO A.

Assignment of the C2 resonances of α50His, α72His, and α89His in Deoxy-Hb A. Figure 5 shows the C2 proton resonances of the histidyl residues of r deoxy-Hb (α:H50Q), r deoxy-Hb (α:H72Q), and deoxy-Hb A in D_2O in 0.1 M HEPES buffer (chloride-free) at 29 °C. A comparison between the spectra of the recombinant mutants and those of Hb A indicates that resonances 4 and 2 belong to α50His and α72His, respectively. Resonance 9 of r deoxy-Hb (α:H72Q) shifts slightly further downfield from HDO by less than 0.05 ppm compared to resonance 9 of deoxy-Hb A. As

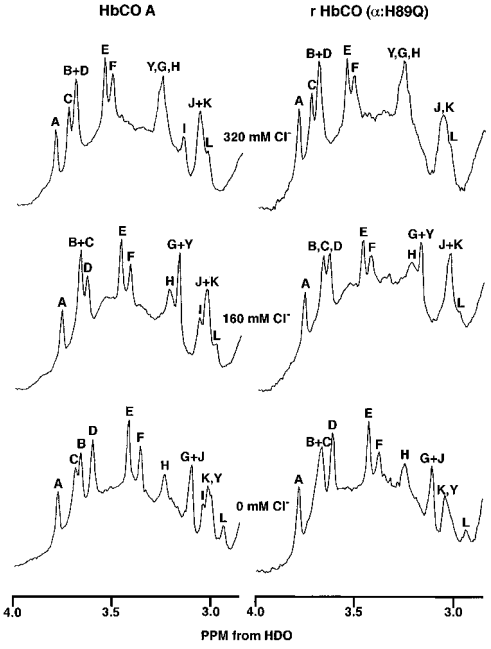

FIGURE 3: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of r HbCO(α:H89Q), and HbCO A in D_2O in 0.1 M HEPES at pH 6.9 with varying concentrations of NaCl at 29 °C.

in the CO case, these assignments have been confirmed at other pH values (results not shown).

Histidine-to-glutamine substitution at the α89 site results in one missing resonance in the spectral region where resonances 8, 9, and 10 of Hb A appear (results not shown). That is, there is one resonance missing in this region in the spectra of r deoxy-Hb (α:H89Q) in D_2O in 0.1 M HEPES. However, it is ambiguous whether the missing resonance is 9 or 10. We have carried out a careful titration experiment to measure the p*K* values of the C2 resonances of the histidyl

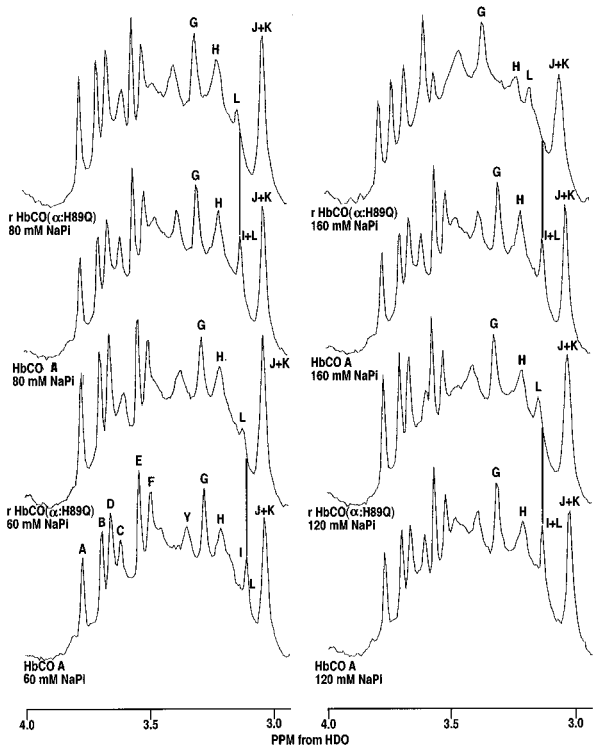

FIGURE 4: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of r HbCO(α:H89Q) and HbCO A in D_2O in 0.1 M HEPES (chloride-free) at pH 6.7 with varying concentrations of sodium phosphate at 29 °C.

residues of r deoxy-Hb (α:H89Q) in D_2O in 0.1 M HEPES. By comparing to the titration behavior of the C2 resonances of the histidyl residues of deoxy-Hb A, we have determined that the missing resonance in the ^{1}H-NMR spectra of r deoxy-Hb (α:H89Q) is resonance 9. Figure 6 shows the titration curves of resonances 7, 8, and 10 of r deoxy-Hb (α:H89Q) and of resonances 7, 8, 9, and 10 of deoxy-Hb A. Resonance 10 of r deoxy-Hb (α:H89Q) has an overall shift of about 0.1 ppm upfield from HDO compared with that of Hb A. Resonances 7 and 8 of the mutant Hb also have overall shifts of about 0.05 ppm. However, the shape of these titration curves of the mutant Hb are similar to those of Hb A. These curves were fitted to a Henderson−Hasselbach type of equation for the variation of chemical shifts as a function of pH. The p*K* values of resonances 8 and 9 of Hb A in D_2O in 0.1 M HEPES at 29 °C were determined by Busch *et al.* (1991). They are 7.10 ± 0.02 and 6.99 ± 0.02, respectively. Independently, we have estimated the p*K* values of resonance 10 of Hb A to be about 6.1 ± 0.8. The large error is due to the fact that the low pH values (<6.0) of HEPES buffer (chloride-free) could not be achieved without the addition of other acid. The p*K* values of resonances 8 and 10 of r Hb (α:H89Q) under the same buffer conditions are 7.17 ± 0.03 and 6.4 ± 0.9, respectively. These results establish the assignment of resonance 9 to α89His in deoxy-Hb A.

Assignment of the C2 Proton Resonances of α20His and α112His in the Deoxy Form. The ^{1}H-NMR spectra of r deoxy-Hb (α:H20Q), r deoxy-Hb (α:H112Q), and deoxy-Hb A are compared in Figure 7. Resonance 6 is missing in the spectra of r deoxy-Hb (α:H20Q) and of r deoxy-Hb (α:H112Q). Resonances 4, 4′, and 5 of both recombinant mutants are better resolved than those of Hb A at the pH values studied. Resonance 10 of r deoxy-Hb (α:H112Q) shifts significantly, by ∼0.15 ppm downfield from HDO, in comparison to that of deoxy-Hb A. In addition, the chemical shifts of resonances 8 and 9 in both r Hb mutants appear to be affected slightly by the mutations as well. We believe that these large spectral perturbations are the result of the single mutations. A careful examination of the local crystal structures of Hb A (the structures of oxy- and deoxy-Hb A are obtained from the Protein Data Bank) around α20His and α112His has led us to believe that, in Hb A, one of the two histidyl residues can make a H-bond with α23Glu. In either r deoxy-Hb (α:H20Q) or r deoxy-Hb (α:H112Q), the remaining histidine maintains this H-bond to α23Glu. This H-bond probably shifts resonance 6 to another location.

In an attempt to compensate for the structural perturbation introduced by the single mutation, we have sought to eliminate the H-bond from α23Glu by replacing the glutamic

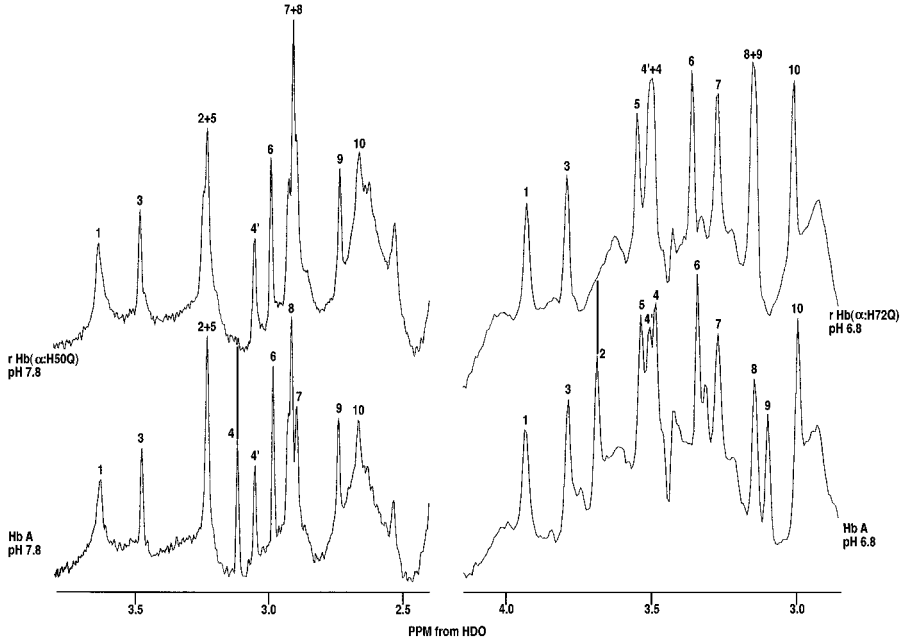

FIGURE 5: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of r deoxy-Hb(α:H50Q), r deoxy-Hb(α:H72Q), and deoxy-Hb A in D_2O in 0.1 M HEPES (chloride-free) at 29 °C.

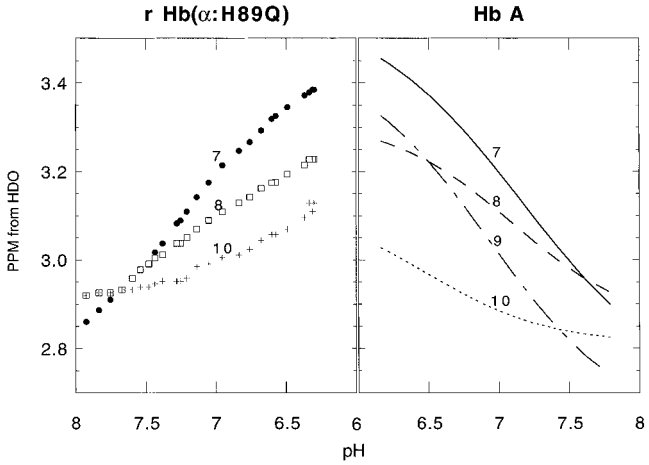

FIGURE 6: Titration of resonances 7, 8, and 10 of r deoxy-Hb(α:H89Q) and resonances 7, 8, 9, and 10 of deoxy-Hb A in D_2O in 0.1 M HEPES (chloride-free) at 29 °C.

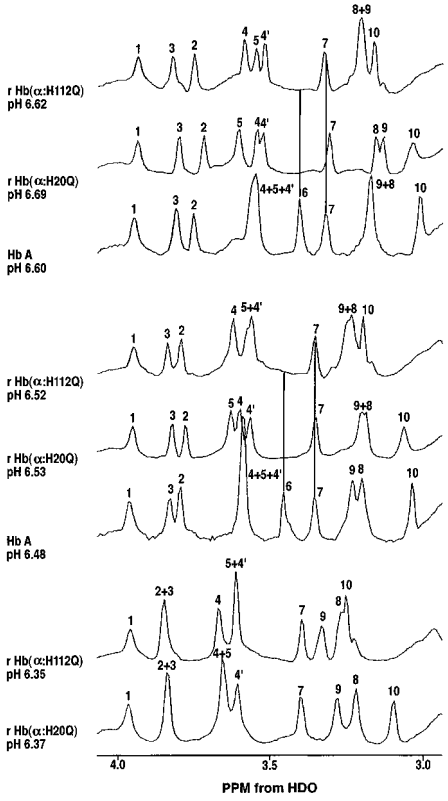

FIGURE 7: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of r deoxy-Hb(α:H20Q), r deoxy-Hb(α:H112Q), and deoxy-Hb A in D_2O in 0.1 M HEPES (chloride-free) at 29 °C.

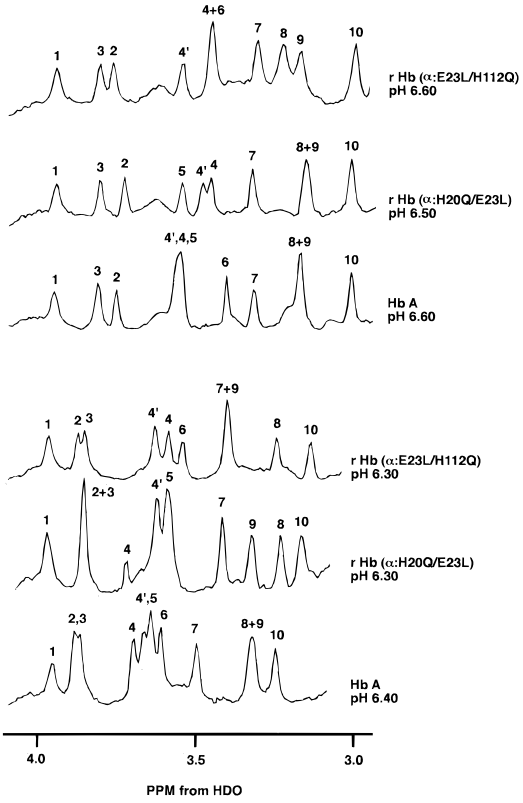

FIGURE 8: 300-MHz ^{1}H-NMR spectra of 4−8% solutions of r deoxy-Hb(α:H20Q/E23L), r deoxy-Hb(α:E23L/H112Q), and deoxy-Hb A in D_2O in 0.1 M HEPES (chloride-free) at 29 °C.

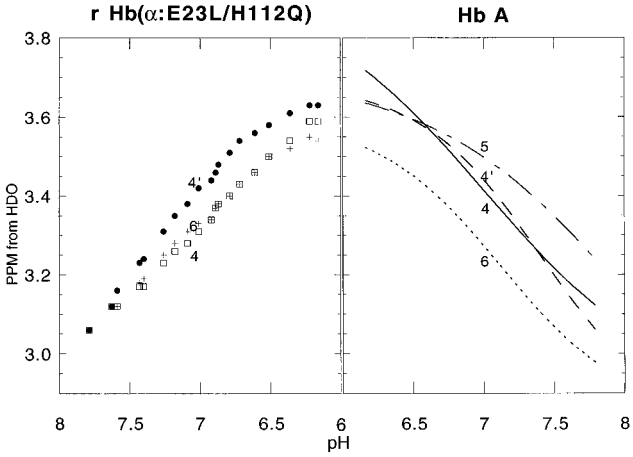

FIGURE 9: Titration of resonances 4, 4′, and 6 of r deoxy-Hb(α:E23L/H112Q) and resonances 4, 4′, 5, and 6 of deoxy-Hb A in D_2O in 0.1 M HEPES (chloride-free) at 29 °C.

acid at the α23 site with a leucine in the single mutants, thus creating two double mutants, r deoxy-Hb (α:H20Q/E23L) and r deoxy-Hb (α:E23L/H112Q). The ^{1}H-NMR spectra of r deoxy-Hb (α:H20Q/E23L) are consistently similar to those of r deoxy-Hb (α:H20Q) at various pH values, in which resonance 6 is missing (Figure 8). The ^{1}H-NMR spectra of r deoxy-Hb (α:E23L/H112Q) show better resolved resonances in the region of resonances 4, 4′, 5, and 6 (Figure 8). We can identify the resonances better by measuring the complete titration curves. Figure 9 shows titration curves of resonances 4, 4′, 5, and 6 of Hb A, and 4, 4′, and 6 of r Hb (α:E23L/H112Q). Though parallel shifts of resonances 4 and 6 of the mutant tend to obscure the assignment at any single pH value, the overall shape, and, thus, the p*K* value for each resonance enables us to identify the true missing resonance, i.e., resonance 5. These results indicate that resonance 5 should be assigned to α112His and resonance 6 to α20His.

The Bohr Effect of Hb A and Contributions from Individual Surface Histidyl Residues. The Bohr effect can be expressed as the number of H^+ ions released per Hb tetramer as the molecule goes from the deoxy conformation (T state) to the oxy conformation (R state) (Wyman, 1948, 1964). The contribution from one binding site is just the difference in the fractional saturation of this site in the R and T states. Using the H^+ dissociation constants, the contribution from the *i*th Bohr group is

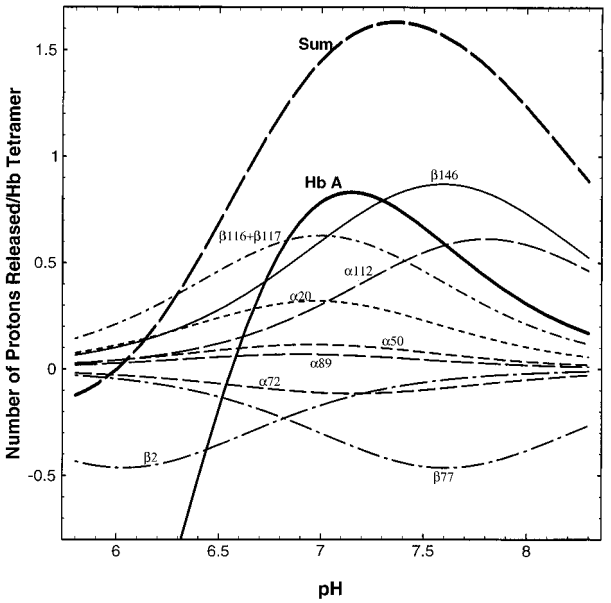

FIGURE 10: Alkaline Bohr effect of Hb A in 0.1 M HEPES (chloride-free) at 29 °C. The Bohr effect of Hb A is measured by oxygen dissociation studies. The contributions from individual histidyl residues are calculated from eq 1 using the p*K* values listed in Table 2.

$$\Delta H_i^+ = 2\left(\frac{K_i^R}{[H^+] + K_i^R} - \frac{K_i^T}{[H^+] + K_i^T}\right) \qquad (1)$$

where the *K*'s are proton dissociation constants and the superscripts denote the R and T states. The factor 2 reflects the fact that there are two identical binding sites in a Hb A tetramer. The contributions from individual histidyl residues are plotted in Figure 10 using eq 1. The sum of the contributions from individual histidyl residues is also plotted in Figure 10.

The net Bohr effect of Hb A can be measured by oxygen dissociation studies. According to Wyman (1948, 1964), the total amount of H^+ released upon oxygenation is $\Delta H^+ = -\partial \log P_{50}/\partial \mathrm{pH}$, where P_{50} is the oxygen pressure at the 50% saturation point. The measured Bohr effect of Hb A is also plotted in Figure 10. The comparison in Figure 10 reveals the contribution of the surface histidyl residues to the Bohr effect. Some histidyl residues contribute positively, while others contribute negatively. The sum of contributions from surface histidyl residues does not account for all of the measured Bohr effect of Hb A. The discrepancy can be accounted for in part by including contributions from α-amino groups of the α1Val, as well as other non-histidyl residues (see below).

DISCUSSION

On the basis of the ^{1}H-NMR data presented, we have compiled a table to summarize the assignment of the C2 proton resonances of the histidyl residues of Hb A and the p*K* values of the resonances under four different experimental conditions (Table 2). The assignments of the surface histidyl residues in the α-chain of Hb A are consistent with most previous assignments except for resonance 9. Previously, resonance 9 was tentatively assigned to β143His (Russu *et al.*, 1982). This resonance is now assigned to α89His. Russu *et al.* (1982) cautioned that the tentative assignment of resonance 9 to β143His could be incorrect from the data they had at the time. β143His is close to the carboxyl terminus of the β-chain, which is involved in salt-bridge formation in the deoxy state (Perutz, 1970). This amino acid residue is also an important component of the chloride binding site in the central cavity of the protein. The local structural features of β143His are unique among all the surface histidyl residues. The C2 resonance of β143His in the deoxy form might not be present in the region where most C2 resonances of surface histidyl residues usually appear. Our assignment of resonance 9 to α89His is confirmed with a complete titration experiment. We have calculated the p*K* values for all C2 resonances of the histidyl residues of r deoxy-Hb (α:H89Q) (results not shown). The comparison of the p*K* values as well as the shapes of the titration curves of these resonances to those of Hb A have established that resonance 9 should be assigned to the C2 proton of α89His.

The assignment of resonance C has been controversial (Russu & Ho, 1986). Craescu *et al.* (1986) and Perutz *et al.* (1985b) have assigned this resonance to β97His using Hb Malmö (β:H97Q) and Hb Wood (β:H97L). However, Russu and Ho (1986) have shown that resonance C is also missing in the ^{1}H-NMR spectra of Hb des-His (β146His deleted), Hb Barcelona (β:D94H), and Hb Abruzzo (β:

Table 2: p*K* Values of C2 Proton Resonances of Histidyl Residues in D_2O under Various Solvent Conditions, Measured by ^{1}H-NMR Spectroscopy

resonance	Bis-Tris and Cl^- [a]	phosphate[b]	Bis-Tris and DPG[c]	HEPES[d]
1, β97	7.85	7.81	7.88	8.16
2, α72	7.34	7.28	7.18	7.10
3, β146	7.80	7.82	8.12	8.00
4, α50	7.27	7.25	7.09	7.01
4′, β77	7.34	7.23	7.05	7.40
5, α112	7.56	7.57	7.71	8.08
6, α20	7.05	7.23	6.98	7.12
7, β116 or 117	7.01	6.91	7.04	7.17
8, β116 or 117	6.60	6.64	6.30	7.10
9, α89	6.69	6.88	6.80	6.93
10, β2	6.13	6.95	7.13	5.83[f]
A, β77	7.73	7.82	7.90	7.81
B, α72	7.30	7.58	7.33	7.20
C	7.71	7.51	8.01	7.78
D, α112	7.52	7.59	7.74	7.53
E, α20	7.13	7.29	7.04	6.84
F, α50	7.00	7.13	7.01	6.91
G, β2	6.46	6.81	6.68	6.24
H	6.22	5.99	6.02	6.00[f]
I, α89	6.45	6.30	5.60	6.87[g]
J, β116 or 117	6.71	6.68	6.48	6.85
K, β116 or 117	6.42	6.59	6.51	6.87
L	5.80	6.30[e]	5.80	6.74
Y, β146	—[h]	7.04	6.87	7.19

[a] Russu *et al.* (1989), 0.1 M Bis-Tris and 0.18 M NaCl in D_2O at 29 °C. [b] Russu *et al.* (1989), 0.1 M sodium phosphate in D_2O at 29 °C. [c] Russu *et al.* (1990), 0.1 M Bis-Tris, with 1:1 ratio of 2,3-DPG to Hb A, in D_2O at 27 °C. [d] Busch *et al.* (1991), 0.1 M anion free HEPES in D_2O at 29 °C. [e] Value not determined by Russu *et al.* (1989). Our study has shown that resonances L and I overlap each other between pH 5.5 and pH 8. Therefore, we estimate the p*K* of resonance L as equal to the p*K* of resonance I. [f] Value not determined by Busch *et al.* (1991) due to the unavailability of low pH values of anion-free HEPES buffer. Our study estimates the upper bound of the p*K* value of resonance 10 to be around 5.83 and of resonance H, about 6.00. [g] Value not determined by Busch *et al.* (1991). Our study has shown that resonance I and K overlap each other between pH 6 and pH 8. Therefore, we estimate the p*K* of resonance I as equal to the p*K* of resonance K. [h] Busch *et al.* (1991), resonance Y cannot be resolved for a range of pH values.

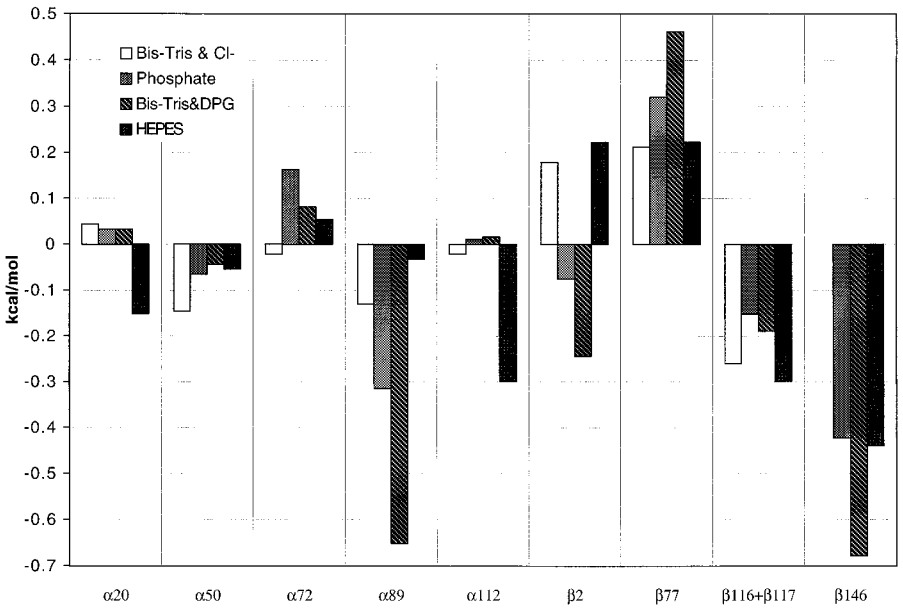

FIGURE 11: Change in free energy of proton dissociation upon oxygenation, $\Delta G = -RT \ln(K_i^R/K_i^T)$ (the constants are as defined in eq 1), of individual surface histidyl residues in 0.1 M Bis-Tris + 0.18 M chloride, 0.1 M sodium phosphate, 0.1 M Bis-Tris + 1:1 2,3-DPG, and 0.1 M HEPES (chloride-free), in D_2O at 29 °C. The datum on β146His in 0.1 M Bis-Tris + 0.18 M chloride, the white bar, is not available (see footnote *h* of Table 2).

H143R). Without further investigation, resonance C cannot be assigned. Similarly, resonance L is missing in the ^{1}H-NMR spectra of Hb Abruzzo (β:H143R), Hb des-His (β146His deleted), and Hb Barcelona (β:D94H) (Russu & Ho, 1986). The assignment of these two resonances requires further studies of the histidyl residues in the β-chain of Hb A.

Alkaline Bohr groups can be classified into three categories: (i) the imidazoles of surface histidyl residues; (ii) the terminal amino acid residues; and (iii) other proton binding sites whose p*K* values are significantly shifted into the physiological pH range due to unique structural and conformational circumstances. The contributions from individual surface histidyl residues to the total Bohr effect of Hb A in 0.1 M HEPES (chloride-free) at 29 °C are plotted in Figure 10 using eq 1 (the results for β97His and β143His are not included, due to insufficient assignment data, see above). The contributions from α20His, α72His, α112His, β77His, β116His, and β117His histidyl residues are all significant. Not only do various surface histidyl residues contribute to the Bohr effect, some amino acid residues also oppose the net Bohr effect (i.e., negative contributions). The contribution from a specific amino acid residue is based on the differential H^+ affinities between the two conformational states of the Hb molecule. Furthermore, contributions from individual amino acid residues vary as different anions are added to the buffer. The histogram in Figure 11 shows the change in proton dissociation energy upon oxygenation, $\Delta G = -RT \ln(K_i^R/K_i^T)$ (the constants are as defined in eq 1), under various buffer conditions. The effect of anions on the Bohr effect of individual histidyl residues can be observed by comparing ΔG in buffers with anions and in anion-free HEPES buffer. Figure 11 shows clearly that the contributions from individual surface histidyl residues are functions of buffer conditions. In some cases, addition of anions diminishes or reverses the contributions of specific histidyl residues. The Bohr effect, therefore, depends on the charge distributions on the Hb molecule in the different solvent media.

The second category of Bohr groups includes the α-amino group of α1Val and the imidazole of β146His. The p*K* value of the α-amino of α1Val shifts higher upon deoxygenation from 7.4 to 7.8 (Kilmartin & Rossi-Bernardi, 1973) and contributes significantly to the Bohr effect. The significance of the β2 and β146 histidyl residues to the Bohr effect of Hb A has been discussed previously (Russu & Ho, 1986; Busch & Ho, 1990; Busch et al., 1991). We have shown that the contribution of β146His varies significantly with the ion composition of the buffer (Busch *et al.*, 1991; also Figure 11). Previously, Perutz and co-workers have suggested that β146His binds and releases H^+ through the formation and breakage of salt bridges and therefore contributes to the chloride independent part of the Bohr effect (Perutz, 1970; Perutz *et al.*, 1980, 1984, 1985a,b; Shih & Perutz, 1987). However, as a form of strong electrostatic interaction, the formation of a salt bridge is influenced by the ionic strength of the buffer. Recently, Perutz *et al.* (1993, 1994) proposed a "new mechanism", in which chloride ions invade the positively charged central cavity of the Hb molecule, thereby influencing the allosteric interactions of the Hb molecule without specific chloride binding. These interactions in the cavity are clearly electrostatic and, thus, do not need specific binding sites for Cl^-. These interactions are also long range and are dependent on the buffer conditions. The results of oxygen binding studies under various concentrations of Cl^- reported by Perutz *et al.* (1994) demonstrate clearly the sensitivity of oxygen binding to the ionic strength of the buffer (anion effect). These results support our model of the Bohr effect, in which the presence of anions alters the electrostatic distributions in the Hb molecule and thereby influences the microscopic mechanism of the Bohr effect (Ho & Russu, 1987; Busch *et al.*, 1991, and the references cited therein) .

The contribution from the third type of Bohr groups is not very well understood, mainly because of a lack of a simple, direct method to determine the p*K* values of amino acid residues other than histidine. Differential titration studies conducted on mutant and chemically modified Hbs

and Hb A can be employed in some cases. These methods are generally much less direct than NMR studies, for the effect of the chemical reactions and of mutation on the electrostatic properties of proton binding sites could not be guaranteed neutral. There is, however, strong evidence to support at least one H^+ binding site in this category. The p*K* values of the side-chain γ-carboxyl group of β43Glu (6.25 for deoxy-Hb A, and 7.0 for oxy-Hb A) have been reported to differ significantly from its usual intrinsic p*K* value of about 4.8 (Rao & Acharya, 1992). β43Glu is located in the $\alpha_1\beta_2$ interface. Its interaction with a hydrophobic and charged environment might have raised its apparent H^+ affinities.

The discrepancy between the measured Bohr effect of Hb A and the sum of the contributions from the histidyl residues can be accounted for, in part, by including the α-amino groups of α1Val and β43Glu. Unfortunately, the solvent conditions under which the p*K* values of these two amino acid residues were previously determined are not strictly identical to each other or to ours. As first proposed by de Bruin and co-workers (de Bruin *et al.*, 1974; Rollema *et al.*, 1974; van Beek *et al.*, 1978, 1979; van Beek & de Bruin, 1980), the anion composition is an important regulating factor of the Bohr effect. Consequently, we cannot integrate these two residues into our calculation with confidence. With a careful comparison of various papers mentioned above, we have found that the most consistent buffer conditions are those containing 0.1−0.2 M chloride. With a degree of uncertainty in mind, we venture into the following accounting: in solvent containing 0.1 M chloride and no phosphate, the maximum Bohr effect of ~2.2 protons per tetramer occurs at pH 7.3 (Kilmartin *et al.*, 1980; Perutz *et al.*, 1980; Shih & Perutz, 1987; Rao & Acharya, 1992). According to the p*K* values reported by Rao & Acharya (1992), β43Glu contributes 0.68 protons, or 31%. According to the p*K* values reported by Kilmartin & Rossi-Bernardi (1973), α1Val contributes 0.52 protons, or 24%. Based on the p*K* values in Table 2, the sum of contributions from the surface histidyl residues excluding β97, β143, and β146 in 0.1 M Bis-Tris and 0.18 M NaCl at pH 7.3 accounts for −0.75 protons per tetramer, or −34%. β97 and β143 are not yet definitively assigned for reasons already discussed. The p*K* values of β146 in the presence of chloride ion are not available in Table 2 due to difficulties in the titration experiment (refer to footnote *h* of Table 2). Using results reported by Kilmartin *et al.* (1973), β146His in 0.2 M phosphate and 0.2 M NaCl accounts for 0.89 protons, or 40%. Consistently, in 0.1 M chloride without phosphate, Kilmartin *et al.* (1980) calculated that 40% of the Bohr effect is due to β146His. Kilmartin *et al.* (1980) also quoted the p*K* values of β143His from Ohe & Kajita (1977a,b). According to these p*K* values, in 0.1 M chloride, β143His accounts for only −0.08 protons, or −4%. This leaves about 25% of the total Bohr effect of Hb A unaccounted for in 0.1 M chloride at pH 7.3.

It is perhaps inappropriate to try to account for all of the Bohr effect by looking at individual H^+ binding sites alone. Under certain solvent conditions, many H^+ binding sites other than those discussed could contribute to the alkaline Bohr effect. Nonetheless, these results illustrate that the heterotropic interaction depends on the intricate arrangement and interactions of all H^+ binding sites in Hb. Thus, the Bohr effect is an excellent example in which the global network of electrostatic interactions plays a dominant role in this important physiological function rather than a few specific amino acid residues in the hemoglobin molecule. There is strong experimental evidence to support the idea that allosteric mechanisms for allosteric proteins, such as Hb A and aspartate transcarbamoylase, require multiple pathways for signal communication (Stevens & Lipscomb, 1992; Lipscomb, 1994; Barrick *et al.*, 1997). The present results on the Bohr effect of Hb A as described in this paper support this idea. It is encouraging that some quantitative measurements and analyses are possible. With the accurate measurement of p*K* values of histidyl residues, the refinement of models describing electrostatic interactions in macromolecules is now possible.

ACKNOWLEDGMENT

We thank Dr. Irina M. Russu and Dr. E. Ann Pratt for helpful discussions. We also extend our gratitude to Dr. Ming F. Tam for carrying out the mass spectrometric and N-terminal sequence analyses of our r Hbs.

REFERENCES

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.
Baldwin, J. M. (1980) *J. Mol. Biol. 136*, 103−128.
Barrick, D., Ho, N. T., Simplaceanu, V., Dahlquist, F. W., & Ho, C. (1997) *Nat. Struct. Biol. 4*, 78−83.
Benesch, R., & Benesch, R. E. (1969) *Nature (London) 221*, 618−622.
Bohr, C., Hasselbalch, K., & Krogh, A. (1904) *Skand. Arch. Physiol. 16*, 402−412.
Busch, M. R., & Ho, C. (1990) *Biophys. Chem. 37*, 313−322.
Busch, M. R., Mace, J. E., Ho, N. T., & Ho, C. (1991) *Biochemistry 30*, 1865−1877.
Craescu, C. T., Schaeffer, C., Mispelter, J., Garin, J., & Rosa, J. (1986) *J. Biol. Chem. 261*, 7894−7901.
de Bruin, S. H., Rollema, H. S., Janssen, L. H. M., & van Os, G. A. J. (1974) *Biochem. Biophys. Res. Commun. 58*, 210−215.
Dickerson, R. E., & Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution, and Pathology*, Benjamin/Cummings, Menlo Park.
Ho, C. (1992) *Adv. Protein Chem. 43*, 153−312.
Ho, C., & Russu, I. M. (1985) in *New Methodologies in Studies of Protein Configuration* (Wu, T. T., Ed.) pp 1−35, Van Nostrand Reinhold Company, New York.
Ho, C., & Russu, I. M. (1987) *Biochemistry 26*, 6299−6305.
Ho, C., Willis, B. F., Shen, T.-J., Ho, N. T., Sun, D. P., Tam, M. F., Suzuka, S. M., Fabry, M. E., & Nagel, R. L. (1996) *J. Mol. Biol. 263*, 475−485.
Kilmartin, J. V., & Rossi-Bernardi, L. (1973) *Physiol. Rev. 53*, 836−890.
Kilmartin, J. V., Breen, J. J., Roberts, G. C. K., & Ho, C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1246−1249.
Kilmartin, J. V., Fogg, J. H., & Perutz, M. F. (1980) *Biochemistry 19*, 3189−3193.
Kim, H.-W., Shen, T.-J., Sun, D. P., Ho, N. T., Madrid, M., Tam, M. F., Zou, M., Cottam, P. F., & Ho, C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11547−11551.
Kim, H.-W., Shen, T.-J., Sun, D. P., Ho, N. T., Madrid, M., & Ho, C. (1995) *J. Mol. Biol. 248*, 867−882.
Kim, H.-W., Shen, T.-J., Ho, N. T., Zou, M., Tam, M. F., & Ho, C. (1996) *Biochemistry 35*, 6620−6627.
Lindstrom, T. R., & Ho, C. (1973) *Biochemistry 12*, 134−139.
Lipscomb, W. N. (1994) *Adv. Enzymol. 68*, 67−151.
Matthew, J. B., Friend, S. H., & Gurd, F. R. N. (1981) *Biochemistry 20*, 571−580.
Matthew, J. B., Friend, S. H., and Gurd, F. R. N. (1982) in *Hemoglobin and Oxygen Binding* (Ho., C., Eaton, W. A., Collman, J. P., Gibson, Q. H., Leigh, J. S., Jr., Margoliash, E., Moffat, K., & Scheidt, W. R., Eds.) pp 231−241, Elsevier North-Holland, New York.

Matthew, J. B., Gurd, F. R. N., Garcia-Moreno, E. B., Flanagan, M. A., March, K. L., & Shire, S. J. (1985) *CRC Crit. Rev. Biochem. 18*, 91−197.
Ohe, M., & Kajita, A. (1977a) *J. Biochem. (Tokyo) 81*, 431−434.
Ohe, M., & Kajita, A. (1977b) *J. Biochem. (Tokyo) 82*, 839−845.
Perutz, M. F. (1970) *Nature 228*, 726−739.
Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J. G., & Rollema, H. S. (1980), *J. Mol. Biol. 138*, 649−670.
Perutz, M. F., Fermi, G., & Shih, D. T.-b. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4781−4784.
Perutz, M. F., Gronenborn, A. M., Clore, G. M., Shih, D. T.-b., & Craescu, C. T. (1985a) *J. Mol. Biol. 186*, 471−473.
Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H., & Shih, D. T.-b. (1985b) *J. Mol. Biol. 183*, 491−498.
Perutz, M. F., Fermi, G., Poyart, C., Pagnier, J., & Kister, J. (1993) *J. Mol. Biol. 233*, 536−545.
Perutz, M. F., Shih, D. T.-b., & Williamson, D. (1994) *J. Mol. Biol. 239*, 555−560.
Plateau, P., & Guéron, M. (1982) *J. Am. Chem. Soc. 104*, 7310−7311.
Rao, M. J., & Acharya, A. S. (1992) *Biochemistry 31*, 7231−7236.
Rollema, H. S., de Bruin, S. H., Janssen, L. H. M., & van Os, G. A. J. (1974) *J. Biol. Chem. 250*, 1333.
Russu, I. M., & Ho, C. (1986) *Biochemistry 25*, 1706−1716.
Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043−1052.
Russu, I. M., Ho, N. T., & Ho, C. (1982) *Biochemistry 21*, 5031−5043.
Russu, I. M., Lin, A. K.-L. C., Ferro-Dosch, S., & Ho, C. (1984) *Biochim. Biophys. Acta 785*, 123−131.
Russu, I. M., Wu, S.-S., Ho, N. T., Kellogg, G. W., & Ho, C. (1989) *Biochemistry 28*, 5298−5306.
Russu, I. M., Wu, S.-S., Bupp, K. A., Ho, N. T., & Ho, C. (1990) *Biochemistry 29*, 3785−3792.
Shen, T.-J., Ho, N. T., Simplaceanu, V., Zou, M., Green, B. N., Tam, M. F., & Ho, C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8108−8112.
Shih, D. T.-b., & Perutz, M. F. (1987) *J. Mol. Biol. 195*, 419−422.
Shih, D. T.-b., Luisi, Ben F., Miyazaki, G., Perutz, M. F., & Nagai, K. (1993) *J. Mol. Biol. 230*, 1291−1296.
Stevens, R. C., & Lipscomb, W. N. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5281−5285.
Van Beek, G. G. M., & de Bruin, S. H. (1980) *Eur. J. Biochem. 105*, 353−360.
Van Beek, G. G. M., Zuiderweg, E. R. P., & de Bruin, S. H. (1978) *Eur. J. Biochem. 92*, 309−316.
Van Beek, G. G. M., Zuiderweg, E. R. P., & de Bruin, S. H. (1979) *Eur. J. Biochem. 99*, 379−383.
Wyman, J. (1948) *Adv. Protein Chem. 4*, 407−531.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223−286.

BI963121D