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Assessment of Roles of β146-Histidyl and Other Histidyl Residues in the Bohr Effect of Human Normal Adult Hemoglobin†

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ABSTRACT: The contribution of the carboxyl-terminal histidines of the β chains, β146(HC3), to the alkaline Bohr effect of human normal adult hemoglobin has been shown by this laboratory to depend upon the solvent composition. Using high-resolution proton nuclear magnetic resonance spectroscopy, we have found that the pK_a value of the β146-histidine is 8.0 in the deoxy form, while in the carbonmonoxy form it ranges from 7.1 to 7.85 depending upon the concentration of inorganic phosphate and chloride ions present. These conclusions have been questioned by Perutz and co-workers on the basis of biochemical, structural, and proton nuclear magnetic resonance studies of mutant and enzymatically or chemically modified hemoglobins [Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J., & Rollema, H. S. (1980) *J. Mol. Biol. 138*, 649-670; Kilmartin, J. V., Fogg, J. H., & Perutz, M. F. (1980) *Biochemistry 19*, 3189-3193; Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H., & Shih, D. T.-b. (1985) *J. Mol. Biol. 183*, 491-498]. In this work, we use proton nuclear magnetic resonance spectroscopy to assess the effects of structural modifications on the histidyl residues and on the overall conformation of the hemoglobin molecule in solution. The structural perturbations investigated all occur within the tertiary domains around the carboxyl-terminal region of the β chain as follows: Hb Cowtown (β146His → Leu); Hb Wood (β97His → Leu); Hb Malmö (β97His → Gln); Hb Abruzzo (β143His → Arg). Our results demonstrate that the conformational effects of single-site structural modifications upon the conformation and dynamics of hemoglobin depend strongly on their location in the three-dimensional structure of the protein molecule and also on their chemical nature. Furthermore, in normal hemoglobin, the spectral properties of several surface histidyl residues are found to depend, in the ligated state, upon the nature of the ligand. Our present findings do not support the recent spectral assignments proposed by Perutz et al. (1985) for the proton resonances of the β146- and β97-histidines and their suggestion that the enzymatic removal of the carboxyl-terminal β146-histidyl residues induces a conformational equilibrium for the β97-histidines in the des-β146His hemoglobin molecule in the carbonmonoxy form.

A main functional property of the hemoglobin (Hb)[1] molecule is to release hydrogen ions upon oxygenation at pH above 6.0. This property, known as the alkaline Bohr effect, results from the conformational changes induced in the Hb molecule by ligand binding. These structural alterations affect the ionization properties of a number of amino acid residues such that they release or capture hydrogen ions upon ligation. The identification of those amino acid residues whose pK_a values are altered in going from deoxy- to oxy- (or carbon-

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; Hb des-His, Hb A with the β146-histidyl residues deleted; met-Hb, methemoglobin; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; NOE, nuclear Overhauser effect.

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monoxy-) Hb has been the subject of intensive experimental and theoretical research during the past 15 years [Perutz, 1970; Kilmartin et al., 1973, 1980; Perutz et al., 1980; Russu et al., 1980, 1982, 1984; Matthew et al. (1985) and references cited therein].

In his stereochemical mechanism for Hb function, Perutz (1970) proposed that the carboxyl-terminal histidine, β146-(HC3), could be one of the residues contributing to the alkaline Bohr effect of Hb. According to the X-ray diffraction results, the imidazole of β146His of deoxy-Hb A forms an intrachain salt bridge with the carboxyl group of β94(FG1)-aspartate (Perutz, 1970; Fermi, 1975). This local electrostatic interaction would greatly increase the pK_a value of the β146His residue. In ligated Hb, β146His is found to move ~10 Å away from the β94Asp (Perutz, 1970; Baldwin, 1980; Shaanan, 1983), and thus, the pK_a value of this His residue should revert to a lower value.

The contribution of β146His to the alkaline Bohr effect has been first investigated by functional studies of Hb molecules in which this histidyl residue is altered either by enzymatic or by chemical modification or replaced by mutation. For all these modified Hbs, the alkaline Bohr effect is reduced compared to that of human normal adult hemoglobin (Hb A) [for example, see Perutz et al. (1980) and Kilmartin et al. (1980)]. The amount of this reduction, however, depends on the nature of the alteration or of the replacing amino acid residue as well as on the experimental conditions. For example, the β146His → Asp substitution in Hb Hiroshima and the β146His → Pro substitution in Hb York result in reductions of the alkaline Bohr effect by about 50% (Imai, 1968; Bare et al., 1976). On the other hand, the β146His → Arg substitution in Hb Cochin-Port Royal reduces the Bohr effect by only ~25% (Wajcman et al., 1975). Similarly, the substitution of His for the β94Asp (the partner residue of β146His in the salt bridge) in Hb Barcelona lowers the alkaline Bohr effect by only 20–30% (Wajcman et al., 1982; Phillips et al., 1983). When the β146His residues are simply removed by carboxypeptidase B digestion as in Hb des-His, the corresponding reduction in the alkaline Bohr effect varies from 40 to 60% depending upon the concentration of chloride ions in the solvent (Kilmartin & Wootton, 1970; Kilmartin et al., 1980). Analysis of the pH dependence of the number of hydrogen ions released by Hb des-His upon oxygenation has shown that the reduction in the Bohr effect of this modified Hb cannot be ascribed to the simple removal of a Bohr group, namely, β146His (Saroff, 1972; Matsukawa et al., 1984). The results from Hbs containing single-site structural perturbations have suggested that additional mechanisms should be involved in the alterations of the Bohr effect in these modified Hbs (Russu et al., 1982; Ho & Russu, 1985).

^{1}H NMR spectroscopy is the only technique presently available that can monitor the conformation and environment of individual amino acid residues in the Hb molecule in solution [for a review, see Ho & Russu (1981)]. Among those of special interest are the 22 surface histidyl residues of Hb that can be observed individually by ^{1}H NMR spectroscopy in both deoxy and ligated forms (Russu et al., 1982). Several of these histidyl residues have been found to be sensitive to the transition from the deoxy to the carbonmonoxy or oxy state of the Hb molecule. The changes in the ionization properties of all the histidyl residues of Hb upon ligation can account for the Bohr effect observed experimentally (Russu et al., 1982). A detailed understanding of the molecular mechanism(s) of the Bohr effect requires the identification of the histidyl residues of Hb that contribute to the hydrogen ions released or captured upon ligation. This information can be readily obtained by ^{1}H NMR spectroscopy provided that the proton resonances of each histidyl residue can be identified. Given the important role proposed for the β146His residue in the Bohr effect (Perutz, 1970), considerable efforts have been devoted, during the last decade, to assigning and studying the proton resonances of this residue.

The proton resonances of β146His in deoxy-Hb A have been first identified on the basis of a comparison between the ^{1}H NMR spectra of deoxy-Hb A and deoxy-Hb des-His (Kilmartin et al., 1973; Russu et al., 1980). The ^{1}H NMR titration of these resonances indicated that, regardless of the experimental conditions, the pK_a value of β146His in deoxy-Hb A is raised to ~8.0 at 29 °C (Kilmartin et al., 1973; Russu et al., 1980). These results have supported the prediction made on the basis of the X-ray diffraction results that, in deoxy-Hb A, the β146His residue forms an intrachain salt bridge with β94Asp (Perutz, 1970).

The C2-proton resonance of β146His in HbCO A was first identified in our laboratory on the basis of a comparison between HbCO A and HbCO des-His in 0.2 M potassium phosphate plus 0.2 M NaCl in D_2O at 30 °C (Kilmartin et al., 1973). The ^{1}H NMR titration of this resonance indicated that, under these experimental conditions, the pK_a value of β146His in HbCO A is 7.1, and thus, these histidyl residues contribute ~50% (or 0.8 hydrogen ion) to the Bohr effect (Kilmartin et al., 1973). More recently, we have extended the ^{1}H NMR investigation of the histidyl residues of Hb A to a wide range of experimental conditions (Russu et al., 1980, 1982; Bupp et al., 1983; Lin et al., 1985). The rationale of our further studies is based on the large effect of anions, such as chloride and inorganic phosphates, on the alkaline Bohr effect of Hb (de Bruin et al., 1974a,b; Rollema et al., 1975; Van Beek et al., 1979). As predicted by these biochemical studies, we have found that the ^{1}H resonances of the surface histidyl residues of Hb A, including those of the β146His residues, are greatly affected by the concentrations of anions present in solution. For example, we have found that, in the presence of 0.1 M Bis-Tris buffer (with chloride ion concentrations ranging from 5 to 60 mM) at 27 °C, the pK_a value of β146His residues in HbCO A is 7.85 (Russu et al., 1980). These results indicate that, in the absence and/or at low concentrations of anions, β146His residues contribute ~15% to the Bohr effect. Hence, we have concluded that the role of the β146His residues in the Bohr effect depends on the concentration of anions present in solution and, thus, the detailed molecular mechanism of the Bohr effect is not unique (Russu et al., 1980, 1982).

The validity of our conclusions on the molecular mechanism of the Bohr effect has been questioned by Perutz and coworkers on the basis of X-ray diffraction and biochemical studies of various mutant and enzymatically or chemically modified Hbs (Kilmartin et al., 1980; Perutz et al., 1980, 1984; Phillips et al., 1983). Kilmartin et al. (1980) have reported that the β146His–β94Asp salt bridge is missing from both salt-free horse methemoglobin (met-Hb) crystals and crystals of the same Hb precipitated from ammonium sulfate. On the basis of these results, they have concluded that this salt bridge should also be absent from HbCO A regardless of the solvent composition and that the 40–60% reduction in the alkaline Bohr effect of Hb des-His is solely due to the missing β146His residues. Phillips et al. (1983) have postulated that in Hb Barcelona (β94Asp → His), in the deoxy form, a new salt bridge is formed between β146His and β90Glu. This proposal has been used to support the role of the β146His residues in

the Bohr effect and to explain the small reduction in the alkaline Bohr effect of this mutant Hb. The X-ray diffraction method has also been applied to investigate the structure of Hb Cowtown, a newly discovered mutant Hb in which the β146His residues are replaced by Leu (Shih et al., 1984; Perutz et al., 1984). The X-ray analysis, carried out only for the deoxy form of this mutant Hb, has indicated that the structural perturbations induced by the β146His → Leu substitution are centered around the C-terminal fragments of the β chains. These results have been interpreted as support for the role of β146His residues in the Bohr effect by attributing the reduced Bohr effect of Hb Cowtown simply to the loss of the β146His residues and not to conformational perturbations of the Hb molecular induced by the mutation.

More recently, Perutz et al. (1985a) have used Hb Cowtown to investigate the ^{1}H NMR resonances of the histidyl residues in ligated Hb A in "stripped" conditions (i.e., 0.1 M Bis-Tris or 0.2 M HEPES buffers, in the absence of phosphate ions and in 5–60 mM chloride ions). They have found that the histidyl C2-proton resonance previously observed by this laboratory to be missing in HbCO des-His [namely, the resonance labeled C in Russu et al. (1980)] is present in HbCO Cowtown. On the basis of this result, Perutz et al. (1985a) have questioned our assignment of resonance C to the β146His C2 proton. Instead, Perutz et al. (1985a) have assigned a different resonance to β146His (namely, the resonance labeled H in our previous publications) on the basis that this resonance was found to be missing in HbCO Cowtown. According to this assignment, the pK_a value of β146His in HbCO A in the presence of low concentrations of anions should be 6.2, and these histidyl residues should contribute 1.5 hydrogen ions to the Bohr effect at pH 7.4 (or 74 to 135% of the effect measured experimentally, depending on the exact chloride ion concentrations). In the latter investigation, Perutz et al. (1985a) have also found that resonance C is missing from the ^{1}H NMR spectra of HbCO Wood (β97His → Leu) and HbCO Malmö (β97His → Gln) and, thus, have assigned this resonance to the C2 proton of β97His. The high pK_a value predicted by this assignment for β97His (i.e., pK_a = 7.85) has been suggested to result from the dipole moment of the F helix of the Hb molecule (Perutz et al., 1985a). The absence of resonance C from the spectra of HbCO des-His was rationalized by proposing that a conformational equilibrium of the β97His residues exists in this enzymatically modified Hb (Perutz et al., 1985a).

In this paper, we report our recent ^{1}H NMR studies of Hb Cowtown (β146His → Leu) as well as other mutant Hbs containing amino acid replacements within the tertiary structures around the C-terminal regions of the β chains. Our results demonstrate that the assignments proposed by Perutz et al. (1985a) for the C2-proton resonances of β146His and β97His in HbCO A are questionable. Furthermore, our present findings emphasize the limitations of using mutant and enzymatically (or chemically) modified Hbs to understand the detailed molecular mechanism of the Bohr effect in Hb A. These limitations arise from the effects of chemical modifications or mutations upon the overall conformation of the Hb molecule in solution. Our results are relevant to the effects of single-site structural perturbations upon the conformation and the dynamics of protein molecules in solution. For a recent review on this topic, see Ho & Russu (1985).

Experimental Procedures

Materials. Hb A was prepared by the usual procedures used in our laboratory from blood samples obtained from the local Blood Bank (Lindstrom & Ho, 1972). Hb Cowtown (β146His → Leu) and Hb Barcelona (β94Asp → His) were kindly provided to us by Dr. Gary K. Ackers and Dr. Henri Wajcman, respectively. Hb Wood (β97His → Leu), Hb Malmö (β97His → Gln), and Hb Abruzzo (β143 His → Arg) were purified in our laboratory on CM-52 columns equilibrated with 10-20 mM phosphate buffer from blood samples provided to us by Dr. Virgil F. Fairbanks (Hb Malmö and Hb Wood) and Dr. John B. Maxwell (Hb Abruzzo).

The purity of all Hb samples was checked by isoelectric focusing on acrylamide gel. In the case of Hb Cowtown, we have also checked the purity of the samples by two additional techniques. The amino acid composition of the Hb Cowtown sample was determined on a Durrum D-50 amino acid analyzer. The results confirmed the presence of two additional Leu residues/Hb tetramer and the loss of two His residues/Hb tetramer. Confirmation of the Hb Cowtown sequence was also made by standard digestions of Hb A and Hb Cowtown with carboxypeptidases A and B (Sigma) in separate experiments. The digestions were run at a Hb concentration of 0.2% in 0.2 M phosphate buffer at pH 7.6, and the enzyme to protein ratio in each experiment was set at 100. Carboxypeptidase A treatment yielded only Leu and Tyr for Hb Cowtown and no free amino acids for Hb A, which is consistent with blockage of digestion at the basic amino acids. Carboxypeptidase B treatment yielded only arginine for Hb Cowtown and arginine and histidine for Hb A, which is consistent with the specificity of this enzyme for basic amino acids.

To remove organic phosphates, all Hb samples were passed through a Sephadex G-25 column equilibrated with 0.01 M Tris-HCl in 0.1 M NaCl at pH 7.6 (Berman et al., 1971). The Hb samples were exchanged with D_2O 6 times in ultrafiltration membrane cones (Centriflo, Amicon Corp.). A Hb sample at a given pH value was obtained by dialysis against a buffer of the appropriate pH. The pH values are reported throughout this paper as direct pH meter readings without correction for the deuterium isotope effect on the glass electrode, namely, pD = pH + 0.4 (Glasoe & Long, 1960). The deoxygenation of the Hb samples was carried out as described previously (Lindstrom & Ho, 1972).

Methods. All ^{1}H NMR experiments were carried out on a Bruker WH-300 NMR spectrometer operating at 300 MHz and at 29 °C. The aromatic and the ring-current-shifted proton resonances were obtained by accumulating 200 transients at a repetition rate of 0.25 s^{-1}. The signal-to-noise ratio was improved by an exponential multiplication of 1 Hz in the time domain. The ferrous hyperfine-shifted resonances were obtained by using the pulse sequence $(t_1-\pi/2-t_2-\pi-t_2-\pi/2)_n$ (Hochmann & Kellerhals, 1980), with t_1 = 1.5 s and t_2 = 0.2 s. The exchangeable proton resonances for Hb samples in H_2O were obtained by the jump-and-return method (Plateau & Gueron, 1982) at a repetition rate of 2 s^{-1}.

The proton chemical shifts are expressed as parts per million (ppm) relative to the water proton resonance, which is 4.73 ppm downfield from the proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 29 °C. The proton chemical shift scale is defined as positive in the low-field direction with respect to the water proton resonance.

Results

^{1}H NMR Studies of Deoxy-Hb Cowtown. The aromatic proton resonances of deoxy-Hb Cowtown (β146His → Leu) and deoxy-Hb A are shown in Figure 1. Two resonances (labeled 3 and 12) in the spectrum of deoxy-Hb A are missing from the corresponding spectrum of deoxy-Hb Cowtown. The same resonances have been found by this laboratory to be missing in the spectra of deoxy-Hb des-His, and on the basis

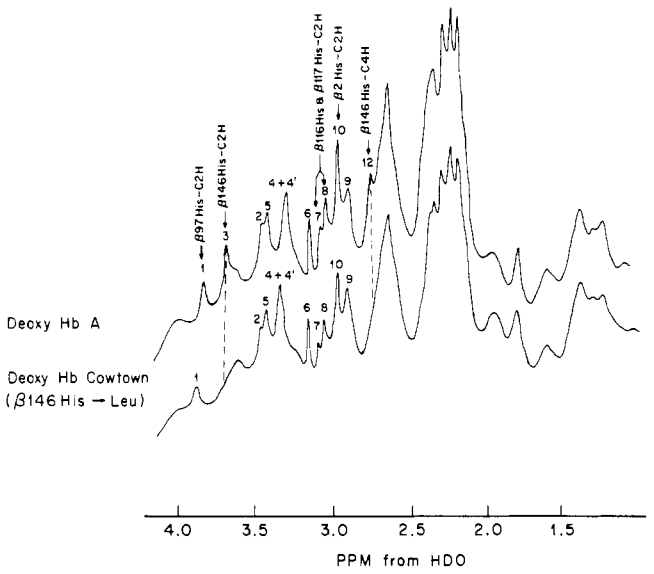

FIGURE 1: 300-MHz aromatic proton resonances of 10% deoxy-Hb A and deoxy-Hb Cowtown (β146His → Leu) in 0.1 M Bis-Tris in D_2O at pH 7.35 and 29 °C.

of these results, they have been assigned to the C2 and C4 protons of β146His in deoxy-Hb A (Kilmartin et al., 1973; Russu et al., 1980). The present ^{1}H NMR spectra of deoxy-Hb Cowtown confirm these assignments. No other significant perturbations of the aromatic proton resonances have been observed in the ^{1}H NMR spectra of deoxy-Hb Cowtown over the entire pH range investigated.

In deoxy-Hb Cowtown, the $N_\delta H$ proton resonances of the proximal histidyl residues in the α and β chains (β92His at 71.0 ppm and α87His at 58.5 ppm) as well as the ferrous hyperfine-shifted resonances of protons situated in the α and β heme pockets (6.0–20.0 ppm) have been found to be very similar to those in deoxy-Hb A (results not shown). The exchangeable proton resonances originating from the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces (6.0–10 ppm) have also been found to be the same in deoxy-Hb Cowtown and in deoxy-Hb A (results not shown). These findings indicate that, in spite of the absence of the β146His–β94Asp salt bridge, the overall conformation of the deoxy-Hb Cowtown molecule in solution is very similar to that of deoxy-Hb A [for reviews on ^{1}H NMR studies of Hb, see Ho & Russu (1981, 1985)]. These results are in accord with the X-ray diffraction analysis of Hb Cowtown crystals in the deoxy form (Perutz et al., 1984).

^{1}H NMR Studies of HbCO Cowtown. The aromatic proton resonances of HbCO Cowtown and HbCO A have been obtained in 0.2 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) or 0.1 M [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane (Bis-Tris) over the pH range from 6.0 to 9.5. No significant effects upon the aromatic proton resonances of HbCO A or HbCO Cowtown have been observed when the buffer was changed from 0.1 M Bis-Tris to 0.2 M HEPES. Representative ^{1}H NMR spectra of HbCO A and HbCO Cowtown are shown in Figure 2. Two spectral features of these two Hbs are of special interest. First, the resonance labeled C in HbCO A is present in HbCO Cowtown over the pH range investigated (for example, see Figure 2B). This finding is in agreement with the results on HbCO Cowtown reported by Perutz et al. (1985a) under the same experimental conditions. Second, the resonance labeled H in HbCO A is also present in ^{1}H NMR spectrum of HbCO Cowtown over the entire pH range investigated (for example, see Figure 2A). This result is different from that of Perutz et al. (1985a), who found that resonance H is missing in

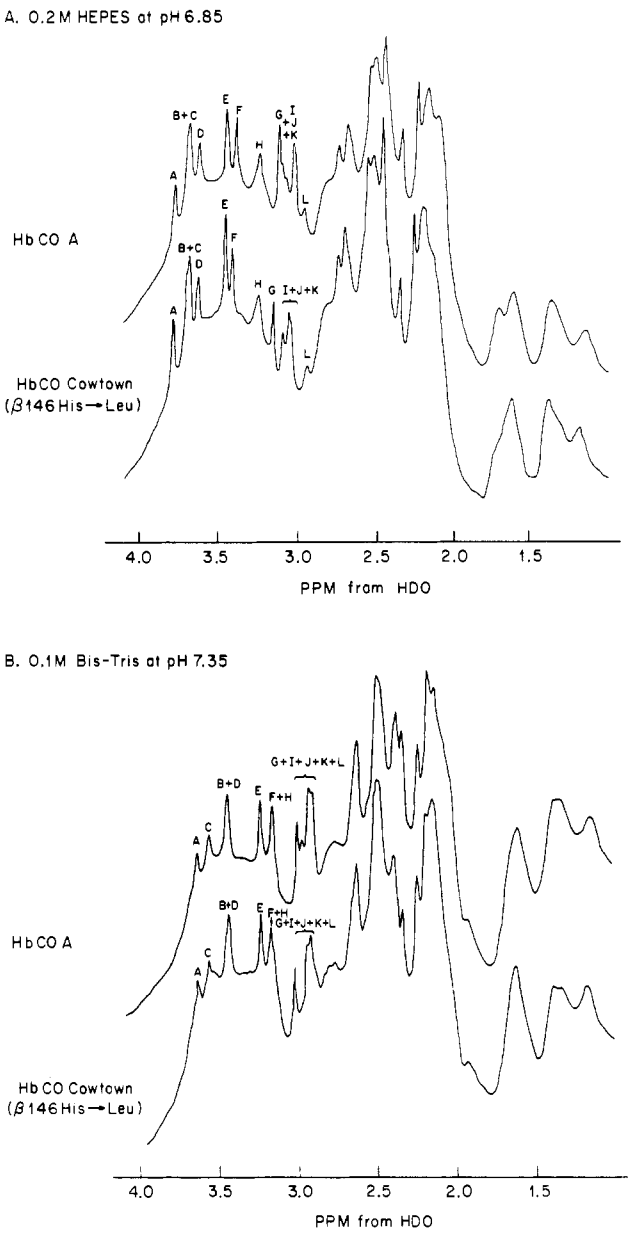

FIGURE 2: 300-MHz aromatic proton resonances of HbCO A and HbCO Cowtown (β146His → Leu): (A) 10% Hb solutions in 0.2 M HEPES in D_2O at pH 6.85 and 29 °C; (B) 10% Hb solutions in 0.1 M Bis-Tris in D_2O at pH 7.35 and 29 °C.

HbCO Cowtown in 0.2 M HEPES [for example, see Figure 2a of Perutz et al. (1985a)].[2] Several other histidyl proton resonances (such as the resonances labeled I, J, K, and L) have been found to be perturbed in HbCO Cowtown as compared to HbCO A [Figure 2 in the present work; Figure 2a,b in Perutz et al. (1985a)].

Given the important effects of anions on the local electrostatic environments of the histidyl residues of Hb and on the Bohr effect, we have also carried out a ^{1}H NMR comparison of HbCO Cowtown and HbCO A in the presence of inorganic phosphate and/or chloride ions. Figure 3A shows the aromatic proton resonances of HbCO Cowtown and HbCO A in 0.2 M phosphate buffer in D_2O at pH 7.05. One aromatic proton resonance of HbCO A is missing in the corresponding spectrum of HbCO Cowtown (i.e., the resonance labeled Y in Figure 3A). The same resonance has been found to be missing

[2] It should be mentioned that resonance H also appears as a shoulder in both HbCO A and HbCO Cowtown at pH 7.4 in Figure 2b of Perutz et al. (1985).

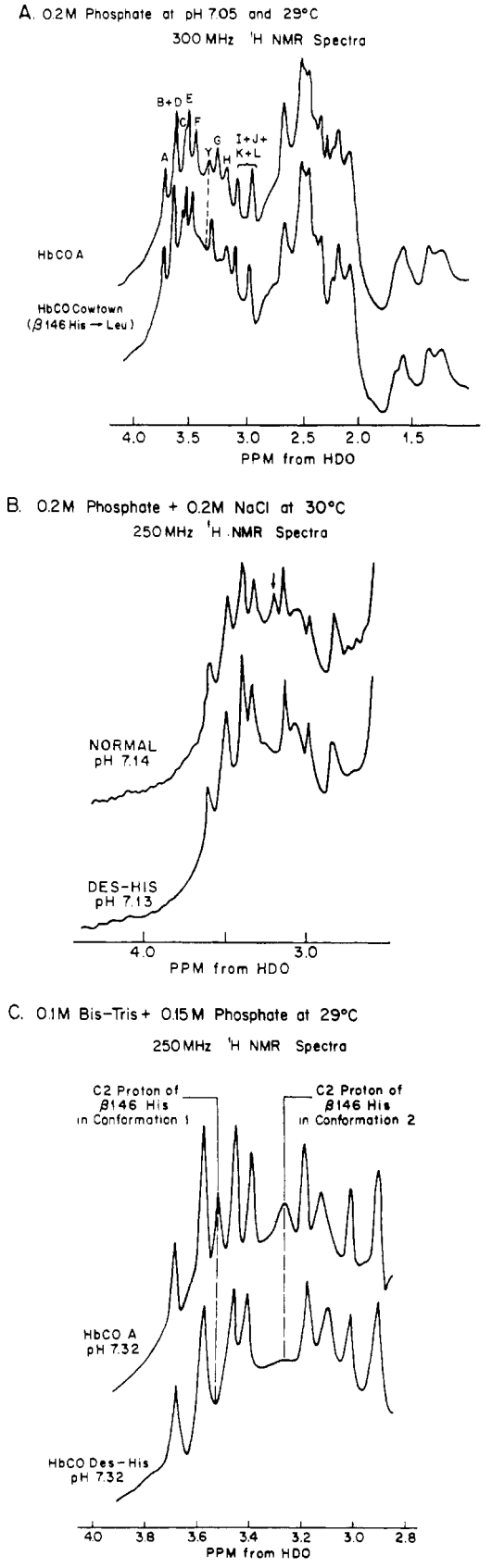

FIGURE 3: ^{1}H NMR spectra of HbCO A, HbCO Cowtown (β146His → Leu), and HbCO des-His: (A) 300-MHz aromatic proton resonances of HbCO A and HbCO Cowtown in 0.2 M phosphate in D_2O at pH 7.05 and 29 °C; (B) 250-MHz aromatic proton resonances of HbCO A and HbCO des-His in 0.2 M phosphate + 0.2 M NaCl in D_2O at pH 7.13 and at 30 °C [reproduced from Figure 1 of Kilmartin et al. (1973)]; (C) 250-MHz aromatic proton resonances of HbCO A and HbCO des-His in 0.1 M Bis-Tris + 0.15 M phosphate in D_2O at pH 7.32 and at 27 °C [reproduced from Figure 7 of Russu et al. (1980)].

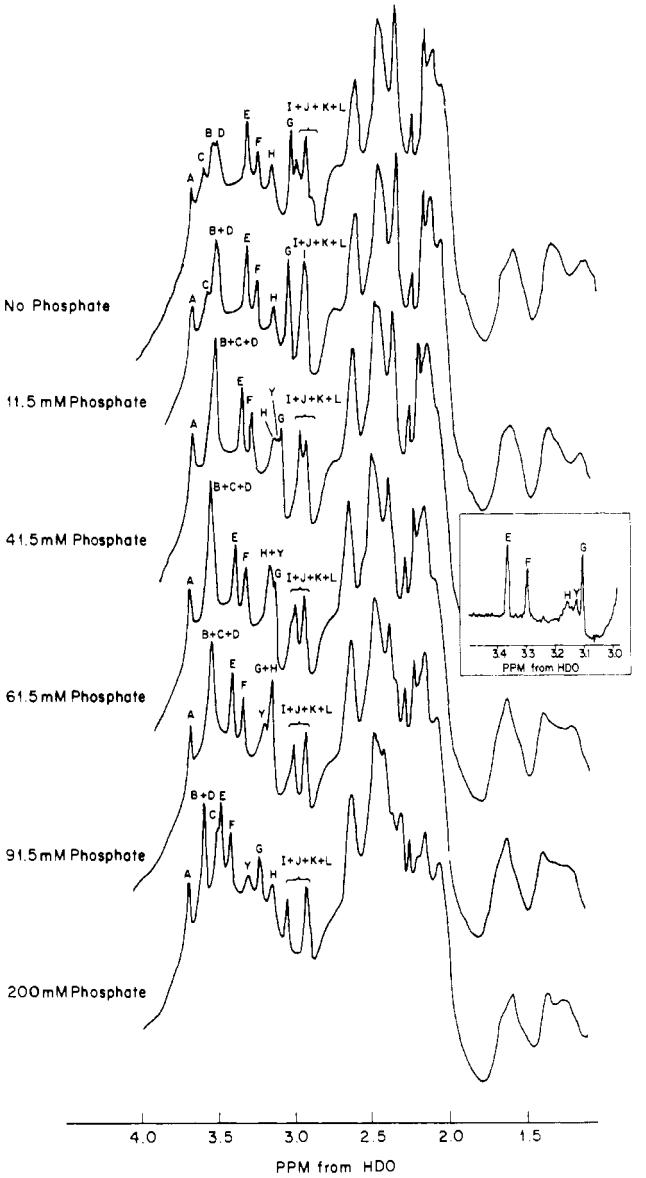

FIGURE 4: 300-MHz aromatic proton resonances of HbCO A in 0.1 M Bis-Tris in D_2O at pH 7.0 and 29 °C as a function of the concentration of inorganic phosphate ions. The insert shows an expansion of resonances E–G for a concentration of inorganic phosphate ions of 41.5 mM. For the spectrum in this insert, a Gaussian multiplication of −7 Hz has been applied in the time domain.

in the ^{1}H NMR spectrum of HbCO des-His in 0.2 M phosphate plus 0.2 M chloride ions [Figure 3B taken from Figure 1 of Kilmartin et al. (1973)] and was assigned to the β146His C2 proton by Kilmartin et al. (1973). Under the experimental condition of intermediate concentrations of inorganic phosphate and/or chloride ions, both resonances Y and C are missing from the ^{1}H NMR spectrum of HbCO des-His [Figure 3C taken from Figure 7 of Russu et al. (1980)]. On the basis of these results, we have proposed that the resonance presently labeled Y originates from the β146His residue of HbCO A in a conformation in which the β146His–β94Asp salt bridge is broken [i.e., conformation 2 of the β146His residues in our earlier paper (Russu et al., 1980)]. The present results on Hb Cowtown add further support to this proposal.

We would like to point out that the aromatic proton resonance of HbCO A presently labeled Y is different from the aromatic proton resonance of HbCO A labeled H. This fact is demonstrated by titration of the aromatic proton resonances of HbCO A as a function of inorganic phosphate ions at a constant pH (Figure 4). At a concentration of inorganic

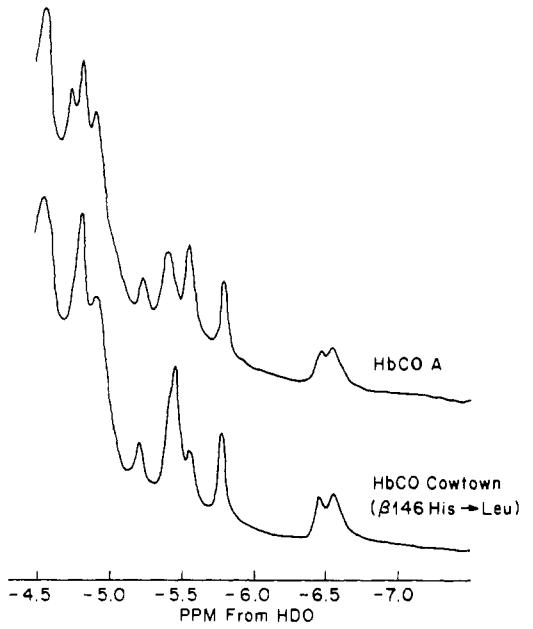

FIGURE 5: 300-MHz ring-current-shifted proton resonances of HbCO A and HbCO Cowtown (β146His → Leu) in 0.1 M Bis-Tris in D_2O at pH 6.5 and 29 °C.

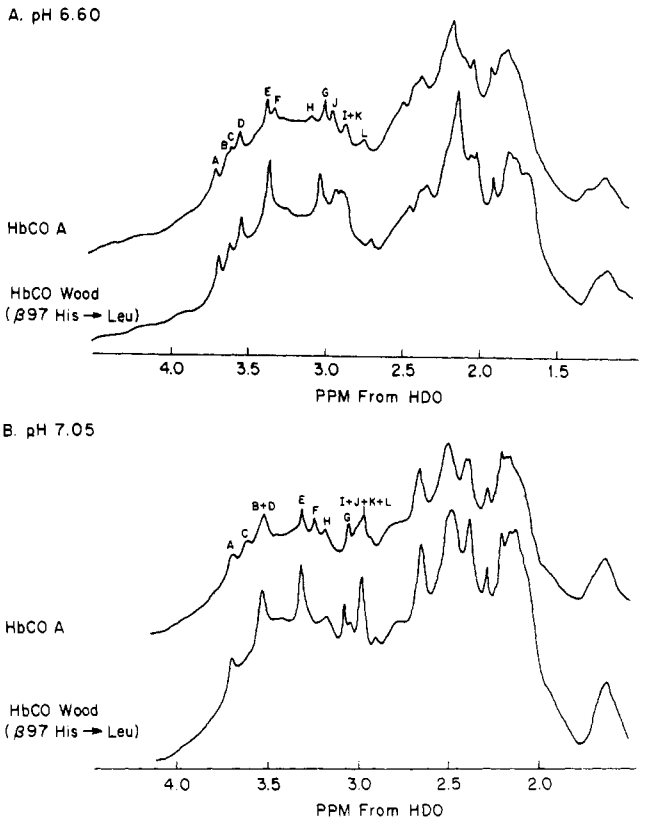

FIGURE 6: 300-MHz aromatic proton resonances of HbCO A and HbCO Wood (β97His → Leu) in 0.1 M Bis-Tris in D_2O and at 29 °C.

phosphate ions of 41.5 mM, resonance Y appears in the NMR spectrum at a position intermediate between resonances G and H (for clarity, see insert in Figure 4). When the concentration of inorganic phosphate ions is increased in small increments, one can follow the downfield shift of resonance Y, and at the final point of the titration (namely, 200 mM inorganic phosphate), this resonance is found between resonance F and resonance G (Figure 4). Parallel to these changes, resonance G [previously assigned by our laboratory to the β2His C2 proton (Russu et al., 1982)] also shifts downfield, a finding consistent with the increase in the pK_a value of the β2His residues upon the binding of inorganic phosphate ions (Lin et al., 1985). During the course of the entire titration, the spectral position of resonance H remains essentially unchanged, and thus, at the final point of the titration, resonance H occurs upfield from both resonance Y and resonance G. The fact that resonances Y and H are different shows that the interpretation given by Perutz et al. (1985a) for resonance H is incorrect. They assumed that resonance H is the same as the resonance presently labeled Y, i.e., the resonance identified by Kilmartin et al. (1973) and by Russu et al. (1980) as originating from the β146His residue in a conformation in which the β146His–β94Asp salt bridge is broken.

In order to fully characterize the overall conformation of Hb Cowtown in solution, in the ligated form, we have also obtained the exchangeable and the ring-current-shifted proton resonances of Hb Cowtown in the CO form. The exchangeable proton resonances (5.5–9.0 ppm) of HbCO Cowtown are very similar to the corresponding ones of HbCO A (results not shown). Hence, in the ligated form, as in the deoxy form, the $\alpha_1\beta_2$ and $\alpha_1\beta_1$ subunit interfaces of Hb Cowtown are not perturbed compared to those in HbCO A.

The ring-current-shifted proton resonances of HbCO Cowtown and HbCO A in 0.1 M Bis-Tris at pH 6.5 are shown in Figure 5. These resonances are present in the ^1H NMR spectrum of Hb, in the ligated state, and they originate from the protons of the amino acid residues situated in or near the α and β heme pockets (Lindstrom et al., 1972; Lindstrom & Ho, 1973; Ho & Russu, 1981; Dalvit & Ho, 1985). At least two of these resonances have been found to be different between HbCO Cowtown and HbCO A. The resonance at −5.56 ppm in HbCO A has a much lower intensity in HbCO Cowtown, probably due to a spectral shift to −5.46 ppm. The resonance at −4.78 ppm in HbCO A is missing in HbCO Cowtown, being probably shifted to −4.82 ppm. These results show that the replacement of the β146His residue by a Leu in Hb Cowtown induces changes in the local conformation of the amino acid residues situated in or near the heme pockets, in the ligated form.

^1H NMR Studies of Mutant Hbs Containing Amino Acid Substitutions near the C-Terminal Regions of the β Chain. We have carried out parallel ^1H NMR studies of the surface histidyl residues in several other mutant Hbs in which the amino acid replacements occur within the tertiary domains around the C-terminal regions of the β chains. These mutant Hbs are Hb Wood (β97His → Leu), Hb Malmö (β97His → Gln), Hb Barcelona (β94Asp → His), and Hb Abruzzo (β143His → Arg).

The aromatic proton resonances of HbCO Wood at two pH values are compared to the corresponding ones of HbCO A in Figure 6. Resonance C is missing from the NMR spectrum of HbCO Wood at pH 7.05 (Figure 6B). Several other His C2-proton resonances are greatly altered in HbCO Wood as compared to those in HbCO A. For example, in HbCO Wood, resonances E and F overlap and resonances I–L change their chemical shifts and intensities (Figure 6). These spectral features indicate that the change from a His to a Leu at the β97 position in Hb Wood affects the local conformation of several other histidyl residues situated over the surface of the Hb molecule. This result is in perfect agreement with our previous finding that the α and β heme pockets in HbCO Wood are significantly different from those in HbCO A (Ho & Russu, 1985).

Changes in the surface conformation occur also in HbCO Malmö (β97His → Gln), a Hb in which the amino acid residue

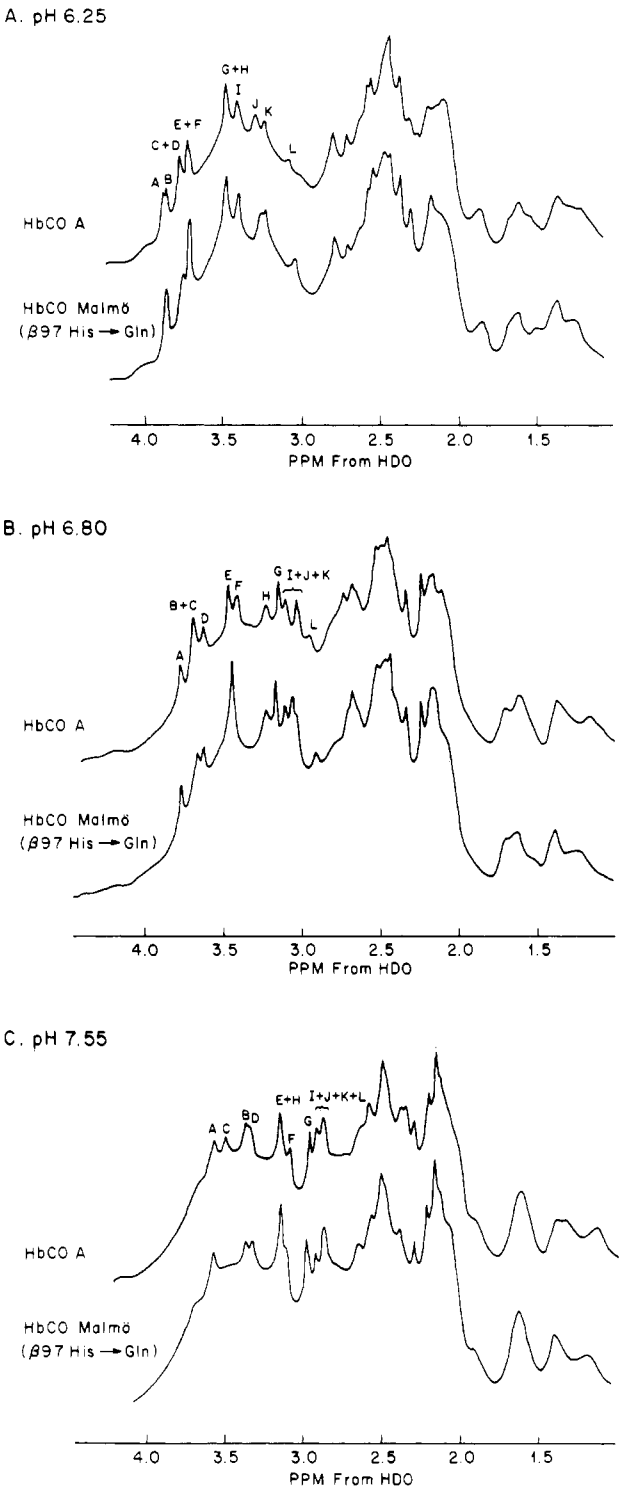

FIGURE 7: 300-MHz aromatic proton resonances of HbCO A and HbCO Malmö (β97His → Gln) in 0.1 M Bis-Tris in D_2O and at 29 °C.

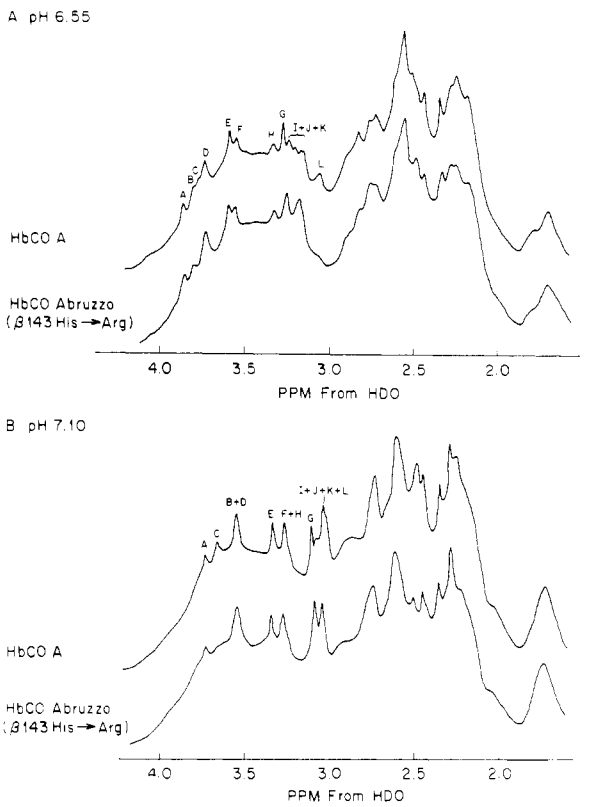

FIGURE 8: 300-MHz aromatic proton resonances of HbCO A and HbCO Abruzzo (β143His → Arg) in 0.1 M Bis-Tris in D_2O and at 29 °C.

replacing β97His differs from that in Hb Wood. As shown in Figure 7C, resonance C is missing in HbCO Malmö at pH 7.55. At lower pH values, the absence of resonance C from the ^1H NMR spectra of HbCO Malmö is more difficult to ascertain since, in HbCO A, this resonance overlaps resonances B and D (parts A and B of Figure 7). The His C2-proton resonances E and F as well as resonances I–L of HbCO Malmö are greatly different from those in HbCO A over the entire pH range investigated (Figure 7). Differences between HbCO Malmö and HbCO A have also been observed in the ring-current-shifted proton resonances of the α and β chains (Wiechelman et al., 1976). Therefore, in Hb Malmö, as in the case of Hb Wood, the effects of the β97 mutation propagate to other regions on the surface of the molecule and to the heme pockets of the α and β chains. Due to these mutation-induced changes in conformation of the Hb molecule, Hb Wood and Hb Malmö are not suitable models for assigning the β97His C2-proton resonances in the ^1H NMR spectrum of HbCO A. For a discussion on the strategy of spectral assignments in Hb, see Ho and Russu (1981, 1985).

Several representative ^1H NMR spectra of HbCO Abruzzo (β143His → Arg) and HbCO Barcelona (β94Asp → His) are shown in Figures 8 and 9, respectively. One spectral change common to both these two mutant Hbs is that resonance C is absent from the spectra of these mutants at all pH values investigated. In the lower pH range, resonance L is also missing from the spectra of the two mutant Hbs. In HbCO Barcelona, at higher pH values (such as pH 7.30), a new resonance occurs upfield from the His C2-proton resonances B and D (Figure 9B). This new resonance very likely originates from the new His residue replacing β94Asp. Spectral changes are also observed in both HbCO Abruzzo and HbCO Barcelona for resonances I–K. A detailed description of our ^1H NMR study of the structure–function relationship in Hb Barcelona will be reported elsewhere (I. M. Russu, H. Wajcman, C. Poyart, and C. Ho, unpublished results).

The spectral changes induced by the enzymatic removal of the β146His residues in HbCO des-His are shown in Figure 10 [taken from Figure 2 of Russu et al. (1980)]. Resonance C is missing from the ^1H NMR spectra of HbCO des-His at all pH values studied. As in the case of HbCO Barcelona and HbCO Abruzzo, in the lower pH range, resonance L is also missing from the spectrum of HbCO des-His. No other spectral changes have been observed for HbCO des-His over the entire pH range investigated. A summary of the ^1H NMR properties of the His C2-proton resonances of histidyl residues in all the β-chain mutant and enzymatically modified Hbs

Table I: ^{1}H NMR Spectral Properties of His C2-Proton Resonances of Interest in a Series of Mutant and Enzymatically Modified Hemoglobins in the CO Form in 0.1 M Bis-Tris or 0.2 M HEPES Buffer in D_2O at 29 °C[a]

Hb	His C2-proton resonance					
	C	E	F	H	I, and J, and K	L
Hb A	+	+	+	+	+	+
Hb Cowtown (β146His → Leu)	+	+	+	+	P	P
Hb des-His (β146His deleted)	-	+	+	+	+	-
Hb Wood (β97His → Leu)	-	P	P	+	P	P
Hb Malmö (β97His → Gln)	-	P	P	+	P	P
Hb Barcelona (β94Asp → His)	-	+	+	+	P	-
Hb Abruzzo (β143His → Arg)	-	+	+	+	P	-

[a] (+) Present in the ^{1}H NMR spectrum at the same spectral position as in HbCO A; (-) absent from the ^{1}H NMR spectrum; (P) perturbed.

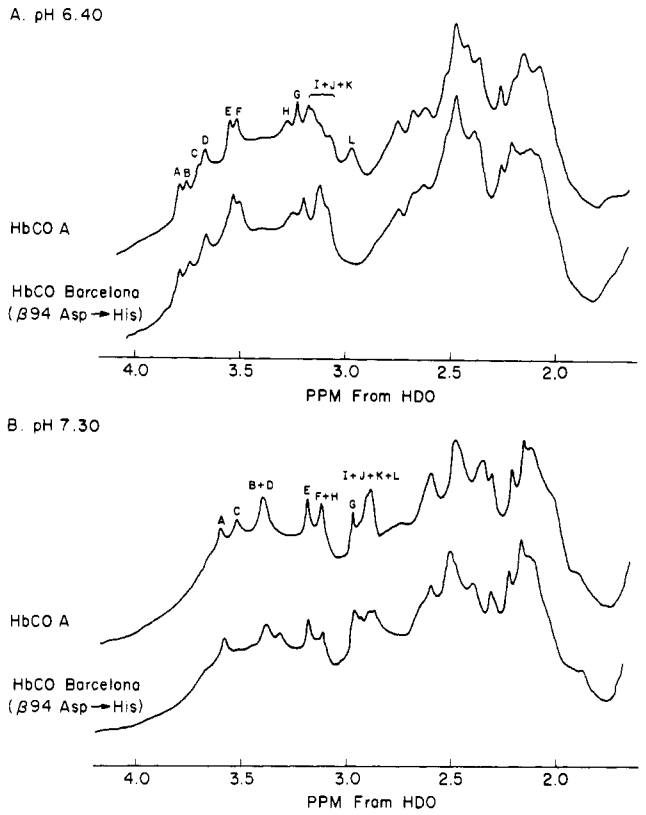

FIGURE 9: 300-MHz aromatic proton resonances of HbCO A and HbCO Barcelona (β94Asp → His) in 0.1 M Bis-Tris in D_2O and at 29 °C.

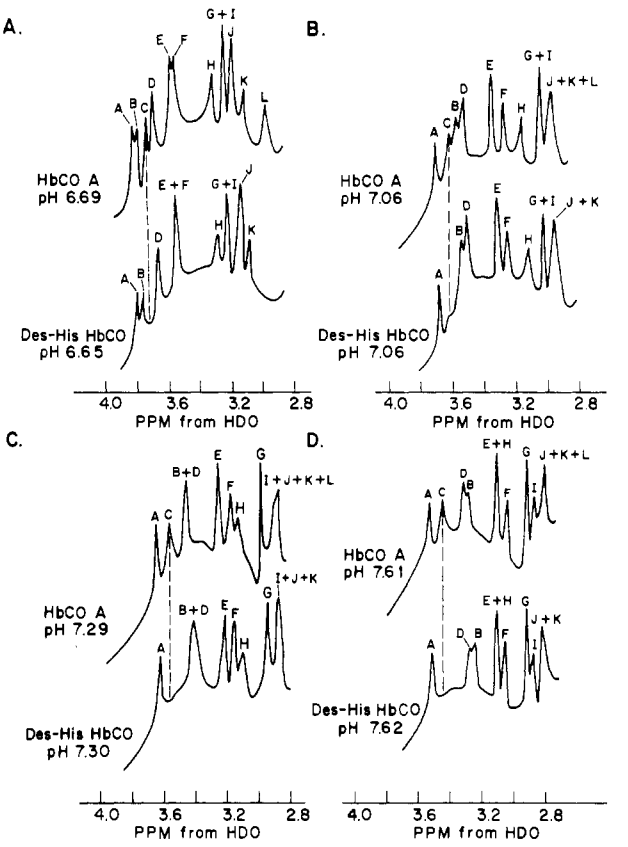

FIGURE 10: 250-MHz aromatic proton resonances of HbCO A and HbCO des-His (β146His deleted) in 0.1 M Bis-Tris in D_2O and at 27 °C [reproduced from Figure 2 of Russu et al. (1980)].

studied in this work is given in Table I.

^{1}H NMR Studies of Surface Conformational Differences between HbCO A and HbO_2 A. We have carried out a comparative ^{1}H NMR study of the surface His C2-proton resonances of Hb A in the carbonmonoxy and oxy forms. Each of the 22 surface histidyl residues (or $11/\alpha\beta$ dimer) has been monitored in a mixture of HbCO A and HbO_2 A as a function of the fraction of HbO_2. The ratio of HbCO A to HbO_2 A was determined for each sample by measuring the intensities of the γ_2-methyl proton resonances of the E11Val residues, which occur at −6.5 ppm in HbCO A and at −7.05 ppm in HbO_2 A (Lindstrom & Ho, 1973; Dalvit & Ho, 1985). Several representative spectra of various mixtures of HbCO A and HbO_2 A are shown in Figure 11. The following spectral changes are observed in going from the carbonmonoxy to the oxy form. First, the intensity of resonance H decreases as the fraction of HbO_2 A increases and is reduced to zero for an approximately equimolar ratio of HbO_2 A to HbCO A. Second, with increasing amounts of HbO_2 A, the intensity of resonance F decreases, and a new resonance (arbitrarily labeled F′) occurs at a spectral position intermediate between resonance E and resonance F. Finally, the spectral pattern of resonances G, I, J, and K is gradually changed as the amount of HbO_2 is increased. These results demonstrate that specific surface histidyl residues of Hb A are sensitive to the conformational differences between the carbonmonoxy and oxy forms of the Hb A molecule.

Discussion

Origin of the His C2-Proton Resonance Labeled H in the ^{1}H NMR Spectra of HbCO A. The ^{1}H NMR spectra of HbCO Cowtown (Figure 2) as well as the spectra of HbCO des-His (Figures 3B,C and 10) clearly show that resonance H is present in both these Hb molecules, over the entire pH range investigated. Furthermore, no significant perturbations of resonance H have been observed in any of the β-chain Hb mutants studied in this work (Figures 6–9 and Table I). All these results suggest that resonance H cannot originate from β146His, and thus, the assignment proposed by Perutz et al. (1985a) is incorrect.

Perutz et al. (1985a) based their assignment of resonance H to the β146His C2 proton on their observation that this resonance is missing from the ^{1}H NMR spectrum of HbCO

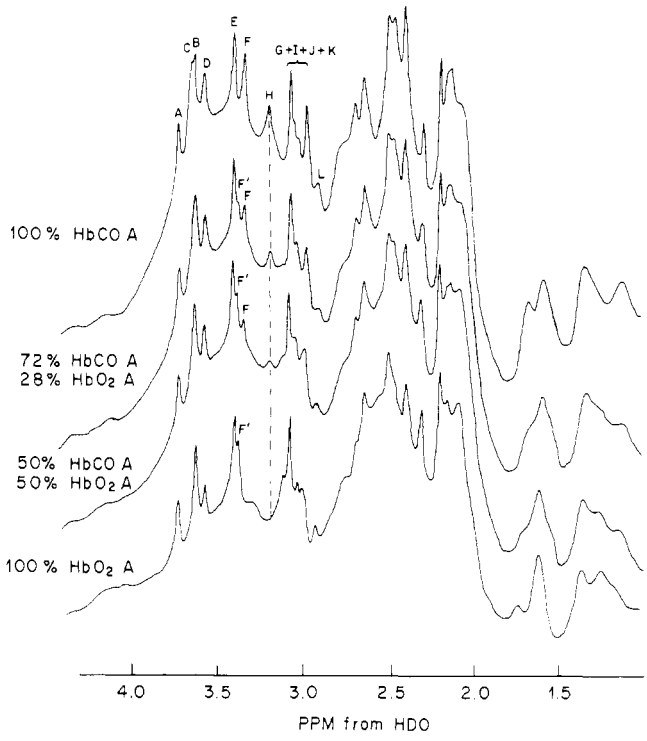

FIGURE 11: 300-MHz aromatic proton resonances of HbCO A in the presence of various fractions of HbO_2 A in 0.2 M HEPES in D_2O at pH 6.85 and 29 °C.

Cowtown at pH 6.95 (see, for example, Figure 2a in their paper).[2] In our work, we are unable to reproduce their results, and at all pH values investigated, resonance H is present in the ^1H NMR spectra of HbCO Cowtown (Figure 2). The reasons for these differences are not completely clear at present. The discrepancy cannot originate from different identities of the samples for the following reasons: (i) both samples were obtained from the same source (namely, from Dr. Richard T. Jones at the Oregon Health Science University); (ii) the identity of our sample was further checked by isoelectric focusing, amino acid analysis, and carboxypeptidase A and B digestions (see Experimental Procedures); (iii) the ^1H NMR spectrum of the same Hb Cowtown sample in the deoxy form showed, as expected, that resonances 3 and 12 are missing (Figure 1). One possible reason for this discrepancy can, however, be found in the unique properties of resonance H in Hb A in the ligated form. We have demonstrated that resonance H is one of the histidyl resonances that are most sensitive to the conformational differences between HbCO A and HbO_2 A (Figure 11). Resonance H is present at full intensity only in the spectrum of pure HbCO A. In HbO_2 A or in equimolar mixtures of HbO_2 A and HbCO A, resonance H is absent from the aromatic proton resonance region of the ^1H NMR spectra, being either broadened or shifted outside this spectral region. Similar changes of the ^1H NMR spectrum of HbCO A have also been observed in the presence of met-Hb (results not shown). Differences between HbCO A and HbO_2 A have been observed by our laboratory for several other amino acid residues in the Hb A tetramer or in isolated α and β chains. For example, in the isolated α and β chains of Hb A, the C2-proton resonances of the distal histidyl residues (E7) (whose chemical shifts in the CO form are very similar to that of resonance H of HbCO A) are shifted upfield by $\sim$2.8 ppm in going from the CO to the oxy form (Dalvit & Ho, 1985). It is also interesting to note that the apparent pK value of the distal histidyl residue of the isolated α chain of Hb A in the CO form is about 6.0, close to that of 6.2 for resonance H of HbCO A. We have neither measured the pK value of the distal histidyl residue of the isolated β chain nor fully identified the proton resonances due to the C2 protons of the distal histidyl residues of HbCO A. It is tempting to speculate that resonance H may originate from the C2 protons of one or both distal histidyl residue(s) of HbCO A. Further work is needed to ascertain the origin of resonance H. However, it is clear that, depending on the nature of the ligand, the proton resonances of specific histidyl residues of Hb A, such as the distal histidyl or the histidyl residue whose C2-proton resonance is labeled H, can be absent from the aromatic proton resonance region of the NMR spectra.

^1H NMR Spectral Properties of Resonance Labeled C. We have previously found that resonance C is missing from the spectra of HbCO des-His (Figure 10). This finding provided the experimental basis for our assignment of this resonance to the β146His C2 protons of HbCO A under "stripped" conditions (Russu et al., 1980). Our present results on a series of β-chain mutations reveal that several other structural perturbations in the tertiary domains around the C-terminal regions of the β chains also affect the presence or absence of resonance C in the NMR spectra. Among those, the β94Asp → His, β143His → Arg, β97His → Leu, and β97His → Gln amino acid substitutions result in resonance C being missing from the ^1H NMR spectra (Table I). The only exception to this trend is HbCO Cowtown (β146His → Leu) in which resonance C appears to be present at all pH values investigated (Figure 2). It is interesting to note that, in HbCO Cowtown, under stripped conditions, no proton resonance is missing from the aromatic proton resonance region in the NMR spectrum, which one would expect from the replacement of the histidyl residue by a nonaromatic amino acid. On the other hand, several other His C2-proton resonances (such as I–L) and the ring-current-shifted resonances of HbCO Cowtown are found to be different from those of HbCO A (Figures 2 and 5). These ^1H NMR results indicate that the β146His → Leu substitution induces changes in several surface histidyl residues as well as in the conformation of amino acid residues situated in the heme pockets of the α and β chains. Thus, HbCO Cowtown is not a suitable mutant to use to assign the proton resonances of the β146His in HbCO A.

The absence of resonance C from the ^1H NMR spectra of HbCO Malmö and HbCO Wood has been used by Perutz et al. (1985) to assign this resonance to the β97His C2 protons. This assignment is questionable in view of the large alterations observed for several surface His C2 protons in these two Hbs [for example, see parts a and b of Figure 2 in Perutz et al. (1985a) and Figures 6 and 7 in this work]. In addition to resonance C being missing, at least five other His C2-proton resonances (labeled E, F, I, J, and K) are also perturbed in HbCO Malmö and HbCO Wood as compared with HbCO A. Moreover, in Hb Wood and Hb Malmö, the conformational effects of the β97His substitutions propagate throughout the Hb molecule, to the heme pockets and to the quaternary interfaces, as demonstrated by our laboratory (Wiechelman et al., 1976; Ho & Russu, 1985). Therefore, the absence of resonance C from the ^1H NMR spectra of HbCO Wood and HbCO Malmö could result from changes in the overall conformations of these two Hb molecules. This possibility is also supported by our finding that resonance C is missing in several other mutant or enzymatically modified Hbs in which the β97His residues are intact (Table I). One of these Hbs is HbCO des-His. Perutz et al. (1985a) have attempted to explain the absence of resonance C in HbCO des-His by postulating that a conformational equilibrium involving β97His

exists in this Hb molecule. The experimental evidence advanced in their work to support this explanation is insufficient for the following reasons. (i) Their ^{1}H NMR spectra contain large differences among the His C2-proton resonances of HbCO A and HbCO des-His [Figure 3 of Perutz et al. (1985a)]. Due to these spectral differences, a one-to-one correspondence between the proton resonances of HbCO des-His and HbCO A cannot be made, and the fractional decrease in the intensity of resonance C postulated by this model in HbCO des-His cannot be measured. Our results on HbCO des-His clearly show that resonance C is consistently missing from the NMR spectra of this enzymatically modified Hb (Figure 10). (ii) In support of their model, Perutz et al. (1985a) have also reported results of transfer of saturation experiments on two His C2-proton resonances (namely, the resonances labeled C^1 and C^2 in Figure 3 of their paper), which suggest a chemical exchange between two conformations of β97His. We have carried out similar transfer of saturation experiments for the His C2-proton resonances of HbCO A under a wide range of experimental conditions. Our results have shown that the irradiation of a given His C2-proton resonance of Hb results in nonspecific changes in a large number of other proton resonances of Hb. These nonspecific effects are due to the large contribution of the spin-diffusion process to the longitudinal relaxation of the Hb protons (Kalk & Berendsen, 1976; Russu & Ho, 1982). The longitudinal relaxation of surface histidyl protons is almost entirely due to the cross-relaxation between these protons and the rest of the protons in the spin system of Hb (Russu & Ho, 1982). Hence, the irradiation of a given histidyl proton should affect the intensities of a large number of other Hb protons. Furthermore, when a His C2-proton resonance is irradiated, a simultaneous irradiation occurs for other aromatic or peptide NH protons contained in the broad envelope of the aromatic proton resonances. The transfer of saturation measurements reported by Perutz et al. (1985a) have been carried out for time intervals of 0.5–1 s, a range that is comparable to the T_1 values of the His C2 protons in Hb [namely, 0.7–1.3 s, Russu & Ho (1982)]. Therefore, these measurements should also be greatly affected by the nonspecific effects of spin-diffusion resulting from the saturation of the His C2-proton and the broad aromatic proton resonances. Thus, the small transfer of saturation effects (5–10%) reported by Perutz et al. (1985a) for resonance C^1 upon irradiation of resonance C^2 do not represent conclusive evidence for an equilibrium specifically involving the β97His residue.

All the NMR results presented in our paper clearly indicate that the NMR spectral properties of the proton resonance labeled C in the spectra of HbCO A are very complex. This resonance, while present in HbCO A and HbCO Cowtown, is absent from the NMR spectra of Hb molecules containing structural perturbations at the β146, β143, and β94 sites. In the latter cases, no additional resonance(s) is (are) present in the ^{1}H NMR spectra as one would expect from (i) a simple shift of resonance C due to conformational changes induced by the mutations or (ii) a conformational equilibrium of the corresponding amino acid residues proposed by Perutz et al. (1985a) for HbCO des-His. Therefore, at present, the mechanisms responsible for the absence or the presence of resonance C in mutant or enzymatically modified Hbs remain an open question. Research is being carried out in our laboratory to clarify this interesting observation.

Conclusions

The ^{1}H NMR results obtained in this work clearly indicate that the substitution and/or chemical modification of single amino acid residues in Hb can result in long-range conformational effects. We have found that some of the single-site structural modifications within the tertiary domains around the carboxyl-terminal regions of the β chains can induce conformational perturbations over the surface of the Hb molecule and into the heme pockets of the α and β chains. The extent of these long-range conformational effects has been found to depend upon (i) the exact location of the single-site modification, (ii) the nature of the amino acid substitution and/or chemical modification at a given site, and (iii) the ligation state of Hb. The present ^{1}H NMR investigation has suggested that long-range conformational effects should be considered when attempting to correlate structural changes in individual amino acid residues with specific spectroscopic perturbations or with global functional properties of the protein molecule.

Added in Proof

After submission of our paper, Perutz et al. (1985b) have reported additional ^{1}H NMR results for the histidyl residues in Hb A and several mutant Hbs, all in the CO form, in different buffers. Their new findings are not in agreement with our ^{1}H NMR results or with their own earlier data (Perutz et al., 1985a). First, we do not find any significant change in the intensity (and/or line width) of the resonance labeled H in HbCO A when HEPES buffer is replaced by Bis-Tris buffer (Figure 2A and the top spectrum in Figure 4 in this paper). In fact, the same results were reported by Perutz et al. (1985a), who stated in that paper "...very similar spectra were obtained on addition of 0.1 M NaCl to our HEPES buffers, or on substitution of Bis-Tris for HEPES...". Our present data clearly demonstrate that the intensity of resonance H in the ^{1}H NMR spectrum of ligated Hb A is mainly determined by the nature of the ligand. Due to this unique property, resonance H is very sensitive to the amount of HbO_2 and/or met-Hb present in the HbCO solution. In their most recent study, Perutz et al. (1985b) observed a change in the intensity of resonance H in going from 0.2 M HEPES to 0.1 M Bis-Tris buffer (see, for example, the two top spectra in Figure 4 of their paper). They attributed this change to the effect of the buffer upon this His C2-proton resonance. However, their spectra contain additional differences such as a large decrease in the intensity of resonance F in 0.1 M Bis-Tris and the appearance of a new, broad resonance (unlabeled) slightly upfield from resonance F. Similar spectral changes are also present in the ^{1}H NMR spectra of HbCO Cowtown reported by Perutz et al. [for example, see Figure 2a in Perutz et al. (1985a) and Figures 1 and 2 in Perutz et al. (1985b)]. As shown in our paper, such spectral changes in the region around resonances F and H are indicative of the presence of HbO_2 and/or met-Hb in the HbCO sample. The ratio of HbCO to HbO_2 can readily be determined for each Hb sample by measuring the intensities of the γ_2-methyl proton resonances of the E11Val residues at −6.5 ppm (for HbCO A) and −7.05 ppm (for HbO A) upfield from HDO. These measurements are essential controls in a ^{1}H NMR study of His C2-proton resonances in Hb, and they are not reported by Perutz et al. (1985a,b).

Second, the experimental evidence provided by Perutz et al. (1985b) to support the presence of resonance C in HbCO Barcelona (β94Asp → His) and HbCO Abruzzo (β143His → Arg) is not conclusive. At pH 6.9, resonance C strongly overlaps resonances B and D [see Figure 1 of Perutz et al. (1985b)]. Hence, at this pH, the absence of resonance C in these two mutant Hbs is difficult to ascertain. In contrast, at pH values above 7.0, resonance C stands alone in the spectra

of HbCO A, and its absence in HbCO Barcelona and HbCO Abruzzo can be more easily observed (see Figures 8 and 9 in this paper).

Third, our present results clearly demonstrate that the spectral position of resonance H does not change as a function of the concentration of inorganic phosphate ions (Figure 4 in this paper). This finding invalidates the interpretation by Perutz et al. (1985b) of the ^{1}H NMR spectra of HbCO A in phosphate buffer (Figure 4 in their paper).

An important conclusion from this paper is the need to control carefully the quality of HbCO samples when the proton resonances of the histidyl residues in Hb are investigated.

ACKNOWLEDGMENTS

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Registry No. Deoxy-Hb Cowtown, 74623-09-9; Hb A, 9034-51-9; Hb A (CO form), 9072-24-6; Hb Abruzzo (CO form), 51668-38-3; Hb Barcelona (CO form), 82391-91-1; Hb Cowtown (CO form), 97501-72-9; Hb Malmö (CO form), 59165-39-8; HbO_2, 9062-91-3; Hb Wood (CO form), 68190-05-6; L-His, 71-00-1.

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