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The pK_a Values of Two Histidine Residues in Human Haemoglobin, the Bohr Effect, and the Dipole Moments of α-Helices

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Studies of abnormal and chemically modified haemoglobins indicate that in 0·1 M-NaCl about 40% of the alkaline Bohr effect of human haemoglobin is contributed by the C-terminal histidine HC3(146)β. In deoxyhaemoglobin, the imidazole of this histidine forms a salt bridge with aspartate FG1(94)β, in oxyhaemoglobin or carbonmonoxyhaemoglobin it accepts a hydrogen bond from its own NH group instead. Kilmartin *et al.* (1973) showed that in 0·2 M-NaCl + 0·2 M-phosphate this change of ligation lowered the pK_a of the histidine from 8·0 in Hb‡ to 7·1 in HbCO, but Russu *et al.* (1980) claimed that in bis-Tris buffer without added NaCl its pK_a in HbCO dropped no lower than 7·85, and that in this medium the C-terminal histidine made only a negligible contribution to the alkaline Bohr effect.

We have compared the histidine resonances of HbCO A with those of three abnormal haemoglobins: HbCO Cowtown (His HC3(146)β → Leu), HbCO Wood (His FG4(97)β → Leu) and HbCO Malmø (His FG4(97)β → Gln). Our results show that the resonance assigned by Russu *et al.* to His HC3(146)β in fact belongs to His FG4(97)β. Although in Hb the pK_a of His HC3(146)β is 8·05 ± 0·05 independent of ionic strength, in HbCO its pK_a drops sharply with diminishing ionic strength, so that in the buffer employed by Russu *et al.* it has a pK_a of 6·2 and makes a contribution to the alkaline Bohr effect that is *57% larger* than in the phosphate buffer employed by Kilmartin *et al.* (1973).

In HbCO A, His FG4(97)β does not contribute to the Bohr effect, but in HbCO from which His HC3(146)β has been cleaved (HbCO des-His), His FG4(97)β is in equilibrium between two conformations with different pK_a values. This equilibrium varies with ionic strength and pH, and presumably also with degree of ligation of the haem moiety.

In HbCO A, His FG4(97)β has a pK_a of 7·8 compared to the pK_a value of about 6·6 characteristic of free histidines at the surface of proteins. This high pK_a is accounted for by its interaction with the negative pole at the C terminus of helices F and FG. It corresponds to a free energy change of the same order as that observed in the interaction of histidines with carboxylate ions and confirms the strongly dipolar character of α-helices, which manifests itself even when they lie on the surface of the protein.

1. Introduction

The oxygen affinity of mammalian haemoglobins falls with increasing hydrogen ion concentration from pH 9 down to about pH 6. Conversely, uptake of oxygen by haemoglobin liberates hydrogen ions, and oxygen release causes uptake of hydrogen ions. These effects, now generally bracketed as the alkaline Bohr effect, are physiologically important,

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‡ Abbreviations used: Hb, haemoglobin; HbO_2, oxhaemoglobin; HbCO, carbon monoxyhaemoglobin; HbCO des-His, HbCO with His146β removed enzymatically; HEPES, *N*-2-hydroxymethyl-piperazine-*N'*-2-ethanesulphonic acid; n.m.r., nuclear magnetic resonance.

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as they raise the fraction of the oxygen carried that can be released in the tissues and help to convert metabolic CO_2 into soluble bicarbonate that can be carried to the lungs. The Bohr effect is exhibited only by the haemoglobin tetramer and depends upon the transition between its two alternative structures: the deoxy (or T), and the oxy (or R) forms. Thus, the oxygen affinities of myoglobin, of separate α or β-chains and of tetrameric haemoglobin in the R-structure are all pH-independent. Interpretation of the alkaline Bohr effect in stereochemical terms represented the first successful attempt to account for the physiological properties of haemoglobin in terms of its three-dimensional structure.

X-ray analysis had shown that, in Hb†, the imidazole side-chain of the C-terminal histidine HC3(146)β forms a salt bridge with an aspartate FG1(94) of the same β-chain, while in HbO_2 and HbCO it accepts a hydrogen bond from its own main-chain NH group (Figs 1 and 5) (Fermi, 1975; Baldwin, 1980; Shaanan, 1983). In 1969, Perutz *et al.* suggested that formation of the salt bridge raises

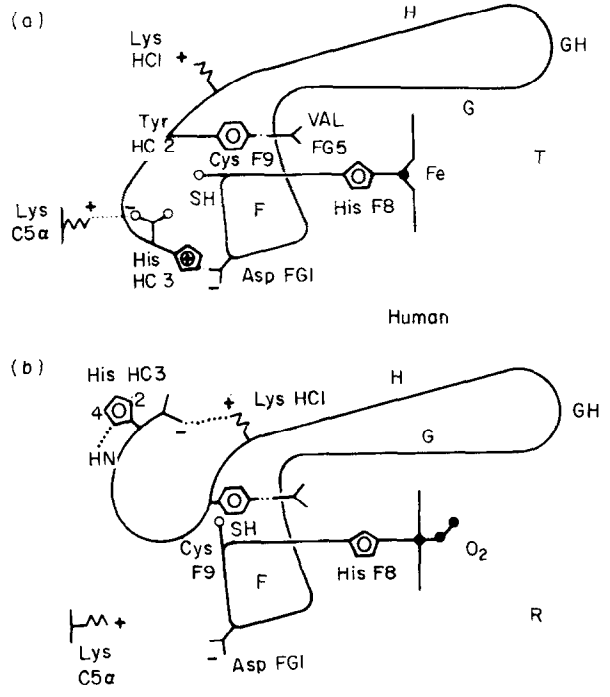


Figure 1. Mechanism of alkaline Bohr effect in haemoglobin. Changes in conformation near the C terminus of the β-chain on going from (a) the deoxy or T-structure to (b) the oxy, CO or R-structure. In the T-structure the imidazole of His HC3(146)β forms a hydrogen bond (salt bridge) with Asp FG1(94)β and one of its carboxylate oxygens forms a hydrogen bond with Lys C5(40)α. The side-chain of Cys F9(93)β is external, but screened by His HC3(146)β. In the R-structure, Lys C5(40)α and Asp FG1(94)β are free; the carboxylate of His HC3(146)β is hydrogen bonded to Lys HC1(144)β and its imidazole accepts a hydrogen bond from the histidine's main-chain NH group (not shown). The side-chain of Cys F9(93)β is internal, wedged against the phenyl ring of Tyr HC2(145)β. In both structures the OH group of Tyr HC2(145)β donates a hydrogen bond to the CO group of Val FG5(98)β.

† See footnote to p. 491.

the pK_a of the histidine, thus causing it to take up protons when oxygen is discharged by the haem. Kilmartin & Wootton (1970) then showed that, in 0·1 M-NaCl in the absence of 2,3-diphosphoglyceric acid, this salt-bridge mechanism accounts for about 40% of the alkaline Bohr effect. Kilmartin *et al.* (1973) also titrated the histidine by proton magnetic resonance and showed that in 0·2 M-phosphate buffer + 0·2 M-NaCl its pK_a equals 7·1 in HbCO and 8·0 in Hb. The difference accounted quantitatively for its contribution to the alkaline Bohr effect found by Kilmartin & Wootton (1970). Russu *et al.* (1980) recently repeated the n.m.r. titrations in 0·1 M-bis-Tris buffer at chloride concentrations ranging from 5 to 60 mM. They found the same pK_a as Kilmartin *et al.* (1973) in Hb, but found the pK_a in HbCO to be 7·85 instead of 7·1, hardly different from the pK_a of 8·0 in Hb. They concluded that in 0·1 M-bis-Tris at low chloride concentration the salt bridge between His HC3(146)β and Asp FG1(94)β remains intact in HbCO, despite the fact that the alkaline Bohr effect is practically the same as it is in the presence of phosphate. They tried to explain this inconsistency by suggesting that the detailed mechanism of the Bohr effect depends upon the experimental conditions.

Any mutation that leads to a replacement of either His HC3(146)β or Asp FG1(94)β causes a drastic reduction of the alkaline Bohr effect (Perutz *et al.*, 1984). The same applies to any chemical modification that inactivates the salt bridge between these two residues in Hb (Perutz *et al.*, 1969). These reductions are observed regardless of phosphate or chloride concentration. In the bis-Tris buffer used by Russu *et al.* (1980) the reduction amounted to 60% (Kilmartin *et al.*, 1980). Moreover, the fact that in oxyhaemoglobin His HC3(146)β *accepts* a hydrogen bond from its own NH, shows that it must have a low pK_a value. These findings convinced us that Russu *et al.* (1980) must have made a mistake, and we decided to track it down.

Russu *et al.* (1980) titrated His HC3(146)β by comparing the n.m.r. spectra of HbCO A with those of an HbCO from which one of us (J.H.F.) had cleaved the C-terminal histidine by digestion with carboxypeptidase B (HbCO des-His). We started the present work by comparing the n.m.r. spectra of HbCO A and HbCO des-His with those of an abnormal HbCO (Cowtown), the Bohr effect of which is halved when His HC3(146)β is replaced by Leu (Shih *et al.*, 1984). The abnormal HbCO exhibited the resonance that is lacking in HbCO des-His and lacked the one assigned to His HC3(146)β by Kilmartin *et al.* (1973). Faced with this paradoxical result, we wondered whether the liberation of the carboxylate of Tyr HC2(145)β in HbCO des-His had perhaps shifted the resonance of another histidine that normally has a high pK_a, but we could find no other nearby histidine with a salt bridge to a carboxylate. Our suspicion then fell on His FG4(97)β; we surmised that this might have a high pK_a in HbCO A because its imidazole caps the C terminus of a helix that, according to Hol *et al.*

(1978, 1981), should correspond to the negative end of a dipole. That dipole might interact with the histidine. On the other hand, we could see no good reason why the pK_a of that histidine should be influenced by the liberation of the carboxylate of Tyr HC2(145)β, because the two residues lie 11 Å apart. All the same, we decided to examine the n.m.r. spectra of an abnormal HbCO in which His FG4(97)β is replaced by a leucine. The result was so astonishing that we proceeded to examine the spectra of another abnormal haemoglobin in which that same histidine is replaced by a glutamine. Our findings led us to conclude that the original assignment of Kilmartin *et al.* (1973) applies regardless of the presence of phosphate or chloride, and that the detailed molecular mechanism of the alkaline Bohr effect is indeed dependent upon experimental conditions, but in a way different from that proposed by Russu *et al.* (1980, 1982).

Our results have also led to the discovery that a histidine that caps the C-terminal end of an α-helix can have a pK_a as high as 7·85, and that this can be lowered by perturbation from a newly created negative charge that lies 11 Å away from its N_ε or N_δ.

2. Materials and Methods

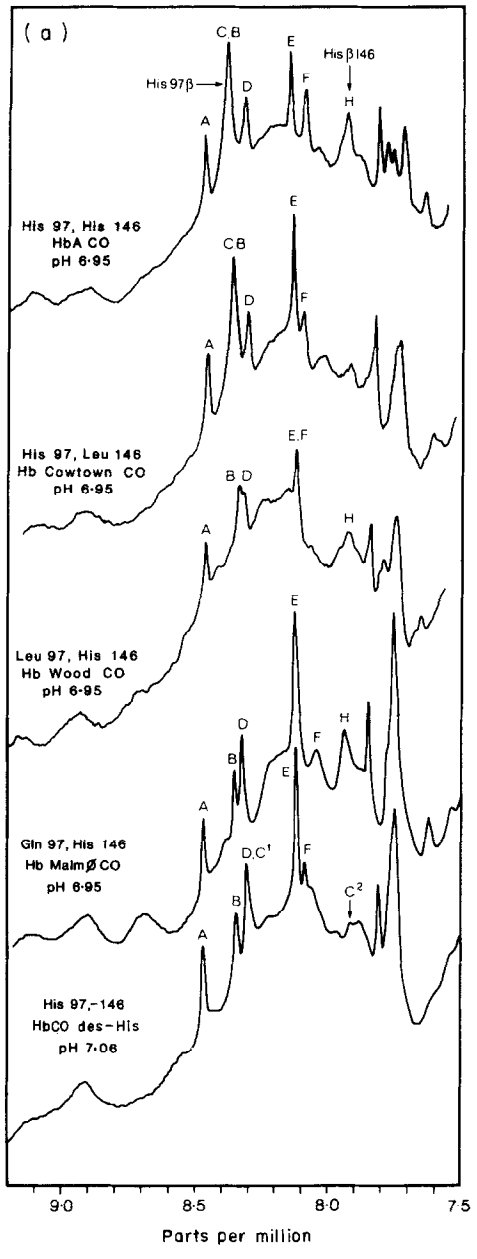
Haemoglobins A, des-His, Cowtown and Wood and Malmø were prepared as described by Perutz (1968), Kilmartin & Wootton (1970), Shih *et al.* (1984), Taketa *et al.* (1975) and Lorkin *et al.* (1970), respectively. We replaced H_2O with unbuffered salt-free ^2H_2O, either by dialysis through tubing or by repeated pressure dialysis through Amicon filters. To avoid possible artefacts due to the binding of chloride or phosphate, we initially used HEPES, a mild buffer widely used in cell culture; its pH is adjusted by addition of NaOH or [^{2}H]NaOH. The pH values of the Hb solutions were adjusted by addition of 1 vol. 0·6 M-HEPES buffer to 2 vol. HbCO solution, with final HbCO concentrations varying between 4 mM and 8 mM haem. The purity of HbCOs Cowtown, Wood, Malmø and des-His was checked by citrate agar gel electrophoresis and by analysis on isoelectric focusing gels (not shown). Absorption spectra showed less than 5% methaemoglobin. The pH values were measured with a Radiometer glass electrode filled with saturated KCl in ^2H_2O and a Radiometer digital pH meter. To be consistent with Kilmartin *et al.* (1973) and Russu *et al.* (1980), we made no correction for the isotope effect on the glass electrode, because the pK_a values of histidine determined by n.m.r. in H_2O agree within experimental error with those determined in ^2H_2O on the basis of uncorrected glass electrode pH meter readings. Thus, the isotope effect of −0·4 pH unit at the glass electrode seems to be equal and opposite to the isotope effect on the pK_a of the histidine (Markley, 1975). The 500 MHz ^{1}H n.m.r. spectra were recorded on a Bruker AM500 spectrometer at 30°C. Two hundred transients were averaged for each spectrum. The spectra were recorded with a 70° observation pulse, an acquisition time of 0·5 s and a relaxation delay of 1·5 s. Prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. The transfer of saturation experiments was recorded by directly collecting the difference free induction decay by interleaving 8 transients of on-resonance saturation with 8 transients of off-resonance saturation, negating the memory between 8 transient cycles; a total of 8000 transients was averaged. Chemical shifts are quoted relative to 4,4-dimethyl-silapentane-1-sulphonate (DSS).

3. Results

Figure 2(a) compares the aromatic regions of the n.m.r. spectra of HbCOs A, Cowtown, Wood, Malmø and des-His at pH 6·95. We will focus on two resonances present in HbCO A: the overlapping peaks C, B and the resonance H. On substitution of His HC3(146)β by Leu (HbCO Cowtown), resonances C, B are unchanged but resonance H disappears (the small broad peak left in its place in HbCO Cowtown and the similar one immediately to the left of it probably arise from unexchanged main-chain NH groups; note that both these peaks also appear in the spectrum of HbCO A. Their chemical shifts do not titrate with pH. On substitution of His FG4(97)β by Leu (HbCO Wood) or Gln (HbCO Malmø), resonances C, B are halved in intensity, but resonance H is unchanged. At pH 7·4 (Fig. 2(b)) we see a similar pattern. Resonance C is distinct in HbCO A and HbCO Cowtown, but absent in HbCO Wood and HbCO Malmø. Resonance H appears as a shoulder in HbCO A and HbCO Malmø, is absent in HbCO Cowtown and distinct in HbCO Wood. (Note that at this pH resonance H overlaps with other resonances and that these others still remain in HbCO Cowtown, so that a residual shoulder persists in the position of resonance H, but with much reduced intensity.) Very similar spectra were obtained on addition of 0·1 M-NaCl to our HEPES buffers, or on substitution of bis-Tris for HEPES (not shown), and indeed our spectra of HbCOs A and des-His closely match those of Russu *et al.* (1980). These results lead to the quite unequivocal assignment of resonance C to His FG4(97)β and H to His HC3(146)β, which matches the assignment of Kilmartin *et al.* (1973), but contradicts that of Russu *et al.* (1980).

In order to determine the pK_a values of histidines FG4(97)β and HC3(146)β, we compared the spectra of HbCOs Cowtown, Wood, Malmø and A at a series of pH values; this enabled us to assign the correct resonance to each of the two histidines in HbCO A and to titrate their chemical shifts (Fig. 4). For His FG4(97)β we obtained curve C and for His HC3(146)β curve H. These are identical to the corresponding titration curves in Figure 4 of Russu *et al.* (1982), even though we discovered the existence of that Figure only after we had traced our own curves. For resonance C we found a pK_a of 7·80, the same (within experimental error) as the pK_a of 7·85 found by Russu *et al.* (1982). Curve H of His HC3(146)β titrates with a pK_a of 6·2, substantially lower than the pK_a of 7·1 of that same histidine in 0·2 M-phosphate + 0·2 M-NaCl (Kilmartin *et al.*, 1973), but identical to the pK_a of resonance H found by Russu *et al.* (1982) in bis-Tris buffer without added NaCl. Our titration curves for the unassigned resonances A, B, D, E and F also superimpose on the corresponding ones in Figure 4 of Russu *et al.* (1982).

We also examined the spectra of HbCO des-His



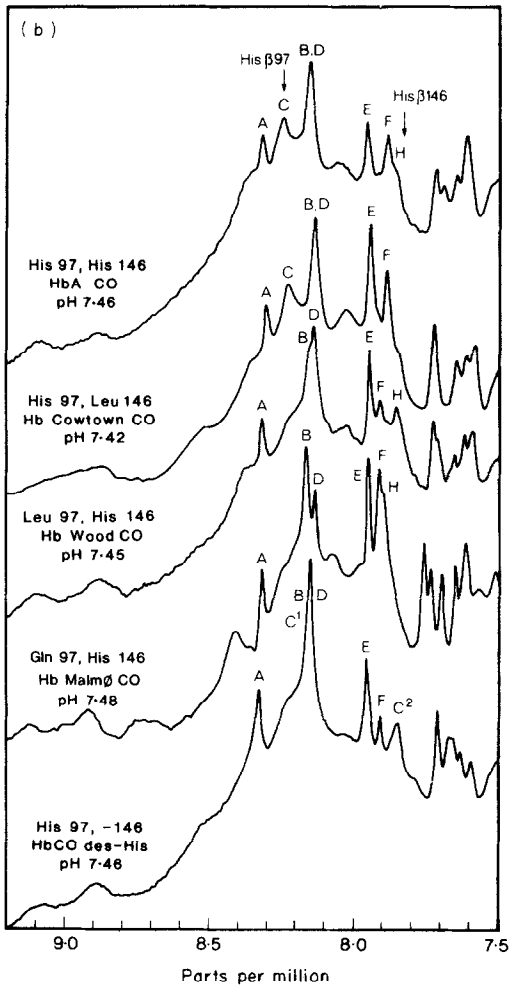


Figure 2. (a) and (b) 500 MHz ^{1}H n.m.r. spectra of the histidine C-2 proton resonance region of HbCO A, HbCO Cowtown (His HC3(146)$\beta \rightarrow$ Leu), HbCO Wood (His FG4(97)$\beta \rightarrow$ Leu), HbCO Malmø (His FG4(97)$\beta \rightarrow$ Gln), and HbCO des-His (His 146β removed enzymatically) at 30°C. The labelling of the histidine C-2 proton resonances is the same as that of Russu *et al.* (1980, 1982). Resonance H is absent in HbCO Cowtown and resonance C is absent in HbCO Wood and Malmø. In HbCO des-His, resonance C is split into two resonances C^1 and C^2: resonance C^2 is approximately at the position occupied by resonance H in HbCO A. Experimental conditions: 5 to 10% HbCO in ^2H_2O containing 0·2 M-HEPES of the appropriate pH.

(Figs 2 and 3), i.e. the derivative that formed the basis of the assignment of Russu *et al.* (1980). As in the spectra of Russu *et al.* (1980), resonance C in HbCO A is no longer present in HbCO des-His. However, careful examination of the spectra of HbCO des-His between pH values 5·8 and 8·4 revealed that the C-2 proton of His FG4(97)β is split into two resonances, C^1 and C^2, of approximately equal intensity, half that of the other histidine resonances (see Figs 2 and 3), which titrate with pK_a values of 7·6 and 6·0, respectively (see Fig. 4). By a remarkable coincidence, the position and pK_a of resonance C^2 is virtually identical to that of resonance H arising from His HC3(146)β in HbCO A; moreover, resonance C^1 in HbCO des-His is superimposed on resonances B and D above pH 7·6 and on resonance D between pH values 6·6 and 7·6; only between pH 5·8 and pH 6·3 is resonance C^1 clearly visible as a separate peak of approximately half intensity just to high field of resonance D (see, e.g. Fig. 3 at pH 6·29). It is this combination of shifts that misled Russu *et al.* (1980). They probably failed to detect resonance C^1 as well as C^2 in HbCO des-His because they processed their spectra with resolution enhancement that distorted the intensities of their resonances and because their spectra were measured at a lower field strength, 250 MHz as opposed to 500 MHz. In contrast, at the high ionic strength used by Kilmartin *et al.* (1973), the upfield shifts and splitting of the resonance of His FG4(97)β in HbCO des-His did not occur.

In order to check that the two resonances C^1 and C^2 in HbCO des-His really do arise from one histidine in equilibrium between two conformations, we carried out transfer of saturation experiments at pH 6·29 and pH 6·98. At both pH values we found a small (5 to 10%), but distinct and specific, transfer of saturation of resonance C^1 on irradiating

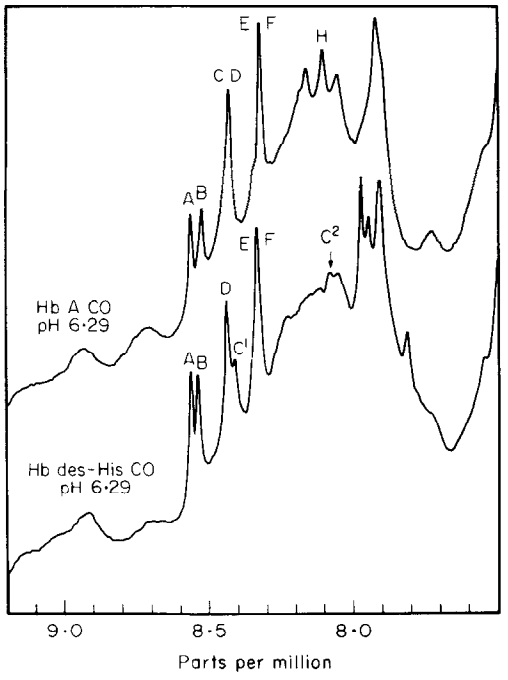


Figure 3. Spectra (500 MHz ^{1}H n.m.r.) of histidine C-2 proton resonances of HbCO A and HbCO des-His. Conditions as for Fig. 2.

resonance C^2 for 0·5 second and 1 second (data not shown). (Note that this cannot have arisen through a nuclear Overhauser effect, as there are no two C-2 histidine protons less than 5 Å apart in haemoglobin.) Assuming a spin lattice relaxation time of 0·1 to 0·2 seconds for the C-2 proton of His FG4(97)β, the exchange rate between the two conformation states of His FG4(97)β lies in the range 0·5 to 2 per second in salt-free HEPES buffer. This exchange rate and the equilibrium between C^1 and C^2 depend on ionic strength: thus, in NaCl-free bis-Tris or HEPES buffers, both C^1 and C^2 are present (Figs 2 and 3); in 0·1 M-bis-Tris +0·15 M-phosphate, neither resonance C^1 nor C^2 is visible (Fig. 7 of Russu *et al.*, 1980); finally, in 0·2 M-phosphate + 0·2 M-NaCl, resonance C^1 is present but C^2 is absent (Kilmartin *et al.*, 1973; and unpublished spectra of Kilmartin *et al.*, 1973, which were shown to us).

4. Discussion

(a) *Contribution of His HC3(146)β to the alkaline Bohr effect*

The pK_a values of this histidine have been measured by several investigators (Table 1). Regardless of ionic strength, its pK_a in Hb is 8·0 to 8·1, which shows that, contrary to popular belief, the free energy of salt bridges at the protein surface is hardly influenced by salt in the solvent. In HbCO, on the other hand, its pK_a rises sharply with ionic strength. Table 1 shows that, in salt-free medium, this histidine releases more protons on oxygenation than the total found to be released by Hb experimentally. This implies that its increased contribution to the alkaline Bohr effect must be partly compensated for by the increased contributions of other residues to the acid Bohr effect.

What is the stereochemical basis of that ionic

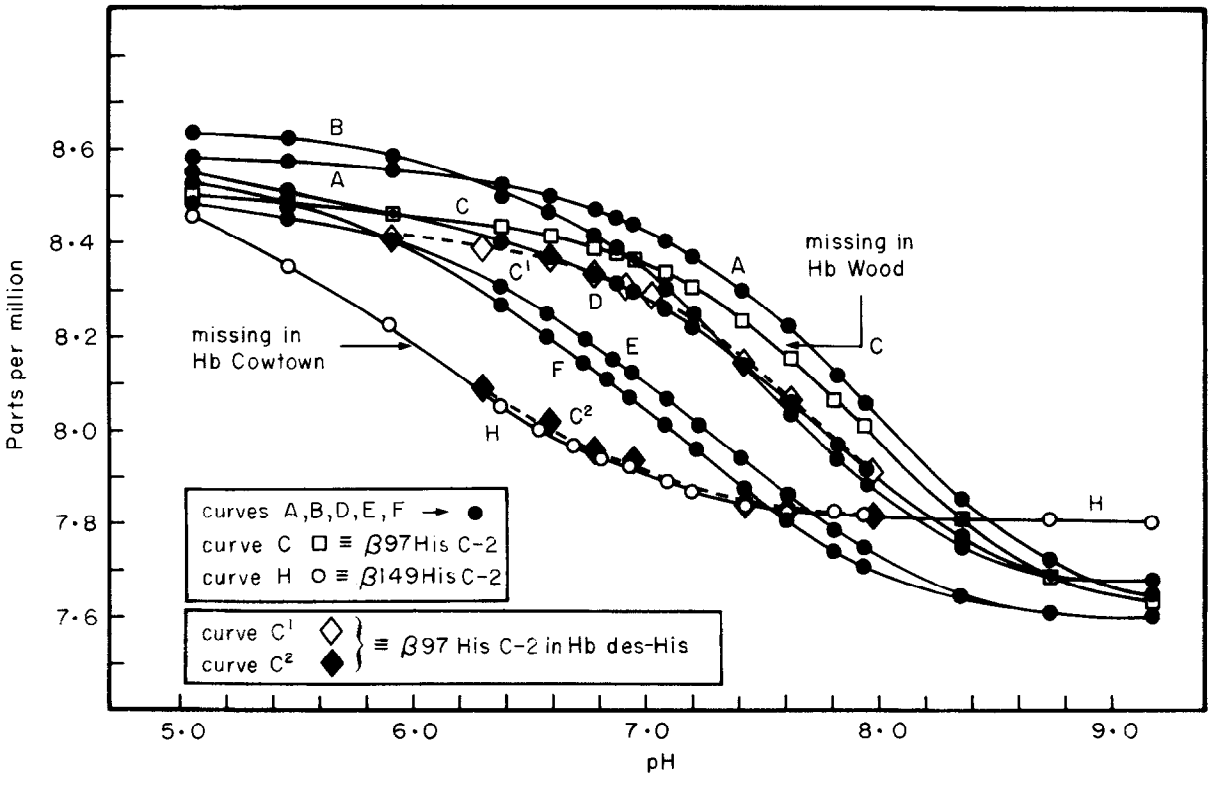


Figure 4. Titration of C-2 proton resonances of histidines in human HbCO A and HbCO des-His at $[Cl^-] \leqslant 60$ mM. The titration curves for resonances A, B, D, E and F are identical in Hb A and Hb des-His. In Hb des-His, however, the C-2 proton of His FG4(97)β is split into 2 resonances C^1 and C^2 with pK_a values of 7·6 and 6·0, respectively, compared to the pK_a of 7·8 for the single resonance C in Hb A.

Table 1
pK_a values of histidine HC3(146)β in human carbonmonoxyhaemoglobin

Method of measurement	Buffer	[Cl^-]	pK_a	Number of protons per tetramer released by His HC3(146)β at pH 7·4 (calculated)	Total number of protons per tetramer released at pH 7·4 (observed)‡	Reference
n.m.r. titration of C-2 proton resonances	0·1 M bis-Tris or Tris	5–60 mM	6·2†	1·48	1·2 at [Cl^-] = 5 mM 2·0 at [Cl^-] = 60 mM	Russu *et al.*, 1980
n.m.r. titration of C-2 proton resonances	0·2 M HEPES	0	6·2	1·48	0·9	This work
Titration by deuterium exchange	0·1 M bis-Tris or Tris with acetate	0·1 M	6·5	1·38	2·0	Matsukawa *et al.*, 1984
n.m.r. titration of C-2 proton resonances	0·2 M phosphate	0·2 M	7·1	0·96	2·0	Kilmartin *et al.*, 1973

† Titration by Russu *et al.* (1980) when given the correct assignment.
‡ The total numbers of protons per tetramer released as a function of [Cl^-] were taken from de Bruin *et al.* (1973), except for the measurement at [Cl^-] = 0, which is from Perutz *et al.* (1980).

strength dependence? Shaanan's structure of HbO_2 at 2·1 Å resolution is isomorphous with that of HbCO and was determined in 3·0 M phosphate buffer of pH 8·5, where all histidines would be uncharged (Shaanan, 1983). It shows the N_δ of His HC3(146)β accepting a hydrogen bond from its own main-chain NH group, and the carboxylate accepting a hydrogen bond from N_ζ of the neighbouring Lys HC1(144)β. Re-examination of Baldwin's (1980) electron density map of human HbCO, a structure determined in phosphate buffer of pH 6·6, showed that His HC3(146)β occupies the same position. The histidine lies in a surface area of the molecule where cationic groups predominate (Val NA1(1), His NA2(2), Lys EF6(82), His H21(143) and Lys HC1(144)β). At low ionic strength these cationic groups evidently depress the pK_a of His HC3(146)β; at high ionic strength their charges are screened and their distant effect may vanish, but the close C-terminal carboxylate takes over and raises the pK_a. The large dependence of the pK_a of this histidine in HbCO upon ionic strength makes one wonder what is its pK_a at the Hb concentration of 20 mM-haem in the red cell. The titration curves of the resonances of HbO_2 in red cells have been recorded, but without any assignments (Brown *et al.*, 1976). They look quite different from those in more diluted buffer solution, suggesting that many of the pK_a values are markedly altered by the high HbO_2 concentration and possibly also by the presence of 2,3-diphosphoglycerate.

(b) *Abnormally high pK_a of a histidine residue capping the C terminus of a helix*

How significant is the raised pK_a of His FG4(97)β? The latest n.m.r. titrations of free histidine in 0·1 M-NaCl at 23°C yielded pK_a values for its three titratable groups of 1·72, 6·24 and 9·24 (Tanokura *et al.*, 1978), not very different from the values of 1·82, 6·0 and 9·17 at 20°C, which were determined 49 years earlier from measurements of electromotive force in cells with liquid junctions (Schmidt *et al.*, 1929; Cohn & Edsall, 1943). Since the pK_a of the imidazole in histidine is likely to be shifted by its neighbouring carboxylate and ammonium ions, *N*-acetyl-L-histidine-methylamide is a better model for the behaviour of the imidazole side-chain on the surface of globular proteins. In 0·1 M-NaCl at 30°C, the temperature of our measurements, this has a pK_a of 6·54 in ^2H_2O, and of 6·43 in H_2O. Absence of NaCl reduced the pK_a values by only 0·05 (Tanokura *et al.*, 1978). For comparison, His105 in ribonuclease, which has a normal reactivity and is exposed to solvent, has a pK_a of 6·72 (Markley, 1975). Formation of salt bridges, i.e. hydrogen bonds between the imidazoles of histidine and carboxylate or phosphate ions at the protein surface has been found to raise the pK_a values of imidazoles by about one unit, corresponding to a free energy change of about 1·4 kcal (5·9 kJ) at 30°C. For example, 2,3-diphosphoglycerate binds to human Hb by forming four pairs of salt bridges, two of them with His NA2(2)β and His H21(143)β. Formation of the salt bridges raises the pK_a values of these histidines from 6·7 to 7·6 (Ferridge *et al.*, 1979). The pK_a values of 7 of the 12 histidines in sperm whale myoglobin have been measured. All but one have pK_a values ranging from 6·73 to 5·43, but His CD6(36), which forms a salt bridge with Asp E3(60), has a pK_a of 7·97 (Botelho *et al.*, 1978; Carver & Bradbury, 1984). Critics have argued that hydrogen bonding to an uncharged carbonyl might produce a rise in pK_a comparable to that produced by a hydrogen bond to a carboxylate. If that were true, then substitution of asparagine for aspartate at position FG1(94)β should hardly influence the magnitude of the alkaline Bohr effect. In fact, that substitution halves it (Como *et al.*, 1983). Values of pK_a below 6·0 are found among histidines that interact with each other (Markley & Finkenstadt, 1975) or with other cationic groups, or when they

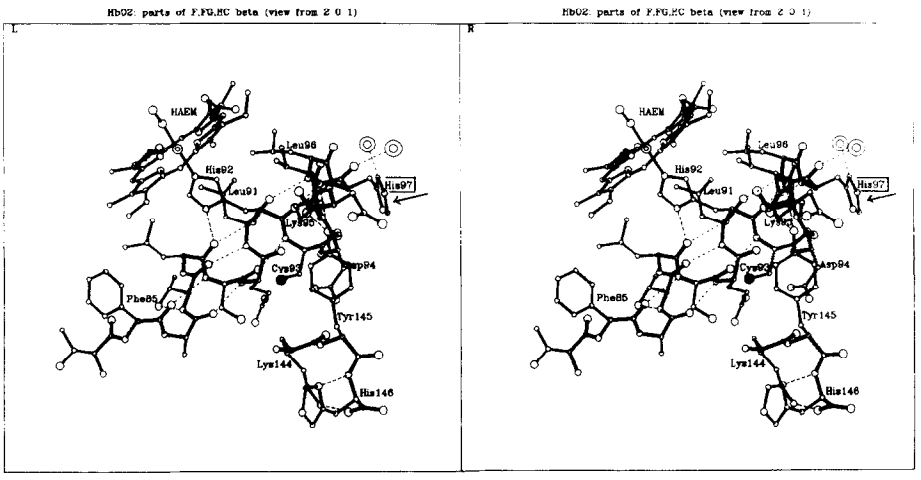


Figure 5. Stereo-diagram showing the haem-linked helix F with His FG4(97)β at its C terminus (arrow). The double circles are water molecules. The hydrogen bond from the main-chain NH to the imidazole of His HC3(146)β and from its carboxylate to Lys HCl(144)β are shown as broken lines at the bottom of the diagram.

are buried. For example, the distal histidines buried in the haem pockets of myoglobin and haemoglobin have pK_a values of about 5·5 (Ikeda-Saito *et al.*, 1977). Since we could find no previous instance of a histidine with a pK_a above 7·5 that is not hydrogen-bonded to an anion, our discovery of a histidine (FG4(97)β) with a pK_a of 7·8 that lies 7·7 Å (from N_ε to O_δ) away from the nearest carboxylate (Asp FG1(94)β) surprised us. The imidazole of this histidine caps the C terminus of the π-helix FG1–FG4, which is the continuation of the α-helix F1–F9. Its N_δ is hydrogen-bonded to a water molecule (Shaanan, 1983) (Fig. 5).

Polypeptide helices are dipolar, with the negative pole at the C terminus. For example, the apparent dipole moment of α-helical poly-L-glutamate in 1,2-dichloroethane or *o*-cresol is 3·4 D ($3{\cdot}4 \times 10^{-18}$ F cm) per residue (apparent, because the internal field of the helix was assumed to equal the externally applied one) (Wada, 1976). This large moment arises, because the dipoles of the main-chain amide groups are aligned at angles of 12° with the axis of the helix. Hol *et al.* (1978) have pointed out that "the field of such a helix dipole is equal to the field of a positive charge at the amino end and a negative charge at the carboxyl end, each of a magnitude of $0{\cdot}8 \times 10^{-19}$ C or half an elementary charge"; they argued that, as a consequence, ionizable groups should interact strongly with the ends of helices. Our discovery of a histidine with a pK_a of 7·8 at the C-terminal end of a helix bears this out and suggests that in a surface crevice of a protein this interaction has a free energy of about 1·5 to 2 kcal mol^{-1}, depending upon the exact value assumed for the pK_a of free histidine. In a recent review, Hol (1985) gives examples of interactions between ionizable groups and the fields at the ends of helices, including one of a cysteine residue in the enzyme rhodanese that is in contact with the N termini of two helices and has a pK_a of 6·5, three units below normal and equivalent to a free energy change of 2 kcal mol^{-1} per helix (Schlesinger & Westley, 1974; Ploegman *et al.*, 1979). This is close to our estimate of 1·5 to 2 kcal mol^{-1}. Similar free energy changes are observed in salt bridges between unit charges in surface crevices of proteins, which suggests that the original estimate of a field equivalent to half an elementary charge is too conservative, and that the true field is closer to the higher estimate of 0·75 e given in Hol's review.

(c) *Nature of conformational equilibrium in HbCO des-His*

The n.m.r. spectra show that HbCO des-His is in equilibrium between two conformations; in one of them His FG4(97)β has a pK_a of about 7·6, close to that of 7·8 in HbCO A. This conformation is most stable at high ionic strength. As the ionic strength is lowered, a second conformation comes into equilibrium with the first. In this conformation the histidine has a pK_a of about 6·0. We have crystallized HbCO des-His from concentrated phosphate buffers and found the crystals to be isomorphous with HbCO A. Since HbCO does not crystallize at low ionic strength, we have no direct evidence about its structure there and must therefore search for indirect clues.

We have seen that in HbCO A the imidazole of His FG4(97)β caps the C terminus of helices F and FG. Cys F9(93)β, which is one turn of a helix below His FG4(97)β, has its side-chain *cis* to the main-chain NH and wedged into a pocket between helix F and Tyr HC2(145)β (Baldwin, 1980; Shaanan, 1983). Cleavage of the Tyr—His bond liberates the carboxyl group of Tyr HC2(145)β that lies about 7 Å from the β-carbon of Cys F9(93)β; we suggest that its negative field switches the sulphydryl side-chain to its external (*cis* to CO) position, where it forms a hydrogen bond with one of the carboxylate oxygens. We further suggest that the switch of the SH group causes some change in conformation of the helices F and FG, thus causing the change in pK_a of His FG4(97)β.

Our interpretation, if true, would explain one observation that has long puzzled us. The oxygen affinity of Hb des-His is higher than that of Hb A at low oxygen saturation and *vice versa*, so that the equilibrium curves cross (Kilmartin & Wootton, 1970); this is unusual as the curves of different Hb

derivatives generally converge at the top. Switching the SH group from its internal to its external position, for instance with sulphydryl reagents, is known to lower the oxygen affinity of free β-chains (Antonini & Brunori, 1971) and of the quaternary R-structure. If liberation of the carboxylate of Tyr HC2(145)β switched the SH group to its external position, this would explain the crossing of the curves: at low fractional saturation with oxygen, there is dominance of the T-structure, in which the SH group is always external; its oxygen affinity is *raised* by the absence of the salt bridge between His HC3(146)β and Asp FG1(94)β. At high fractional saturation with oxygen, the R-structure dominates; in low spin Hbs, the SH group is normally internal, so its oxygen affinity would be *lowered* by the switching of the SH group to the external position. More convincing than the interpretation of a past observation is the prediction of a new one: formation of the hydrogen bond between the SH group and the carboxylate of Tyr HC2(144)β should lead to an observable lowering of the SH-stretching frequency of Cys F9(93)β. This prediction will be tested.

The ΔH^+ *versus* pH curve of Hb des-His differs from that of Hb A by more than the calculated absence of the contribution of His HC3(146)β to the alkaline Bohr effect. For example, Hb des-His shows a substantial positive Bohr effect at pH 6·0 and a negative one at pH 5·0 (Kilmartin & Wootton, 1970; Saroff, 1972; Matsukawa *et al.*, 1984). Our results suggest that this is probably due to the pH-dependent changes in the equilibrium between the two different conformations of His FG4(97)β, which make this histidine contribute to the Bohr effect of Hb des-His even though it does not contribute to the Bohr effect of Hb A (Perutz *et al.*, 1980).

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