previously reported results (Oi et al., 1970; Reed & Swindell, 1969).

## Discussion

The results reported here show that addition of benzylamine to plasma amine oxidase under anaerobic conditions results in the formation of 1 mol of benzaldehyde and 1 mol of $NH_4^+$ per mol of enzyme subunit (active site). These results are consistent with the mechanism that we have proposed involving the oxidation of the substrate to an enzyme-bound Schiff base, which is hydrolyzed prior to release from the enzyme (Scheme I). On the other hand, the results reported here do not agree with the proposal that the immediate product of the oxidation is the aldehyde and pyridoxamine. Therefore, these results together with previous experiments (Suva & Abeles, 1978) make the pyridoxal mechanism for bovine plasma amine oxidase untenable. We wish to point out that the reports, referred to above, that $NH_4^+$ is not released under anaerobic conditions were based on experiments with enzymes other than the beef plasma enzyme. It is, therefore, possible that amine oxidase from other sources operates through a different mechanism. In view of the many similar properties which these amine oxidases show, we consider that possibility unlikely. We believe that none of the amine oxidases is a pyridoxal enzyme.

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# Role of C-Terminal Histidine in the Alkaline Bohr Effect of Human Hemoglobin†

John V. Kilmartin, Jan H. Fogg, and Max F. Perutz*

ABSTRACT: We have determined the alkaline Bohr effect in concentrated hemoglobin solution (~100 mg/mL) at low ionic strength (10 mM $Cl^-$) in the absence of phosphate in two chemically modified hemoglobins (desHis-146$\beta$ hemoglobin and hemoglobin *N*-ethylsuccinimide) in which only the contribution of the C-terminal histidine to the alkaline Bohr effect is inhibited. We found that either of these modifications results in the inhibition of 60% of the alkaline Bohr effect. We have also compared the structure of salt-free methemoglobin crystals with that of methemoglobin crystals precipitated from ammonium sulfate. A difference electron-density map shows the salt bridge between the C-terminal histidine and the nearby aspartate FG1(94)$\beta$ to be absent in both. These results disprove the conclusions of Ho & Russu [Ho, C., & Russu, I. M. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 179–194, Academic Press, New York], Russu et al. [Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043–1052], and Matthew et al. [Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979) *Biochemistry 18*, 1928–1936] that in the absence of phosphate and at low concentrations of chloride the salt bridge remains intact in liganded hemoglobins and that the C-terminal histidine therefore makes only a minor contribution to the alkaline Bohr effect. On the contrary, our results show that the fractional contribution of the histidine increases at low chloride concentration. The p*K* changes calculated by Matthew et al. [Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979) *Biochemistry 18*, 1919–1928] are based on an incorrect position of this residue in oxyhemoglobin; when this residue is positioned correctly, the equations of Matthew and co-workers predict the major p*K* change which we have observed.

"Love people, slay errors."—St. Augustine.

One of the successes of the X-ray analysis of hemoglobin was a structural interpretation of most of the alkaline Bohr effect (Perutz et al., 1969; Kilmartin & Rossi-Bernardi, 1969; O'Donnell et al., 1980). In deoxyhemoglobin, which has the quaternary T structure, the terminal residues form the salt bridges shown in Figure 1, whereas in liganded hemoglobins, which have the R structure, they are free. In the light of this evidence we suggested that the p$K_a$ values of the C-terminal histidine [HC3(146)$\beta$] and the N-terminal valine [NA1(1)$\alpha$] are normal in liganded hemoglobins but are raised through their linkage to anionic groups in deoxyhemoglobin. This was confirmed by chemical studies which showed that the histidine contributes ~40% and the valine 20–30% to the alkaline Bohr effect (Kilmartin, 1977). The contribution of the histidine was

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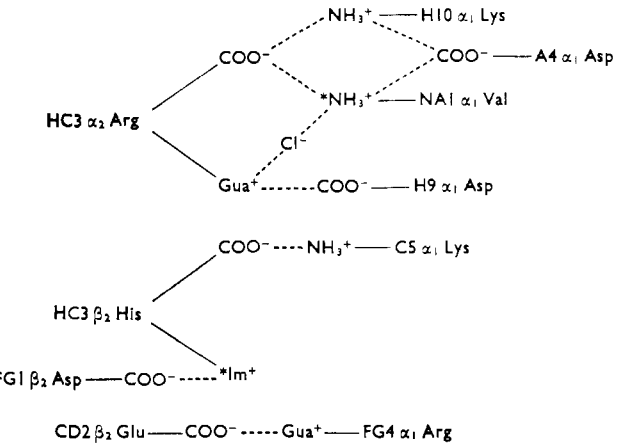


FIGURE 1: Salt bridges in human or horse deoxyhemoglobin [reproduced with permission from Baldwin (1976)].

further proved by titrating it with proton NMR in 0.2 M chloride and 0.2 M phosphate, yielding p$K_a$ values of 8.0 in deoxyhemoglobin and 7.1 in carbonmonoxyhemoglobin (Kilmartin et al., 1973).

We now wish to discuss two studies, one experimental and the other theoretical, which question these findings. Ho & Russu (1978) and Russu et al. (1980) tried to measure the p$K_a$ values of all histidines in human hemoglobin by proton NMR in 0.1 M Bistris or 0.1 M Tris-HCl in $D_2O$ with [$Cl^-$] ranging from 5 to 60 mM, in the absence of phosphate. They report p$K_a$ values for His HC3(146)$\beta$ of 8.0 in deoxy- and 7.9 in carbonmonoxyhemoglobin, which lead them to conclude that under their conditions, i.e., at low [$Cl^-$] and in the absence of phosphate, its salt bridge is not broken in going from the deoxy to the carbonmonoxy form and does not contribute significantly to the alkaline Bohr effect. Matthew et al. (1979a,b) applied a modification of the discrete-charge electrostatic theory of Tanford & Kirkwood (1957) and Tanford (1957) to the p$K_a$ values of all ionizing groups in deoxy- and oxyhemoglobin. Their calculations also suggest that at 10 mM $Cl^-$, i.e., under the conditions of Ho & Russu's NMR titrations, many groups contribute to the alkaline Bohr effect, each of them, including His HC3(146)$\beta$, undergoing only a small change of p$K_a$. In 0.1 M $Cl^-$, i.e., the conditions used in most of our Bohr titrations, they attribute the alkaline Bohr effect to differential chloride binding by Val 1$\alpha$ and 1$\beta$ and by His G19(117)$\beta$, with His HC3(146)$\beta$ contributing only about 15%.

Ho & Russu (1978) and Russu et al. (1980) used desHis-146$\beta$ hemoglobin (desHisHb)[1] supplied by us to try to identify the proton resonance given by this histidine, but they did not check whether under their conditions (10% hemoglobin and 5–60 mM $Cl^-$) the alkaline Bohr effect of desHisHb is nearly as large as that of hemoglobin A, which it must be if their p$K_a$ values are right, or whether it is reduced to about half, as it would be if ours are correct. We have now done this and have also measured, under the same conditions, the alkaline Bohr effect of a hemoglobin in which the blocking of Cys F9(93)$\beta$ with *N*-ethylsuccinimide inhibits the formation of the C-terminal salt bridge (NES-Hb; Perutz et al., 1969). Our results show that at 100 mg/mL hemoglobin and 10 mM $Cl^-$ the contribution of His HC3(146)$\beta$ to the alkaline Bohr effect is ~60%. We have also done an X-ray study of horse methemoglobin crystals in salt-free solution which shows that the C-terminal histidine is free, proving that low ionic strength does not cause the salt bridge to form in liganded hemoglobins.

Table I: Alkaline Bohr Effect of Hb A, NES-Hb, and desHisHb

| deriv-ative | final concn[a] [Hb] (mg/mL) | final concn[a] [$Cl^-$] (mM) | pH | $\Delta H^+$/Fe | ΔpH | % contribn of His-146$\beta$ to $\Delta H^+$/Fe | % contribn of His-146$\beta$ to ΔpH |
|---|---|---|---|---|---|---|---|
| Hb A | 107 | 100 | 7.3 | 0.55 | 0.211 | | |
| | 107 | 10.4 | 6.8 | 0.35 | 0.184 | | |
| | 107 | 10.4 | 7.2 | 0.33 | 0.181 | | |
| | 107 | 10.4 | 7.2 | 0.34 | 0.181 | | |
| | 107 | 10.4 | 7.4 | 0.32 | 0.184 | | |
| | 107 | 10.4 | 7.6 | 0.27 | 0.157 | | |
| NES | 89 | 8.5 | 6.8 | 0.12 | 0.071 | 66 | 61 |
| | 89 | 8.5 | 6.8 | 0.13 | 0.073 | 63 | 60 |
| | 89 | 8.5 | 7.2 | 0.12 | 0.064 | 64 | 65 |
| | 89 | 8.5 | 7.2 | 0.12 | 0.064 | 64 | 65 |
| | 89 | 8.5 | 7.4 | 0.10 | 0.059 | 69 | 68 |
| desHis | 100 | 10.0 | 7.2 | 0.15 | 0.084 | 55 | 54 |
| | 100 | 10.0 | 7.4 | 0.13 | 0.080 | 61 | 57 |
| | 100 | 10.0 | 7.4 | 0.13 | 0.080 | 61 | 57 |
| | 100 | 10.0 | 7.6 | 0.12 | 0.074 | 56 | 53 |
| mean: | | | | | | 62 | 60 |

[a] The final concentrations of hemoglobin and chloride are those after deoxygenation.

## Experimental Procedures

desHisHb and NES-Hb were prepared as described by Kilmartin et al. (1973). After CO and any remaining diphosphoglycerate (Kilmartin & Rossi-Bernardi, 1971) were removed, the Hb's were concentrated to ~200 mg/mL in an Amicon 400 using a UM-10 membrane. HCl (0.06 N) was carefully added to the stirred Hb solution to reduce the pH to 6.6; then 2.5 M KCl and water were added to adjust the final concentration of $Cl^-$ to 10 mM and of Hb to 100 mg/mL. The Hb's were deoxygenated by repeated evacuation and flushing with nitrogen in a glass bottle with a thin rubber bung until a hand spectroscope showed less than 5% $HbO_2$. A Hamilton gastight syringe was used to transfer 0.5 mL of Hb to a glass vessel built to the same size as the plastic Radiometer autoburet sample vessel. This contained the Radiometer circular magnetic stirrer and was closed by a thin rubber bung with one hole for the pH electrode (Beckman 39030) connected to a Radiometer 26 pH meter, a second hole for the humidified nitrogen or oxygen, and a third hole for a lead connected to an Agla micrometer syringe. The vessel was placed inside a Radiometer V526 thermostat jacket maintained at 25 °C. The stirred deoxygenated Hb was adjusted to the appropriate pH with deoxygenated 0.06 N KOH delivered by the micrometer syringe; the pH drift was ~0.002 pH unit/10 min, and oxygenation took 10 min. The volume of 0.06 N KOH needed to titrate back to the original deoxy pH was recorded and the Bohr effect calculated. The metHb content at the end of the experiment was <5%.

Salt-free crystals of horse metHb were prepared by concentrating a deionized solution quickly by pressure dialysis to a final concentration of ~10 mg/mL and letting aliquots stand at room temperature. A drop of toluene was added to each aliquot to prevent infection. The crystals formed after a few days were isomorphous with those grown from concentrated salt solutions (Perutz, 1946). X-ray data were recorded on a Hilger & Watts four-circle diffractometer to a resolution of 3.5 Å and corrected by the usual factors. Reflections of spacing >20 Å were excluded, because their intensities are strongly affected by the density of the liquid between the molecules (Bragg & Perutz, 1952). We used the structure

[1] Abbreviations used: Hb, hemoglobin; metHb, methemoglobin; $HbO_2$, oxyhemoglobin; deoxyHb, deoxyhemoglobin; HbCO, carbonmonoxyhemoglobin; desHisHb, desHis-146$\beta$ hemoglobin; NES, *N*-ethylsuccinimide.

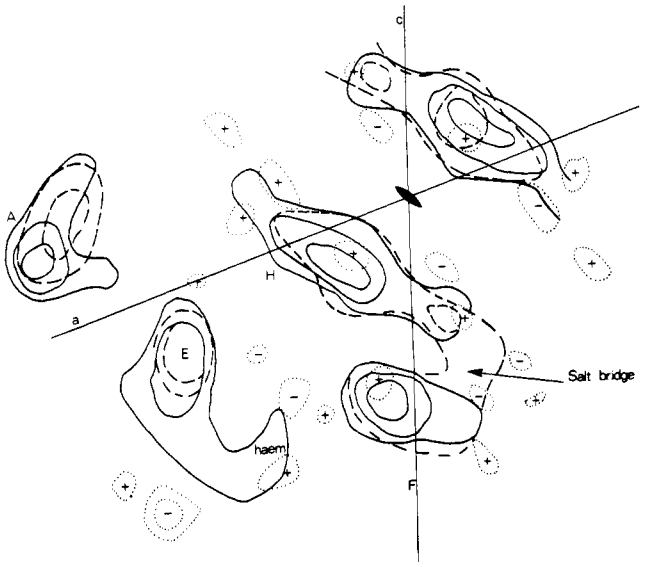


FIGURE 2: Difference electron-density map of salt-free minus salty crystals of horse metHb (dotted contours with + and − marking positive and negative peaks) superimposed on electron-density maps of horse metHb (full contours) and human deoxyHb (broken contours). The section shows parts of helices F and H and the salt bridge linking them in deoxy but not in metHb.

factors and phases of the salty crystals determined by Perutz et al. (1968). Difference electron-density maps were calculated in sections at intervals of $b/60$ = 1.06 Å.

## Results

Our results are shown in Table I. In Hb A at pH 7.3, 100 mM $Cl^-$, and 25 °C, $\Delta H^+/Fe$ = 0.55 and $\Delta pH$ = 0.211, in agreement with accepted standard values. On reduction of the initial $[Cl^-]$ to 10.4 mM, these values drop to 0.33 and 0.18, respectively, in agreement with de Bruin et al. (1974). In NES-Hb and desHisHb the average $\Delta H^+/Fe$ drops to 38% and the average $\Delta pH$ to 40% of their values in Hb A, showing that at this low chloride concentration the C-terminal histidine contributes as much as 60% of the alkaline Bohr effect, compared to 40% in 100 mM $Cl^-$. This decrease was to be expected since it is known that much of the remaining Bohr effect is due to differential chloride binding. The observed reduction of the alkaline Bohr effect might have been spurious if in 100 mM $Cl^-$ desHisHb and NES-Hb had remained in the R structure even when fully deoxygenated; in that case Hill's coefficient would have been unity. In fact, the oxygen equilibrium curve of NES-Hb in 0.05 M Bistris of pH 7.4 (7 mM $Cl^-$) at 25 °C shows a Hill's coefficient of 1.44 which proves that the R to T transition is normal (K. Imai, personal communication).

Figure 2 shows a difference electron-density map of salt-free minus salty crystals of horse methemoglobin at 3.5-Å resolution superimposed on a map at 5.5-Å resolution in which the $\beta$ chains of horse metHb and human deoxyHb are superimposed. The section includes parts of helices F and H. In deoxyHb these are joined by a region of density representing the C-terminal salt bridge. In metHb this density is absent. Figure 3 shows the same section with the difference map of human deoxyHb NES minus deoxyHb A superimposed. This map contains a negative peak (2) due to the displacement of the C-terminal histidine from its salt-bridged position. If that salt bridge were present in the salt-free crystals of horse metHb, then the difference map of Figure 2 should show a positive peak in the corresponding position. In fact, the map is a blank; none of the peaks inside the molecule exceed in

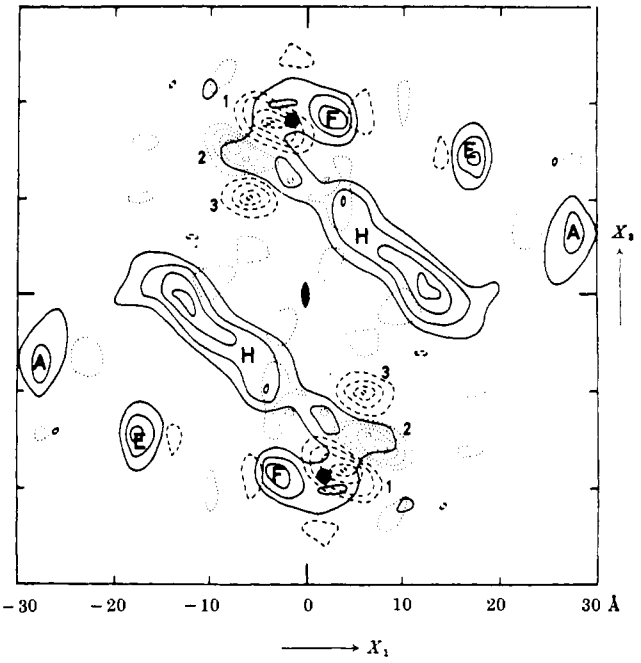


FIGURE 3: Difference electron-density map of human deoxyHb NES minus deoxyHb A (broken contours denote positive peaks and dotted contours denote negative peaks) superimposed on an electron-density map of human deoxyHb A. The regions shown are the same as in Figure 2. Positive peak 1 marks the *N*-ethylsuccinimide residue attached to SH-93$\beta$, negative peak 2 the position of His HC3(146)$\beta$ in deoxyHb A, and positive peak 3 its position in deoxyHb NES [reproduced with permission from Perutz et al. (1968)].

magnitude the general background of random peaks in the liquid regions. This shows that the C-terminal salt bridge is absent from horse metHb at zero ionic strength. It might be argued that human HbCO might behave differently from horse metHb, but the two species have identical alkaline Bohr effects and have the same amino acid sequences in the region concerned. Moreover, if the salt bridge is absent from high-spin metHb, it is even more likely to be absent from low-spin HbCO.

It might also be argued that the removal of the C-terminal histidine or the attachment of *N*-ethylsuccinimide produces structural changes in the hemoglobin molecule which inhibit contributions by other residues to the alkaline Bohr effect. This possibility has been excluded by X-ray analysis of these two derivatives. Difference electron-density maps show that their structures are the same as that of Hb A except for the loss or displacement of the histidine (Perutz et al., 1969; Perutz, unpublished experiments). This does not exclude minor structural adjustments of the kind that give rise to small chemical shifts of various proton resonances, but such shifts do not imply structural changes large enough to have a significant influence on the alkaline Bohr effect.

## Discussion

*Steric Opposition to the C-Terminal Salt Bridge in Liganded Hemoglobins.* Why does the C-terminal salt bridge not form in the R structure—not even at zero ionic strength where one would expect it to be favored? One reason is the coupling between the coordination number and spin of the iron on the one hand and the occupancy of the so-called tyrosine pocket between helices F and H on the other hand (Figure 4). When the iron is high spin and five coordinated, the SH group of Cys F9(93)$\beta$ is external, and the side chain of Tyr HC2(145)$\beta$ lies in the pocket with its OH hydrogen bonded to the CO of Val FG5(98)$\beta$. When the iron is low spin and six coordinated, the SH group displaces the tyrosine from its pocket, possibly

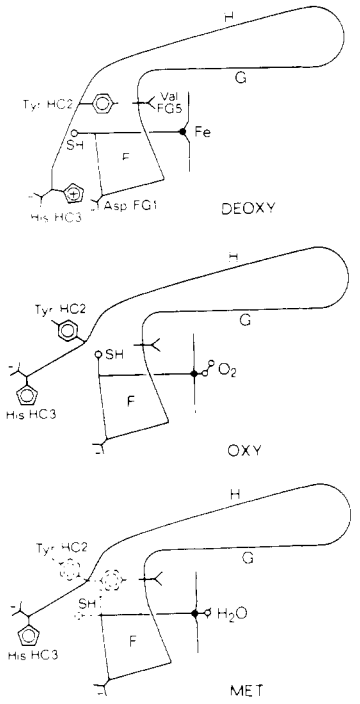


FIGURE 4: Occupancy of tyrosine pocket between helices F and H and position of C-terminal histidine in deoxy Hb, $HbO_2$ or HbCO, and metHb.

because changes in tertiary structure do not allow the crucial hydrogen bond with the valine to be formed, so that the tyrosine and the preceding lysine HC1(144)$\beta$ now hang loose. When the iron is high spin and six coordinated, as in metHb, an equilibrium exists between the two conformations (Heidner et al., 1976). In the T structure Lys C5(40)$\alpha$ lies within reach of the carboxyl group of His HC3(146)$\beta$, so that this residue is tied down by two salt bridges (Figure 1). In the R structure, that lysine is 13 Å further away so that the histidine could form only the single bridge to Asp FG1(94)$\beta$ of its own chain. In the T structure, therefore, the three C-terminal residues are tied down by two salt bridges and one strong internal hydrogen bond, while in the R structure only a single salt bridge is sterically possible, and its free energy may be lower than the thermal energy of the three residues.

*Other Evidence for the Large Contribution of His HC3-(146)$\beta$ to the Alkaline Bohr Effect.* In 0.1 M KCl and at 25 °C, which have been our standard conditions, ~40% of the alkaline Bohr effect is inhibited in desHisHb (Kilmartin & Wootton, 1970), and in abnormal hemoglobins in which the C-terminal histidine is replaced either by aspartate (Imai, 1968; Perutz et al., 1971), by arginine (Wajcman et al., 1975), or by proline (Barem et al., 1976), the Bohr effect is similarly diminished. The contribution of the histidine was confirmed by the NMR titrations already mentioned in the introduction (Kilmartin et al., 1973) and also by deuterium exchange (Ohe & Kajita, 1977a). While the NMR titrations were carried out in the presence of inorganic phosphate, the deuterium-exchange measurements were done in 0.2 M Tris-HCl or 0.2 M acetic acid–sodium acetate buffers free from phosphate and gave $pK_a$ values of 8.2 in deoxyHb and 7.0 in $HbO_2$, in agreement with our values of 8.0 in deoxyHb and 7.1 in HbCO found by proton NMR in 0.2 M phosphate. This proved that the $pK_a$ values are independent of inorganic phosphate. They are also independent of organic phosphates (Ohe & Kajita, 1977b; Kilmartin et al., 1978).

It has been argued that our Bohr titrations were done in pure KCl solutions, while the NMR titrations were done in Tris and Bistris buffers which might have changed the p$K$ of the histidine, but Benesch et al. (1969) have shown that Bistris has no influence on the log $p_{50}$ of hemoglobin, and thus it cannot influence the p$K$ values of the Bohr groups. Further, it has been argued that our X-ray work was done with horse hemoglobin and may therefore not be relevant to the results obtained by Ho and co-workers and Matthews and co-workers on *human* hemoglobin, but there are no amino acid differences between these two species that affect the allosteric mechanism or the Bohr effect. Finally, Ohe's titration of His HC3(146)$\beta$ in oxyhemoglobin has been questioned because it was accompanied by some methemoglobin formation and because their titration curve was unsymmetric. They have now repeated the titration of His HC3(146)$\beta$ with HbCO and obtained a symmetrical curve with a $pK_a$ of 7.0; no methemoglobin was formed (M. Ohe, personal communication).

We now come to the calculations of Matthew et al. (1979a,b). They used the coordinates of Fermi (1975) for human deoxyhemoglobin, which are correct. For $HbO_2$ they applied the transformation matrix of Cox (1967) to reorient the deoxy subunits to their positions in liganded hemoglobins but neglected the changes in tertiary structure which accompany the binding of ligands; thus, their coordinates for the quaternary oxy structure are incorrect, especially for the C-terminal residues of both $\alpha$ and $\beta$ chains which float freely in the solvent and therefore no longer make significant contributions to the electrostatic potential of the molecule. At the C terminus of the $\beta$ chains, rupture of the salt bridge between His HC3(146)$\beta$ and Asp FG1(94)$\beta$ increases the distance between the charges on these residues from 3 Å in deoxyhemoglobin to an estimated mean of ~10 Å, in oxyhemoglobin, a change which was neglected in Matthew and co-workers' calculations. In consequence, they obtained a change in $pK_i$ at pH 7.60 for this histidine on going from deoxy- to oxyhemoglobin of only +0.25 at ionic strength $I$ = 0.10 and +0.48 at $I$ = 0.01. We have recalculated these p$K$ changes by using the change in distance between the two charged groups mentioned above, together with the electrostatic free energies in Figure 1 and eq 4 of Matthew et al. (1979a). Our calculated p$K$ changes are +1.28 at $I$ = 0.10 and +1.35 at $I$ = 0.01. These values are somewhat higher than the observed changes but could be scaled down by applying a solvent-accessibility factor of the kind employed by Matthew and co-workers to the electrostatic free energy or by delocalizing the positive charge over the imidazole ring instead of concentrating it on $N_\epsilon$.

Realizing that part of the alkaline Bohr effect arises from differential chloride binding, Matthew and co-workers fitted chloride ions to Val NA1(1)$\alpha$, where they are known to occur, and also to His G19(117)$\beta$, apparently following a suggestion by Tucker & Perutz (1977) that this might be a differential chloride binding site. However, though Tucker and Perutz raised this possibility in the introduction to their paper, their results showed no evidence for a differential anion binding site in that position. His G19(117)$\beta$ is absent in hemoglobin A2 which has the same Bohr effect as hemoglobin A, and there are no other changes in ionizing residues on going from hemoglobin A to A2 which could compensate for the loss of that histidine's supposed contribution to the Bohr effect (Perutz et al., 1980). Other investigators have found that the "missing" part of the chloride-linked alkaline Bohr effect is, in fact, associated with cationic residues in the internal cavity between the two $\beta$ chains. Matthew et al. (1979b) also predict that His H21(143)$\beta$ makes a contribution to the alkaline Bohr effect at pH 7.6, while Perutz et al. (1980) discovered that

it is one of the residues responsible for about half the acid Bohr effect at pH 5.5.

## Conclusions

Contrary to Ho & Russu (1978) and Russu et al. (1980), the contribution of the C-terminal histidine to the alkaline Bohr effect in 0.1 M chloride is independent of the presence of phosphate, either inorganic or organic; its contribution as a fraction of the total alkaline Bohr effect increases from 40 to 60% as the chloride concentration is lowered from 100 to 10 mM. X-ray analysis shows that in the R structure this histidine forms no salt bridge with Asp FG1(94)$\beta$ even at zero ionic strength. The contributions of this histidine and of several other residues to the alkaline Bohr effect calculated by Matthew et al. (1979a,b) are based on incorrect atomic coordinates of the oxyhemoglobin structure and are at variance with those observed.

## Added in Proof

Ohe & Kajita (1980) have reported titrations by deuterium exchange of all histidines in human deoxyHb and HbCO. They found significant changes in p$K_a$ for His B1(20)$\alpha$, His FG1(89)$\alpha$, His H21(143)$\beta$, and His HC3(146)$\beta$.

| His no. | p$K_a$ HbCO | p$K_a$ deoxyHb | $\Delta$p$K_a$ |
|---|---|---|---|
| B1(20)$\alpha$ | 7.0 | 7.6 | 0.6 |
| FG1(89)$\alpha$ | 5.6 | 7.2 | 1.6 |
| H21(143)$\beta$ | 6.1 | 5.6 | −0.5 |
| HC3(146)$\beta$ | 7.0 | 8.1 | 1.1 |

All the p$K_a$ values for deoxyHb and also those for His H21(143)$\beta$ and His HC3(146)$\beta$ in HbCO are consistent with the atomic models of hemoglobin and with the studies of Perutz et al. (1980), but the p$K_a$ values reported for His B1(20)$\alpha$ and His FG1(89)$\alpha$ in HbCO are not. His B1(20)$\alpha$ forms a salt bridge with Glu B4(23)$\alpha$ in both deoxyHb and in HbCO and must therefore have the same raised p$K_a$ in both structures. It is absent from opossum Hb which has a normal alkaline Bohr effect. His FG1(89)$\alpha$ is external and free and should have the same fairly normal p$K_a$ in both deoxyHb and HbCO. It is absent from cat Hb which has a normal alkaline Bohr effect. For these reasons neither of these two histidines is likely to contribute significantly to the alkaline Bohr effect.

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