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Biochemistry

Structure of deoxyhemoglobin Cowtown [His HC3(146)β→Leu]: Origin of the alkaline Bohr effect and electrostatic interactions in hemoglobin

(abnormal hemoglobin/x-ray analysis)

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ABSTRACT **Hemoglobin Cowtown [His HC3(146)-β→Leu] exhibits high oxygen affinity and a halved alkaline Bohr effect. X-ray analysis shows the COOH-terminal leucine to be in equilibrium between two positions: one with the salt bridge between the terminal carboxyl and Lys C5(40)α intact and the leucyl side chain leaning against main chain atoms of helices F and FG and the other with the terminal salt bridge broken and the leucyl side chain touching Pro C2(37)α. Structural changes are confined to the immediate neighborhood of the COOH terminus, showing the halving of the alkaline Bohr effect to be due directly to the loss of the histidine, without significant contributions from changes in pK values of other ionizable groups due to structural changes elsewhere.**

One of the successes of x-ray analysis of hemoglobin was a structural interpretation of the alkaline Bohr effect (1–5). In deoxyhemoglobin, which has the quaternary T structure, the terminal residues form the salt bridges shown in Fig. 1 whereas, in oxyhemoglobin, which has the quaternary R structure, they do not interact with neighboring subunits. In consequence, oxygen uptake reduces the pK values of several weak conjugated bases, and protons are released. At very low concentrations of chloride, the main contribution to the alkaline Bohr effect comes from His HC3(146)β (5), while at higher chloride concentrations Val NA1(1)α and Lys EF6(82)β between them contribute up to 60%. The contribution of His HC3(146)β in phosphate-free solutions has been questioned by Russu *et al.* (6, 7) on the basis of NMR titrations and by Matthew *et al.* (8, 9) on the basis of electrostatic calculations. Since the appearance of those papers several abnormal hemoglobins have been discovered in which the COOH-terminal salt bridges are broken and the oxygen equilibria are disturbed. One particularly interesting case is hemoglobin Cowtown, in which the normal salt bridge between the imidazole of His HC3(146)β and Asp FG1(94)β is abolished by the substitution His → Leu. This hemoglobin has a high oxygen affinity and a halved alkaline Bohr effect in phosphate-free solutions (10). Are these abnormalities due directly to the replacement His → Leu, which leaves the rest of the molecule unaltered, as work by Kilmartin, Perutz, and co-workers (1, 2, 4, 5) would lead us to believe, and as Shih *et al.* suggested (10), or are they the indirect result of more widespread structural changes caused by this replacement that induce significant alteration in pK of several other ionizable residues, as the proposals of Russu *et al.* (6, 7) and Matthew *et al.* (8, 9) would require? We have carried out an x-ray analysis of Hb Cowtown at 2.8 Å resolution to answer this question.

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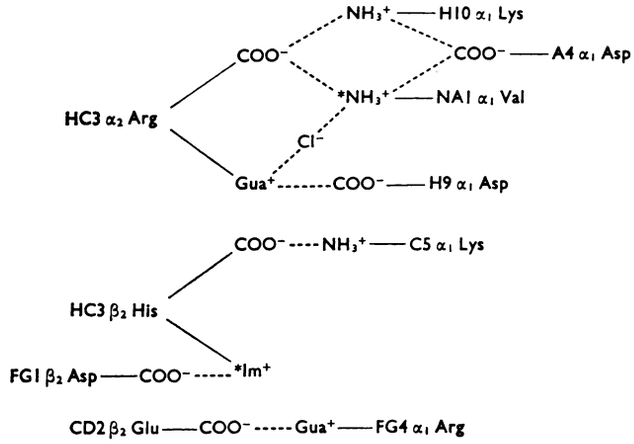

FIG. 1. Salt bridges in human deoxyhemoglobin.

EXPERIMENTAL

Deoxy Hb Cowtown was isolated as described (10) and crystallized according to Perutz (11). Friedel pairs of about 14,000 unique reflections extending to 2.8 Å resolution were measured with one fragment cut from a large crystal on a Nonius Cad 4 x-ray diffractometer and corrected by the usual factors. The R factor between symmetry-related reflections was 5.0% on intensity. The R factor between the Cowtown derivative data (F_{der}) and the native hemoglobin data (14) (F_{nat}) was 6.4% on amplitude. An electron-density difference map was calculated with calculated phases of native Hb. The rms difference density (σ) was 0.016 e/A^3. An electron-density map of Hb Cowtown was also calculated as the transform of $[(2|F_{der}| - |F_c|)\exp i\alpha_c]$ for which the amplitudes (F_c) and phases (α_c) were calculated from the native Hb atomic coordinates (14) with His HC3(146)β omitted.

RESULTS

The electron-density difference map plotted over the whole molecular region (not illustrated) showed significant features ($|\Delta\rho| > 3\sigma$) only in the vicinity of the COOH-terminal regions of the β chains. All significant features were approximately symmetric with respect to the molecular dyad. Hence, symmetry-averaged maps of the β-chain COOH-terminal regions were used for further structural investigation. The main features of the symmetry-averaged difference map (Fig. 2) are a negative peak on the side chain of His HC3(146)β and an extended positive peak on the opposite side of the main chain. These features are consistent with one of the configurations of the COOH terminus of Hb Cowtown illustrated in Figs. 2 and 3, wherein the leucyl side chain is in contact with Pro C2(37)α and the salt bridge between the COOH-terminal

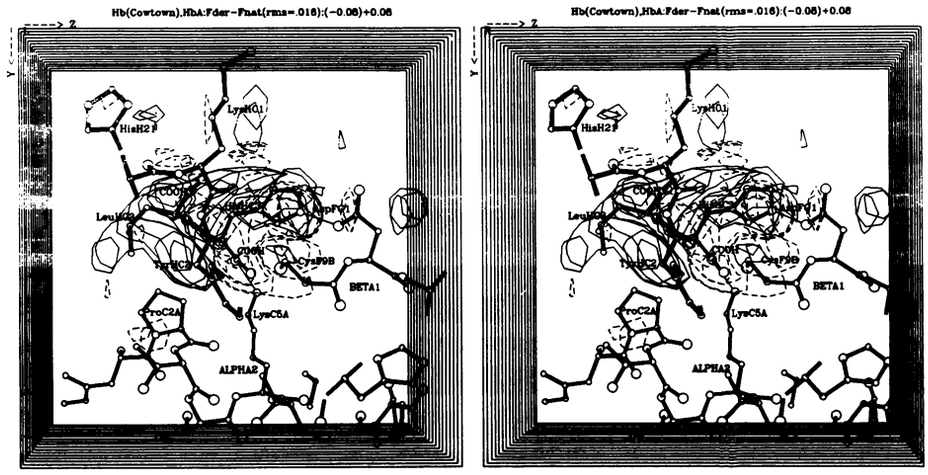

FIG. 2. Symmetry-averaged electron-density difference map of Hb Cowtown − Hb A. Solid contours, +0.08 e/A^3; dashed contours, −0.08 e/A^3. Atomic model: heavy bonds, Hb A; light bonds, possible position of the terminal residue of Hb Cowtown. The salt bridges in Hb A between N^{ϵ} of His HC3β and O^{δ} of Asp FG1β and between COO^- of Asp FG1β, and N^{ζ} of Lys C5α_2 are shown as dotted lines. Viewpoint is along the molecular x-axis (pseudo dyad relating α_2 to β_1). (Scale: 1 Å = 2.7 mm.)

carboxyl group and Lys C5(40)α is broken; to account for the portion of the positive peak under Cβ of Tyr HC2(145)β, one must also assume some shift of the HC2/HC3 β-peptide plane along its normal. However, the electron-density map of Hb Cowtown (Fig. 3) shows that significant density remains, despite the negative peaks in the difference map, for leucine in a configuration unchanged from that of the histidine in native Hb A. Thus, both configurations of the β-chain terminus illustrated in Fig. 2 are occupied. The difference map (Fig. 2) also shows paired positive and negative peaks about the side chain of Asp FG1(94)β, indicating a movement away from its hydrogen-bonded position in Hb A. Slight shifts are also indicated (more evident at lower contour levels than those illustrated) for Pro C2α toward the contacting Leu HC3β and of His H21(143)β and Lys HC1(144)β in a direction opposite to that of the HC2/HC3 peptide plane. No other changes in structure are indicated by the difference map.

DISCUSSION

Since the substitution of leucine for histidine at the COOH terminus leaves the remainder of the molecule essentially undisturbed, the halving of the alkaline Bohr effect in phosphate-free bis(2-hydroxyethyl)imino-tris(hydroxymethyl)-methane (Bistris) cannot be due to alterations of the hemoglobin structure induced elsewhere but must be due directly to loss of the contribution of the COOH-terminal histidine to the alkaline Bohr effect in Hb A. Hb Cowtown displays some other unusual physiological properties. As in Hb A, the first Adair constant K_1 is pH dependent, but the line marking that dependence crosses that of Hb A: in the absence of phosphate, Hb Cowtown has the smaller K_1 above and the larger K_1 below pH 7.6. (K_1 is an association constant, so that a rise means higher oxygen affinity.) K_2 of Hb Cowtown is larger than K_2 of Hb A below pH 7 and the same above pH 7; K_3 of Hb Cowtown is much larger than K_3 of Hb A at all pH values, so that p_{50} is always smaller. K_4 is pH invariant and is the same in the two hemoglobins. Expressed in allosteric terms this means that at physiological pH the substitution of His HC3(146)β by leucine has little influence on K_T and K_R but markedly lowers L. This behavior differs from that of other modified or abnormal hemoglobins, in which any changes in L are coupled to those in K_T so closely that the displacements of the oxygen equilibrium curves can be treated in terms of a single variable (13).

How are these properties to be reconciled with our results? Our electron-density maps show Leu HC3 to be in equilibrium between two positions. In one of them the salt bridge between its carboxylate and Lys C5(40)α remains intact and its side chain leans against atoms of helices F and FG; in the other the carboxylate is free and the side chain touches Pro C2(37)α. The first position is likely to be favored at the low ionic strength of the solutions used in the oxygen equilibrium studies, while the second may become signifi-

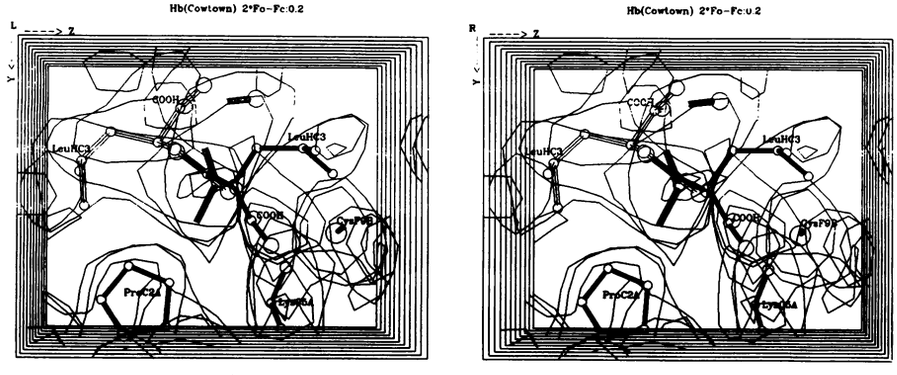

FIG. 3. Symmetry-averaged electron-density map of Hb Cowtown contoured at 0.2 e/A^3. Viewpoint is the same as Fig. 1, but a smaller region is shown. Atomic model: light bonds, as Fig. 1; heavy bonds, as Hb A, except that His HC3 is replaced by leucine with no change in χ_1 angle. The map was calculated as the transform of $(2|F_0| - |F_c|)\exp(i\alpha_c)$, where F_0 are the observed structure amplitudes for Hb Cowtown, F_c are the calculated amplitudes, and α_c, the phases derived from a model of Hb A with His HC3β omitted. (Scale: 1 Å = 4 mm.)

cantly populated only at the high ionic strength of the crystal suspension medium. On this assumption the crossover of the pH dependence of K_1 may arise as follows. In deoxy Hb the imidazole of His HC3 has a pK of 8.0. Hence at the crossover point of pH 7.6 three quarters of the histidines would carry positive charges. At this point the average strength of the constraints placed on the oxygen affinity of the β-hemes by the COOH-terminal salt bridges in Hb A may equal those produced by the van der Waals interactions between the leucyl side chain and helices F and FG in Hb Cowtown. Above pH 7.6 the constraints of the salt bridge are weakened relative to those of the van der Waals interactions, while below that pH they are strengthened. The His → Leu substitution has a similar effect on K_2 and K_3, except that there is no crossover: at pH 6.5, where the histidine salt bridge is strongest, K_2 and K_3 of Hb A are much lower than those of Hb Cowtown, whereas at pH 9 where the salt bridges are weakest, they are only slightly lower. The *R* structure is unconstrained by COOH-terminal salt bridges, whence K_4 is unaffected by either pH or the His → Leu substitution. So far the interpretation has been straightforward, but our results do not explain why in Hb Cowtown, unlike other abnormal Hbs, the effect of the His → Leu substitution on the allosteric constant *L* should be uncoupled from that on K_T.

One particularly significant observation of Shih *et al.* (10) is the coincidence of the oxygen equilibrium curves of Hb A and Cowtown after reaction of Cys F9(93)β with *N*-ethylmaleimide. When His HC3 makes its salt bridge with Asp FG1, it wraps a ring around the sulfhydryl group of Cys F9. Reaction with *N*-ethylmaleimide opens the ring and pushes the histidyl side chain away from Asp FG1, thus breaking the salt bridge (1). Similarly it would push the leucyl side chain away from its contacts with helices F and FG. Suppose the halving of the alkaline Bohr effect in Hb Cowtown and other abnormal and chemically modified hemoglobins that lack the salt bridge between His HC3 and Asp FG1 were due to small changes in pK of several histidines, rather than the absence of one histidine with a large change of pK, then these many small changes are not likely to vanish after reaction of Cys F9 with *N*-ethylmaleimide, so as to produce the same oxygen affinity and Bohr effect in Hb A and Cowtown. This observation is consistent only with the halving of the Bohr effect in Hb Cowtown being due directly and exclusively to the absence of His HC3.

The inconsistency between our results and those of Matthew *et al.* (8, 9) made us wonder whether their theory predicted the contributions of other ionizable residues to the free energy of cooperativity correctly. The experimentally determined contributions of the various components of the COOH-terminal salt bridges are compared with those calculated by Matthew *et al.* in Table 1. For the COOH-terminal carboxyl of Arg HC3(141)α, the calculated contribution is of opposite sign to the observed one. For other components, the calculated free energies have the same sign but are smaller than the observed ones by factors of 4 to 5.

The above inconsistencies are due partly to the inadequacies of their theory, as discussed by Warshel *et al.* (20), and partly to the use of an incorrect HbO_2 structure. Rather than using as their model the known structure of human HbCO (21) which has since proved to be isomorphous with HbO_2 to within experimental error (22), the authors decided to use Fermi's (23) coordinates of deoxy Hb and simply rotate the individual subunits according to Cox (24) to generate the oxy structure. This procedure neglected important changes in tertiary structure and kept the salt bridge between His HC3(146)β and Asp FG1(94)β intact, whereas in fact x-ray analysis shows the N^{ϵ} of the histidine to be 10 Å away from the O^{δ} of the aspartate in HbO_2 (ref. 22; Fig. 3). By putting the histidine in the wrong place, they obtained a ΔpK value for the imidazole on going from Hb to HbO_2 of only −0.25 at $I = 0.1$ compared with the observed one of −0.9 (25). We have recalculated the ΔpK using the observed increase in the distance between N^{ϵ} and O^{δ} from 3 Å in deoxy Hb to 10 Å in HbO_2 and calculated electrostatic free energies according to Fig. 1 and equation 4 of Matthew *et al.* (8). We obtained ΔpK = −1.3; this could be scaled down to 0.9 by manipulating their somewhat arbitrary solvent accessibility factor. Our results and those of Shih *et al.* (10) are also inconsistent with the NMR titrations of His HC3 in human HbCO, which attributes to the imidazoles a pK as high as 7.9 in phosphate-free solutions (6, 7). The halving of the alkaline Bohr effect of Hb Cowtown in phosphate-free buffer could not occur unless the pK were close to 7.0, the same as in 0.2 M phosphate (25).

The halved alkaline Bohr effect of Hb Cowtown is consistent with its reduction in several other abnormal and chemically modified hemoglobins in which the COOH-terminal salt bridges of His HC3(146)β are disturbed. It has been argued that these reductions may be due, not to the rupture of these salt bridges directly, but indirectly to alterations in-

Table 1. Comparison of experimental values of energy contributions to lowering of oxygen affinity by COOH-terminal residues with those calculated by Matthew *et al.* (8, 9)

	E, kcal/mol			
	Exp.	Calc.	Calculated value	Origin of experimental value
Arg HC3α_1 COO^- − Lys H10α_2	−1.3	+0.9	With 2 or more Cl^- bound	Δp_{50} of hydrazide Hb − Hb A in 0.2 M Bistris/0.1 M Cl^-, pH 6.8, 25°C; ref. 15.
Asp H5α_2 − Arg HC3$_1$ Gua^+	−2.7	−0.5	With or without Cl^-	Δp_{50} of Hb Tarrant [Asp H5(126)α → Asn] − Hb A in 0.5 M Bistris, pH 7.4/2 mM DPG, 20°C; ref. 16.
Lys C5α_1 − His HC3$_2$ COO^-	−2.7			Δp_{50} of Hb Kariya [LysC5(40)α → Glu] − Hb A in 0.05 M Bistris/0.1 M Cl^-, pH 7.4, 25°C; ref. 17.
His HC3β_2 Im^+ − Asp FG1β_2	−2.1	−0.4		Δp_{50} of Hb Hiroshima [His HC3(146)β → Asp] − Hb A in 0.01 M Bistris (pH 7.0) with or without 0.3 mM DPG; ref. 18.
Asp HC3β_2 − $^+$Im His HC3β_2	≈1.0			Δp_{50} of Hb Bunbury [Asp FG1(94)β → Asn] − Hb A extrapolated of Δp_{50} of blood or stripped lysate containing 38% Hb Bunbury/60% Hb A and normal blood or stripped lysate at 37°C; ref. 19.

Bistris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; DPG, 2,3-diphosphoglycerate. (One calorie = 4.18 J.)

duced elsewhere in the Hb molecule. However, no such alterations have ever been found, and Hb Cowtown is the seventh instance in which x-ray analyses have proved structural changes to be strictly localized at the COOH terminus. The others are NES-hemoglobin (1), des-Tyr 145-His 146 Hb (26), des-His 146 Hb (unpublished work), Hb Hiroshima [His HC3(146)β→Asp] (27), Hb Rainier [Tyr HC2(145)β→Cys] (28), and Hb Barcelona [Asp FG1(94)β→His] (12). It is true that NMR resonances of protons elsewhere in the Hb molecule are sometimes slightly perturbed by modifications or substitutions at the COOH terminus, but these resonances are sensitive to atomic shifts of <0.2 Å, which are below the power of resolution of the x-ray analyses and also too small to cause significant changes in pK values of ionizable groups.

The cooperative interactions of Hb Cowtown under conditions of moderate ionic strength are similar to those of Hb A, not because the salt bridge of the COOH-terminal imidazole fails to contribute significantly to the free energy difference between T and R states, as proposed by Shih *et al.* (10). Any bond that exists in the T state and is broken in the R state must contribute to the free energy of cooperativity. A thermodynamically tenable explanation is that the van der Waals interactions made between the leucyl side chain and other atoms in the T structure of Hb Cowtown are energetically equivalent to the salt bridge in the T structure of Hb A.

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