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## Stereochemistry of Cooperative Mechanisms in Hemoglobin

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Hemoglobin (Hb) is the respiratory protein of the red blood cells which carries O<sub>2</sub> from the lungs to the tissues and facilitates the return transport of CO<sub>2</sub> from the tissues to the lungs. The physicist J. J. Hopfield has called it the hydrogen atom of biochemistry because the understanding of its functions is so fundamental to proteins generally. One of these functions is allosteric, the switching of proteins between active and inactive structures in response to chemical stimuli. Another is the formation of coordination complexes with transition metals for a great variety of catalytic and other actions. Hemoglobin may be unique in exploiting changes in Fe-N bond lengths accompanying the transition from

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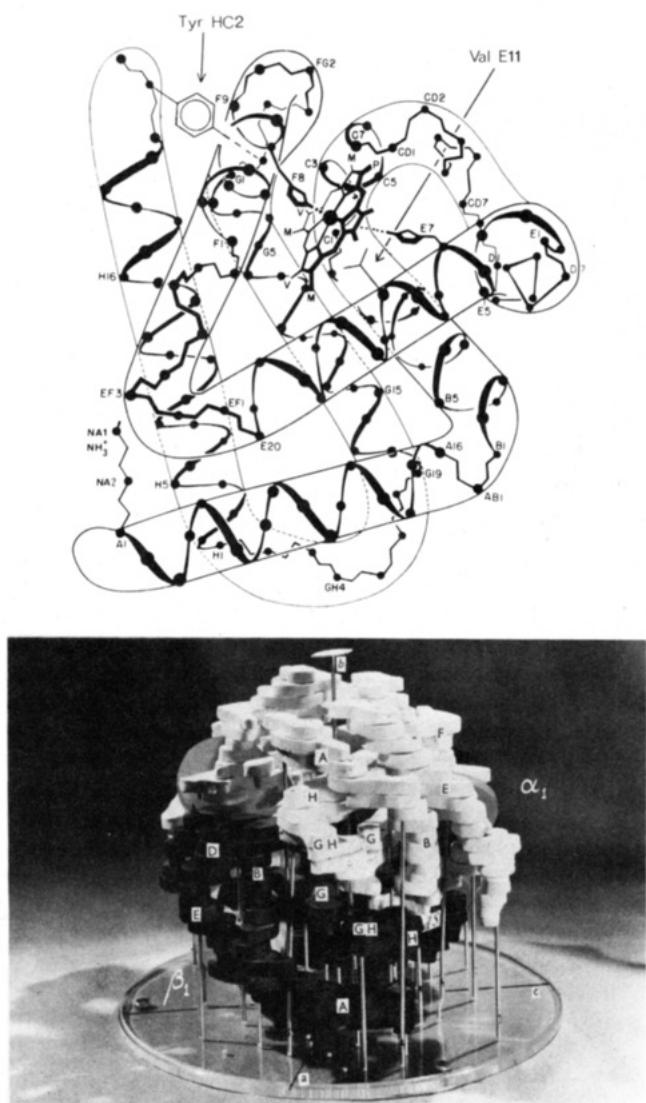
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high-spin to low-spin ferrous iron for the purpose of efficient oxygen transport: without use of that spin transition fast-moving animals could not have evolved. The isolation of the iron atoms in separate pockets of the globin prevents collisions between them, which means that their reactions with ligands can be studied without any of the elaborate apparatus of matrix isolation needed for investigating the reaction mechanisms of simpler metal complexes. Finally, hemoglobin shows how Nature uses conjugated bases with pK's in the physiological pH range to sensitize proteins to small pH changes in their environment.

Hemoglobin (MW = 64 500) is a tetramer made up of two  $\alpha$ -chains and two  $\beta$ -chains, each containing 141 and 146 amino acid residues, respectively. Each chain carries one heme. The  $\alpha$ -chains contain seven and the  $\beta$ -chains eight helical segments, interrupted by non-helical ones. Each chain also carries short nonhelical segments at the N and C termini. Myoglobin (Mb) is a similar protein consisting of a single chain of 153 amino acid residues and one heme. It is found in muscle where it stores the O<sub>2</sub> transferred to it from hemoglobin and liberates the O<sub>2</sub> to the mitochondria for oxidative phosphorylation of adenosine diphosphate. As shown in Figure 1a, the hemes in Mb and Hb are held in pockets formed by several helical and nonhelical segments CD and FG; their Fe's are 5-coordinated to N<sub>6</sub> of histidines F8, also known as proximal, and to the four porphyrin nitrogens (N<sub>porph</sub>); the porphyrin is in van der Waals contact with another histidine on the



**Figure 1.** (a, top) Secondary and tertiary structure characteristic of the hemoglobins. Starting from the N terminus the helical segments are denoted A to H, and the nonhelical ones NA, AB, BC, etc., to HC, which denotes a segment of three nonhelical residues at the C terminus. Residues within each segment are numbered from the amino end, A1 to A16, etc. We label each residue by its structural position followed by its position in the sequence, e.g., Glu A3(6)β. The diagram shows the helical and nonhelical segments, the heme, the proximal His F8, the distal His E7, and the distal Val E11. (b, bottom) Model of oxy-hemoglobin. The tetramer has pseudotetrahedral symmetry. A true dyad relating the chains  $\alpha_1$  to  $\alpha_2$  and  $\beta_1$  to  $\beta_2$  runs along a 50-Å-long and ~10-Å-wide water-filled central cavity. Pseudo-dyads at right angles to the true dyad and to each other relate  $\alpha_1$  to  $\beta_1$  and  $\alpha_1$  to  $\beta_2$ . The  $\alpha$ -chains are white and the  $\beta$ -chains black; the gray disks represent the hemes and the sign at the top represents the dyad axis. On transition to the deoxy structure the  $\alpha_1\beta_1$  dimer at the back turns relative to the  $\alpha_2\beta_2$  dimer at the front.

distal side (E7) and also makes contact with 18 other amino acid side chains, most of which are nonpolar. The propionate side chains of the porphyrin protrude into the solvent and form hydrogen bonds with basic side chains of the globin [His CD3(45)α and Lys E10(66)β].<sup>1,2</sup>

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The heme irons in Hb and Mb combine reversibly with  $O_2$  to form dioxygen complexes and, in the process, change from high-spin Fe(II) to low-spin Fe(II). The reaction of  $O_2$  with Mb is a simple bimolecular one, but the reaction with Hb is cooperative, with a free energy of cooperativity, under conditions that mimic physiological ones, of 3.6 kcal mol<sup>-1</sup> (15 kJ mol<sup>-1</sup>) heme<sup>-1</sup>. The oxygen affinity of Mb is independent of pH and ionic strength, but that of mammalian Hb's is lowered by  $H^+$ ,  $Cl^-$ ,  $CO_2$ , and 2,3-diphosphoglycerate (DPG), all of which are present in the red cell. They are known collectively as the heterotropic ligands, while ligands that combine with the heme iron are called homotropic. The interactions between the various ligands reacting with Hb are known as the cooperative effects. They are needed to ensure efficient respiratory transport.

The cooperative binding of  $O_2$  is manifested by its sigmoid equilibrium curve and ensures uptake and release of  $O_2$  over the comparatively narrow range of partial  $O_2$  pressures that distinguishes the lungs ( $p_{O_2} \sim 100$  mmHg) from the tissues ( $p_{O_2} \sim 30-40$  mmHg). Hb binds one  $H^+$  for every two  $O_2$ 's released; this uptake biases the equilibrium of the reaction between  $CO_2$  and  $H_2O$  in the direction of  $HCO_3^-$ , thus promoting the transport of  $CO_2$  by the blood serum. Conversely, the protons released by the metabolic products, lactic and carbonic acids, facilitate the release of  $O_2$  to the tissues.

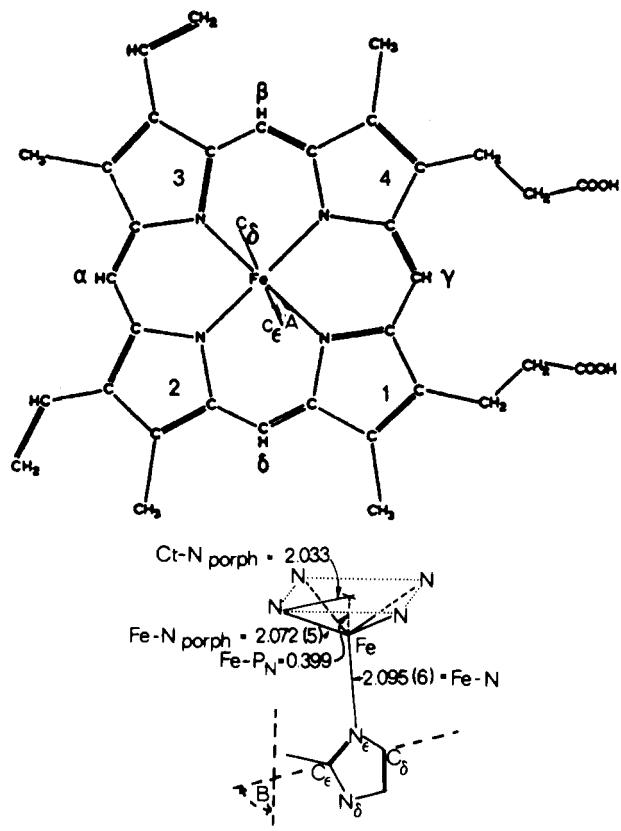
In the hemoglobins of bony vertebrates the cooperative effects arise from an equilibrium between two alternative Hb structures, the oxy or R (relaxed) and deoxy or T (tense) structure. The  $O_2$  affinity of the R structure is slightly larger than the average of free  $\alpha$ - and  $\beta$ -subunits; that of the T structure is lower by the equivalent of the free energy of cooperativity. The  $O_2$  equilibrium of Hb can be described by the  $O_2$  association constants  $K_T$  and  $K_R$ , usually expressed in mmHg<sup>-1</sup>, and by the equilibrium constant  $L_0 = [T]/[R]$  in the absence of  $O_2$ . Imai has shown empirically that  $\log K_T/K_R = A - 0.25 \log L_0$ , where  $A$  is a constant, which leaves  $K_R$  and  $K_T$  as the only independent variables.  $K_T$  varies over a wide range as a function of  $[H^+]$ ,  $[Cl^-]$ ,  $[CO_2]$ , and  $[DPG]$ ;  $K_R$  varies as a function of  $[H^+]$  below pH 7 but is little affected by the other ligands.<sup>3,4</sup>

The T and R structures differ in the arrangement of the four subunits, referred to as the quaternary structure, and the conformation of the subunits, referred to as the tertiary structure. The quaternary R  $\rightarrow$  T transition consists of a rotation of the dimer  $\alpha_1\beta_1$  relative to the dimer  $\alpha_2\beta_2$  by 12–15° and a translation of one dimer relative to the other by 0.8 Å (Figure 1b). The  $\alpha\beta$  dimers move relative to each other at the symmetry-related contacts  $\alpha_1\beta_2$  and  $\alpha_2\beta_1$  and at the contacts  $\alpha_1\alpha_2$  and  $\beta_1\beta_2$ ; the contacts  $\alpha_1\beta_1$  and  $\alpha_2\beta_2$  remain rigid.

At the  $\alpha_1\beta_2$  interface the nonhelical segment  $FG\alpha_1$  is in contact with helix  $C\beta_2$  and helix  $C\alpha_1$  with  $FG\beta_2$ . During the R  $\rightarrow$  T transition, the contact  $FG\alpha_1-C\beta_2$  acts as a ball and socket joint, while the contact  $C\alpha_1-FG\beta_2$  acts as a two-way switch that shifts  $C\alpha_1$  relative to  $FG\beta_2$  by about 6 Å, like the knuckles of one hand moving over those of the other. Intermediate positions of the switch are blocked by steric hindrance. The gaps along the

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**Figure 2.** (a, top) Heme with imidazole of His F8 projected onto its plane. (b, bottom) Interatomic distances around Fe in (2-methylimidazole)(tetrakis(pivalamidophenyl)porphyrinato)iron (II). The diagrams illustrate the meanings of angles A and B in Table I.

central cavity between  $\alpha_1$  and  $\alpha_2$  and between  $\beta_1$  and  $\beta_2$  narrow on transition from T to R. The shape of the  $\alpha_1\beta_1$  and  $\alpha_2\beta_2$  dimers is altered by changes in tertiary structure: for example, on oxygenation the distance between the  $\alpha$ -carbons of residues FG1 $\alpha_1$  and  $\beta_1$  shrinks from 45.6 to 41.3 Å. These changes make an  $\alpha_1\beta_1$  dimer that has the tertiary oxy structure a misfit in the quaternary T structure and an  $\alpha_1\beta_1$  dimer that has the tertiary deoxy structure a misfit in the quaternary R structure.<sup>5,69</sup>

The key questions for the understanding of Hb function are these: How does the reaction with  $O_2$  affect the stereochemistry at and around the heme so as to trigger the transition from the T to the R structure? What are the constraints of the T structure, and how do they lower the  $O_2$  affinity? By what mechanisms do the heterotropic ligands influence the  $O_2$  affinity? Single-crystal X-ray analyses of deoxy- and oxyhemoglobin, and of analogues of intermediates in the reactions with  $O_2$  or CO, together with chemical, spectroscopic, and magnetic studies have furnished some of the answers. Table I lists the structures on which our analysis of the cooperative mechanism rests. We shall now review these in turn.

### Structures of Deoxy- and Oxyhemoglobin<sup>6,9</sup>

**Changes on Oxygenation in Stereochemistry of the Hemes.** Table I and Figures 2-4 summarize the stereochemistry of the hemes in deoxyhemoglobin

(DHb) and oxyhemoglobin (HbO<sub>2</sub>). In DHb the porphyrins are domed, as predicted by Gelin et al.,<sup>16</sup> and the Fe's are displaced from the planes of the porphyrin nitrogens toward the proximal histidines. On oxygenation the porphyrins flatten and the Fe-N<sub>porph</sub> bond lengths contract, thus moving the Fe's toward the porphyrin planes. In consequence, the proximal histidines come 0.5-0.6 Å closer to the porphyrin planes in HbO<sub>2</sub> than in DHb. A water molecule that is hydrogen bonded to the distal histidines (E7) of the  $\alpha$ -subunits in DHb dissociates in HbO<sub>2</sub>.

Are these stereochemical changes at the hemes intrinsic, or are they influenced by the globin or the crystal lattice? The Fe-N<sub>porph</sub> distances and the displacements of the Fe's from the planes of the N<sub>porph</sub>'s in DHb and DMb are the same as in two synthetic 5-coordinated Fe(II) porphyrins (Table I).<sup>18,19</sup> The average Fe-N<sub>porph</sub> distances measured in crystals of DHb and HbO<sub>2</sub> also agree with those measured in solution by extended X-ray fluorescence spectroscopy (EXAFS).<sup>20,21</sup> The conformation of the hemes in human DHb is the same within error in crystals of space group P2<sub>1</sub> grown in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solutions and in crystals of space group P2<sub>1</sub>2<sub>1</sub>2 grown in poly(ethylene glycol) (PEG) solutions. The degree of doming of the porphyrin, on the other hand, appears to be influenced by the constraints of the globin and by those of the crystal lattices in the synthetic iron porphyrins.

Hb, Mb, and the model complexes bind  $O_2$  to the Fe's in the bent, terminal  $\gamma'$  mode (Figure 4). The Fe-O-O angle varies over a wide range, depending on the shape and chemical constitution of the heme pocket, but its influence on the strength of the Fe-O<sub>2</sub> bond is unknown. Single-crystal neutron diffraction of MbO<sub>2</sub> has proved that N<sub>7</sub>H of the distal histidine (E7) donates a hydrogen bond to the bound  $O_2$ .<sup>14</sup> N<sub>7</sub>H-O distances in the  $\alpha$ -subunits are similar to those in MbO<sub>2</sub>; in the  $\beta$ -subunits they are longer, indicative of weaker bonds, which may be related to the lower oxygen affinity of the  $\beta$ -subunits (Table I). Other interatomic distances in the heme complexes of HbO<sub>2</sub>, MbO<sub>2</sub>, and the two synthetic Fe porphyrins are very similar, but the displacements of the Fe's from the plane of the N<sub>porph</sub>'s vary, apparently due to steric factors on the proximal side. In HbO<sub>2</sub> and MbO<sub>2</sub> these take the form of variations which

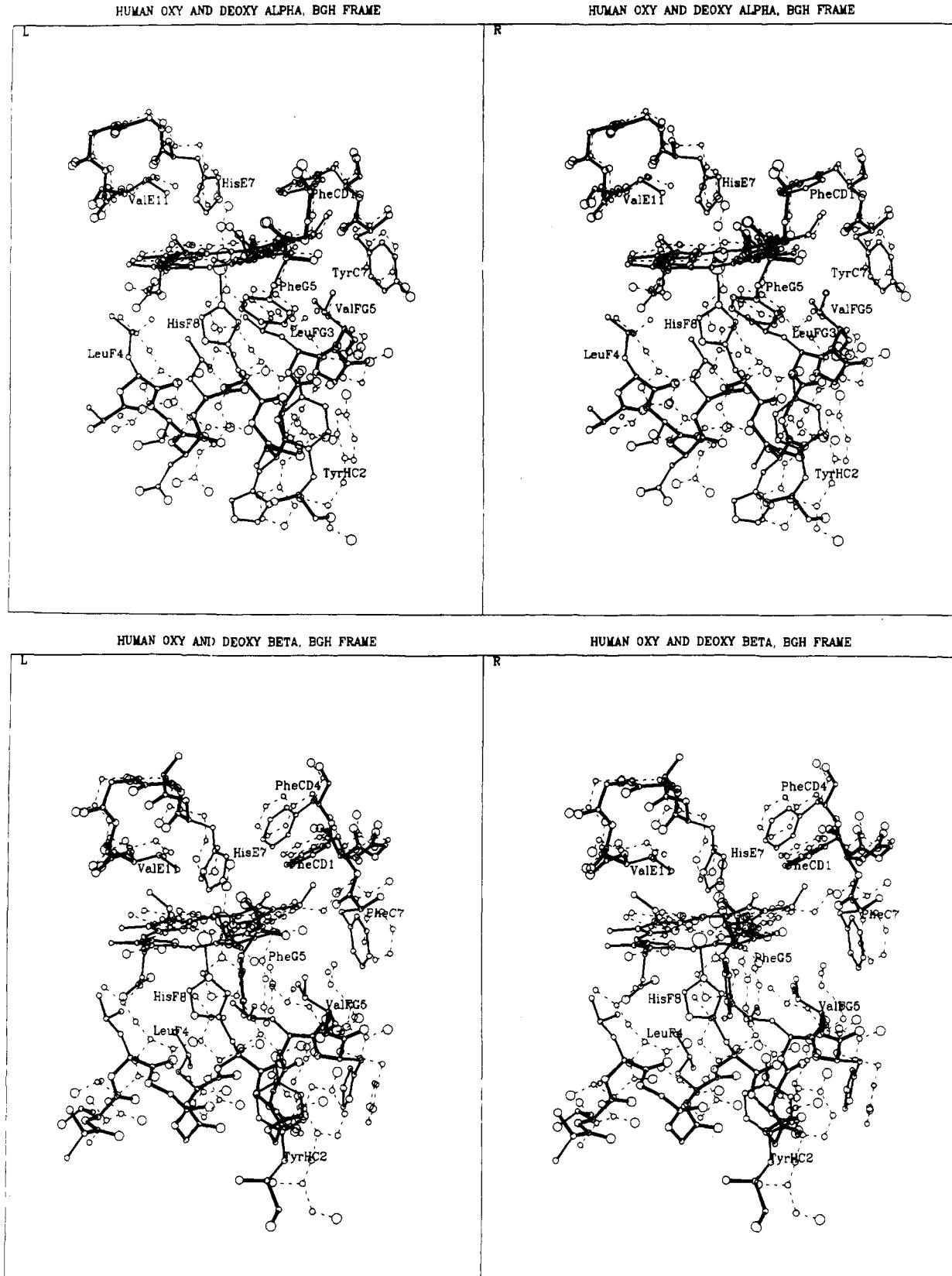
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Table I.  
Heme Stoichiometry in Hemoglobin, Myoglobin and Synthetic Iron Porphyrins

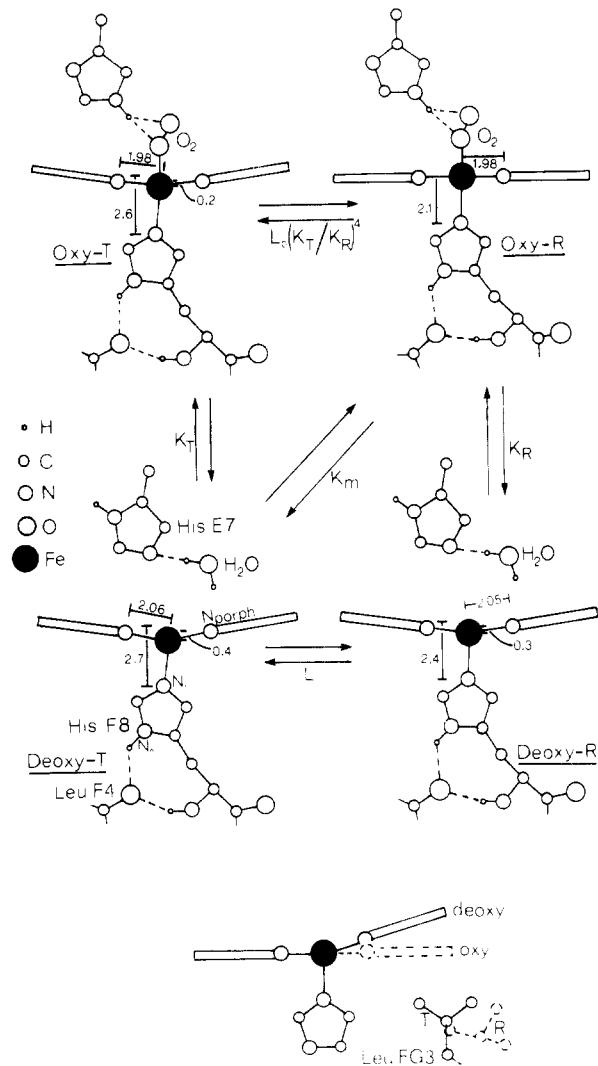
<sup>a</sup>  $P_{\text{heme}} = \text{displacement of Fe from mean plane of porphyrin N's and C's, including the first atom of each side chain. } P_{\text{Fe}} = \text{displacement of Fe from mean plane of porphyrin N's. } P_{\text{N}} - P_{\text{C}} = \text{displacement of plane of porphyrin N's from plane of porphyrin C's. This is the doming parameter. } A = \text{Angle between plane of imidazole of His F8 projected onto the heme and line N<sub>1</sub>-N<sub>3</sub> (Figure 2a). } B = \text{angle between line C-N<sub>6</sub> of His F8 and heme normal (Figure 2b). } 2\text{-MeIm[TpivPP]Fe}^{\text{II}} = (2\text{-methylimidazole})\text{(meso-tetraphenylporphyrinato)iron(II). } 2\text{-MeIm(TPP)} = (2\text{-methylimidazole})\text{(tetraphenylporphyrinato)iron(II). } [\text{Fe}^{\text{II}}(\text{FvI}_2\text{C}_8)(1\text{-MeIm})]\text{Fe}^{\text{II}} = \text{an } \alpha,\alpha,\alpha,\alpha\text{-tetra-}p\text{-amido functionalized tetraphenylporphyrin having } trans\text{-dipivalamido pickets and a } trans\text{-NH-C(O)-}(\text{CH}_2)_6\text{-C(O)-NH-} \text{strep with an unhindered axial ligand 1-methylimidazole.}$



**Figure 3.** Change in stereochemistry of the hemes and the surrounding globin after superposition of the B, G, and H helices of (a, top) the  $\alpha$  and (b, bottom) the  $\beta$  chains of human deoxy (full lines) and oxyhemoglobin (broken lines). Note the flattening of the hemes and the movements of His F8 toward the hemes on oxygenation; also note the rotation of the heme and the large movements of His E7 and Val E11 in  $\beta$ .<sup>6,8</sup> The view in this and subsequent stereo pictures is approximately from the central dyad and perpendicular to it, looking toward the surface of the molecule.

the angle of the imidazole plane of His F8 makes with the plane normal to the heme that contains N(1)<sub>porph</sub>, Fe, and N(3)<sub>porph</sub> (A in Figure 2a). The smaller that angle, the greater the displacement of the Fe, appar-

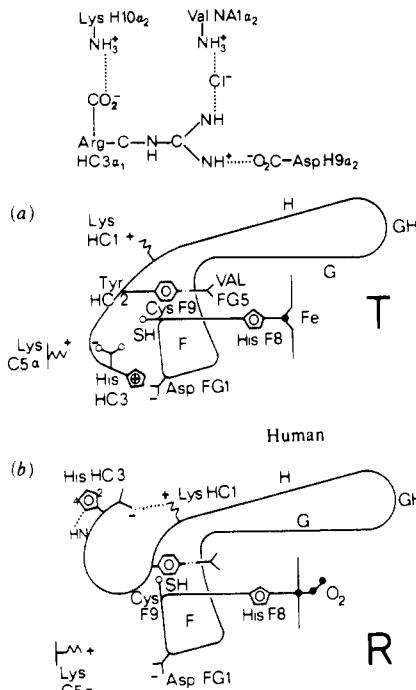
ently due to repulsion of C<sub>ε</sub> and C<sub>δ</sub> of the proximal histidine by the two porphyrin nitrogens.<sup>16,70</sup> Moreover, X-ray studies of synthetic metalloporphyrin structures have shown that whenever two axial imidazoles have



**Figure 4.** Schematic diagram of changes in heme stereochemistry on binding of  $O_2$  by the  $\alpha$ -subunits in the R and T structures. On uptake of oxygen by the T structure, the heme remains domed and the iron remains displaced from the porphyrin plane, due to the constraints by the tightly packed side chains of the globin around the heme; on the other hand, the porphyrin becomes domed on dissociation of oxygen from the R structure, which does not constrain it to the flat conformation. The bottom diagram shows the change in conformation of the heme, seen when residues F1–F6 of DHb and  $HbO_2$  are superimposed. The heme flattens and in so doing pushes down the side chains of Leu FG3 and Val FG5 (hidden behind FG3 in the diagram). This may be one of the ways the change in conformation of the heme is transmitted to the  $\alpha_1\beta_2$  contact, thus triggering the T  $\rightarrow$  R transition.  $L = [T]/[R]$ . At the  $i$ th step of oxygenation  $L_i = L_0(K_R/K_T)^i$ . The numbers in the diagram indicate the distances (in Å) of N<sub>i</sub> from the mean plane of the porphyrin carbons and nitrogens, including the pyrroles and the first carbon of the side chains (N<sub>i</sub>-P<sub>porph</sub>), the mean distance between the iron and the porphyrin nitrogens (Fe-N<sub>porph</sub>), and the displacement of the iron from the plane of the porphyrin nitrogens (Fe-P<sub>N</sub>). In deoxy-R Fe-N<sub>porph</sub> is the mean of  $\alpha$  and  $\beta$ .

different Me–N bonds, the longer one is associated with the smaller angle A. Nevertheless, A tends to be close to zero: its average is  $10.4^\circ$  in 5-coordinated and  $16.2^\circ$  in 6-coordinated metalloporphyrins. Charge-iterative extended Hückel calculations reveal that surprising tendency to be due to an electronic factor that favors bonding between metal and imidazole  $p_z$  orbitals.<sup>76</sup>

**Changes on Oxygenation in Tertiary Structure of the Globin.** (a) The  $\alpha$ -Subunits. Since the  $\alpha_1\beta_1$



**Figure 5.** (a, top) Hydrogen bonds made by ionized groups between subunits  $\alpha_1$  and  $\alpha_2$  in deoxyhemoglobin. (b, bottom) Hydrogen bonds made by His HC3(146) $\beta$  in deoxy (T) and oxyhemoglobin (R). Note also the changing orientations of the side chain of Cys F9(93) $\beta$ . The orientation in T is found in high-spin derivatives, and the one shown in R is found in low-spin derivatives; in mixed-spin derivatives the two orientations are in equilibrium. The salt bridges made by DPG are shown in Figure 10 of ref 1.

contact undergoes no significant changes during the R  $\rightarrow$  T transition, the atoms at this contact can serve as a reference frame for changes in tertiary structure elsewhere; except for residues of G1–G4 and H18–H21, the B, G, and H helices were also found to be static.<sup>5</sup> The largest movements relative to either of these frames occur in helix F, in segment FG, and in residues G1–G4, H18–H21, and HC1–HC3. We shall use the BGH reference frame because it gives the smallest rms deviation for all the other atoms (0.29 Å in the  $\alpha$ -subunits). Figure 3a shows the heme environment of DHb superimposed on that of  $HbO_2$ . It can be seen that on oxygenation helix F $\alpha$  shifts toward the heme and to the right and carries the FG segment with it. In DHb the imidazole of His F8 is tilted relative to the heme normal; in  $HbO_2$  the shift of helix F relative to DHb aligns it with the heme normal. Relative to the BGH frame, the heme turns clockwise by  $1^\circ$  on going from DHb to  $HbO_2$ . Taking as a reference frame residues F1–F8 to which the heme is attached, the heme flattens and turns clockwise by  $10^\circ$ ; the motion of its right-hand edge pushes down Leu FG3(91) $\alpha$  and Val FG5(93) $\alpha$  which form part of the  $\alpha_1\beta_2$  contact where the quaternary switch occurs (Figures 3 and 4). In the T structure the N and C termini form the hydrogen bonds shown in Figure 5a. In the R structure these hydrogen bonds are broken, and the terminal residues are seen only at a low level of electron density ( $0.25$ – $0.5$  e Å<sup>-3</sup>), implying that they are disordered. The map shows indications that the guanidinium of the C terminal arginine is hydrogen bonded to a phosphate or sulfate ion that lies on the dyad symmetry axis in the central cavity, but that ion would be absent in the red cell, where the

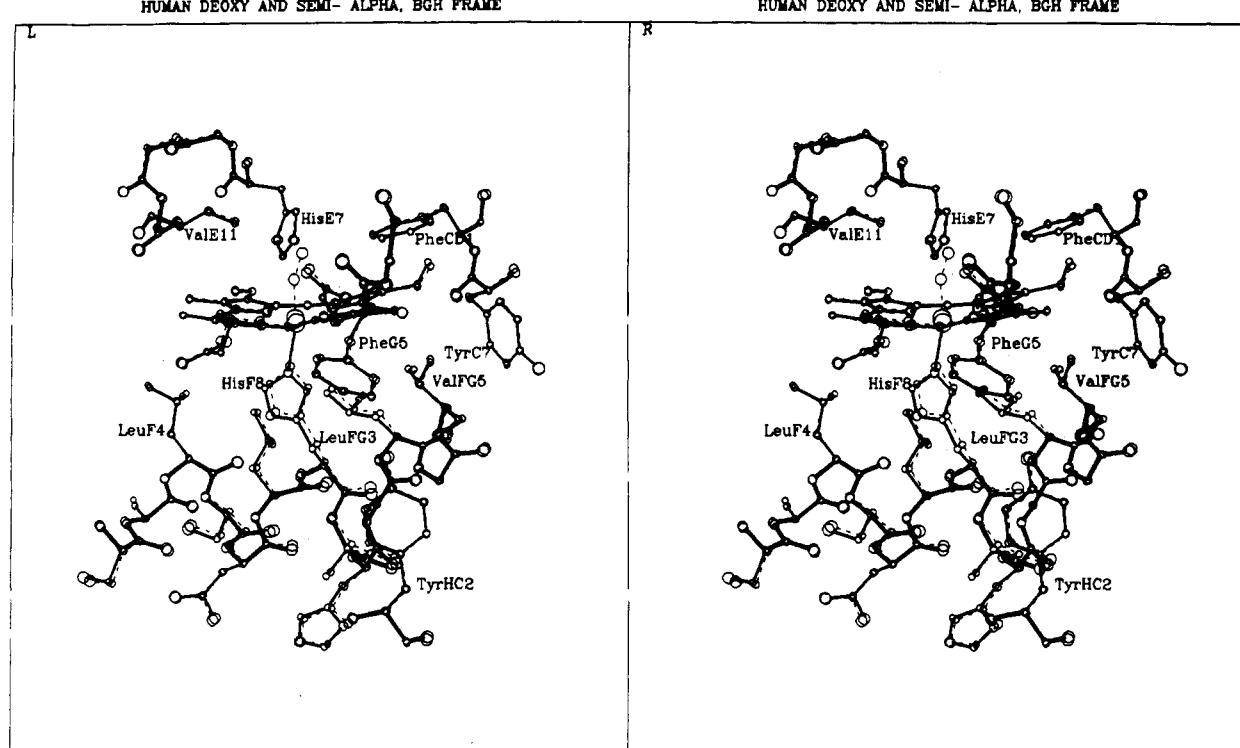


Figure 6. Change in stereochemistry of the heme and the surrounding globin seen after superposition of the B, G, and H helices in one of the  $\alpha$ -chains of human deoxyhemoglobin (full lines) and in  $(\alpha\text{-Fe}^{\text{II}}\text{-O}_2)_2(\beta\text{-Fe}^{\text{II}})_2$  (broken lines). Symbols as in Figure 3.<sup>7,8</sup>

guanidinium groups would be free.

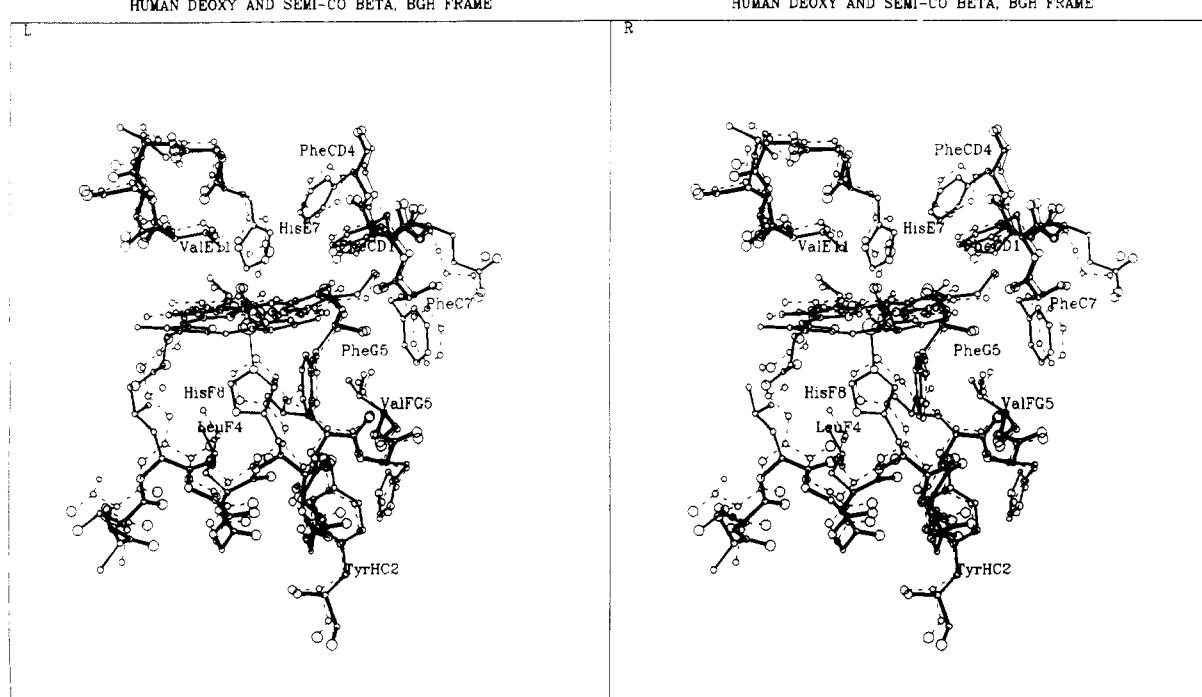
(b) **The  $\beta$ -Subunits.** Figure 3b shows that on oxygenation helix F moves toward the heme and in the direction of the FG segment, carrying that segment with it and aligning His F8 with the heme normal. The movement of F and FG is transmitted to residue G1 and dissipated beyond G5. The center of the heme moves further into its pocket along a line linking porphyrin  $\text{N}_1$  to  $\text{N}_3$ , and the heme rotates about an axis close to the line linking  $\text{N}_2$  to  $\text{N}_4$ . Referred to residues F1 to F6, the iron stays still and the porphyrin becomes coplanar with it, as shown at the bottom of Figure 4. In the T structure  $\text{C}_\gamma\text{H}_3$  of Val E11(67) obstructs the ligand site at the iron; in the oxygenated R structure that obstruction is cleared by a concerted shift of helices D and E and the CD segment together with the beginning of helix B, away from and across the heme.

The C-terminal histidines form different sets of hydrogen bonds in the T and R structures, as a result of which their  $\text{pK}_a$ 's drop on oxygenation from 8.0 to 6.5, and protons are released. The conformation of the reactive sulphydryl groups of Cys F9(93) $\beta$  also changes (Figure 5b).

**Partially Liganded Hemoglobins in the Quaternary T Structure.** (a) **From Deoxyhemoglobin to  $(\alpha\text{-Fe}^{\text{II}}\text{-O}_2)_2(\beta\text{-Fe}^{\text{II}})_2$ .** This derivative was obtained by exposure to air of orthorhombic crystals of DHb grown in PEG (Table I). The molecules lie in general positions in the unit cell, so that subunits  $\alpha_1$  and  $\alpha_2$  make different lattice contacts; their coordinates were refined separately. The refined structure shows full occupancy by  $\text{O}_2$  at the  $\alpha$ -hemes and no evidence of  $\text{O}_2$  bound to the  $\beta$ -hemes, but up to 30% of the  $\beta$ -hemes may have become oxidized to methemoglobin ( $\text{Hb}^+\text{H}_2\text{O}$ ). In both  $\alpha$ -subunits the reaction with  $\text{O}_2$  causes the Fe's to move by 0.15 Å toward the porphyrin which remains domed as in DHb, but the tilt of the proximal histidine is

slightly reduced. The distal residues remain unperturbed; only the water molecule that is hydrogen bonded to His E7(58) $\alpha$  in DHb is displaced by the heme-linked  $\text{O}_2$ . In one of the  $\alpha$ -subunits the motion of the Fe produces a small concerted shift of residues F5-F9, of the main chain of FG4, and of the side chains of FG4 and FG5; in the other  $\alpha$ -subunit the motion of the Fe stretches the  $\text{Fe}-\text{N}_e$  bond by  $\sim 0.2$  Å, with hardly detectable shifts of F or FG (Figures 4 and 6).<sup>7,8</sup> These differences are due, presumably, to the different lattice contacts of the two  $\alpha$ -subunits. However, the important features that are common to both subunits are the doming of the hemes as in DHb and localization of strain to the immediate neighborhood of the Fe's and proximal histidines.

(b) **From Deoxyhemoglobin to  $(\alpha\text{-Ni}^{\text{II}})_2(\beta\text{-Fe}^{\text{II}}\text{CO})_2$  in the T Structure.**<sup>72</sup> This derivative crystallizes with unit-cell dimensions similar to those of the ligand-free T structure, but the molecular packing is so different that the structure had to be solved by molecular replacement. The electron density map shows Ni porphyrin to be planar and the Ni to be 4-coordinated, in agreement with spectroscopic evidence,<sup>75</sup> with a  $\text{Ni}-\text{N}_e(\text{His})$  distance of 3.2 Å; this large distance biases the allosteric equilibrium strongly toward the T structure, just as in the abnormal human Hb Boston, where the replacement His E7  $\rightarrow$  Tyr causes the iron atom to be linked to the distal tyrosine instead of the proximal histidine.<sup>77</sup> These structures behave as if the metal atoms were displaced from the porphyrin plane by over 1 Å instead of the normal 0.55 Å and had pushed the proximal histidines away with them. Figure 7 shows the marked changes in tertiary structure around the liganded  $\beta$ -hemes after superposition of the B, G, and H helices on those of the ligand-free T structure. CO is seen to be accommodated by a change in tilt of the heme and its attached helix F, combined with shifts in



**Figure 7.** Change in stereochemistry of the heme and the surrounding globin seen after superposition of the B, G, and H helices in one of the  $\beta$ -chains of human deoxyhemoglobin (full lines) and in  $(\alpha\text{-Ni}^{\text{II}})_2(\beta\text{-Fe}^{\text{II}}\text{CO})_2$  (broken lines). Symbols as in Figure 3.

the distal residues. The map shows the shift of the distal histidine and valine clearly. The heme pocket is closed as in the ligand-free T structure. The CO occupancy is different in the two  $\beta$ -subunits, apparently due to different lattice constraints. The packing of the residues at the  $\alpha_1\beta_2$  interface is the same as in the normal, fully deoxy, T structure. We shall call  $t_{\beta}$  the tertiary structure of the  $\beta$ -chains in  $(\alpha\text{-Fe}^{\text{II}})_2(\beta\text{-Fe}^{\text{II}})_2$ , where the ligand site is blocked by Val E11, and  $t'_{\beta}$  in  $(\alpha\text{-Ni}^{\text{II}})_2(\beta\text{-Fe}^{\text{II}}\text{CO})_2$ , where the site is occupied by a ligand. The ligand binding properties of this hybrid Hb have been determined by Shibayama *et al.*,<sup>75</sup> who found that it binds oxygen noncooperatively with an equilibrium constant similar to  $k_1$  of native human hemoglobin, which implies that it remains in the T structure, and that the  $\beta$ -hemes bind oxygen in the T structure in solution, contrary to some recent claims.

(c) **From Deoxyhemoglobin to  $(\alpha\text{-Fe}^{\text{II}}\text{-CO})_2(\beta\text{-Mn}^{\text{II}})_2$  and to  $(\alpha\text{-Fe}^{\text{II}}\text{-CO})_2(\beta\text{-Co}^{\text{II}})_2$  in the T Structure.** Crystalline hybrid Hb's carrying iron porphyrin in the  $\alpha$ -chains and a metalloporphyrin that fails to combine with  $\text{O}_2$  or CO in the  $\beta$ -chain are isomorphous with those of normal DHb. A difference electron density map of the hybrid  $(\alpha\text{-Fe}^{\text{II}}\text{-CO})_2(\beta\text{-Mn}^{\text{II}})_2$  minus DHb at 3.0- $\text{\AA}$  resolution showed no significant density in the  $\beta$ -subunits, as 5-coordinated Mn(II) and Fe(II) porphyrins are isomorphous. In the  $\alpha$ -subunits the map has pairs of positive and negative peaks indicative of a movement of the ligated Fe's and of residues F6(85) to FG1(89) toward the porphyrin. Restrained least-squares refinement leads to tentative estimates of 0.3  $\text{\AA}$  for the movements of the Fe and the imidazole of His F8(87) $\alpha$  and of 0.2  $\text{\AA}$  for some of the main-chain atoms.<sup>11</sup>

The Co-N<sub>porph</sub> bond is 0.1  $\text{\AA}$  shorter than the Fe-N<sub>porph</sub> bond; consequently, the displacement of Co from

the plane of the N<sub>porph</sub> in Co<sup>II</sup>(TPP)(1,2-diMeIm) is only 0.15  $\text{\AA}$ <sup>23</sup> compared to 0.42  $\text{\AA}$  in Fe<sup>II</sup>(TPP)(2-MeIm) (TPP, tetraphenylporphyrin; MeIm, methylimidazole). In deoxy( $\alpha\text{-Co}^{\text{II}})_2(\beta\text{-Co}^{\text{II}})_2$  the displacement of Co from the mean plane of the porphyrin nitrogens and carbons is 0.25  $\text{\AA}$  less than in Fe<sup>II</sup>DHb, but this closer approach of the Co to the porphyrin is compensated by a stretching of the Co-N<sub>ε</sub> bond, so that the distance of N<sub>ε</sub> from the porphyrin plane remains the same as in ferrous DHb, and the substitution of Co for Fe causes no perceptible changes in the structure of the globin.<sup>24</sup> The difference electron density map of  $(\alpha\text{-Fe}^{\text{II}}\text{-CO})_2(\beta\text{-Co}^{\text{II}})_2$  minus DHbA at 2.9- $\text{\AA}$  resolution shows the Co atoms in the  $\beta$ -subunits to be flanked by pairs of positive and negative peaks, due to the smaller displacement of Co from the porphyrin planes compared to Fe, but again there is no change in the structure of the globin. Difference peaks appear near both the ligated  $\alpha$ -hemes: a large positive peak on the distal side represents the CO; a negative peak next to it is due to the removal of the H<sub>2</sub>O bound to His E7(58) $\alpha$  in DHb; a negative peak on the proximal side of the iron shows that it has moved closer to the porphyrin, and pairs of positive and negative peaks flanking helix F show that residues F5 to FG1 have also moved closer to the porphyrin. In one of the  $\alpha$ -subunits these movements are transmitted to the FG segments, but in the other they are not. No refinement of this structure has yet been done.<sup>10</sup>

(d) **From Met- to Deoxyhemoglobin in the R Structure.** When crystals of HbO<sub>2</sub> or methemoglobin (Hb<sup>+</sup>H<sub>2</sub>O) are reduced to DHb with Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, they break up and their diffraction pattern is lost, but crystals of horse Hb<sup>+</sup>H<sub>2</sub>O that have been reacted with bis(*N*-maleimidomethyl) ether (BME) remain intact

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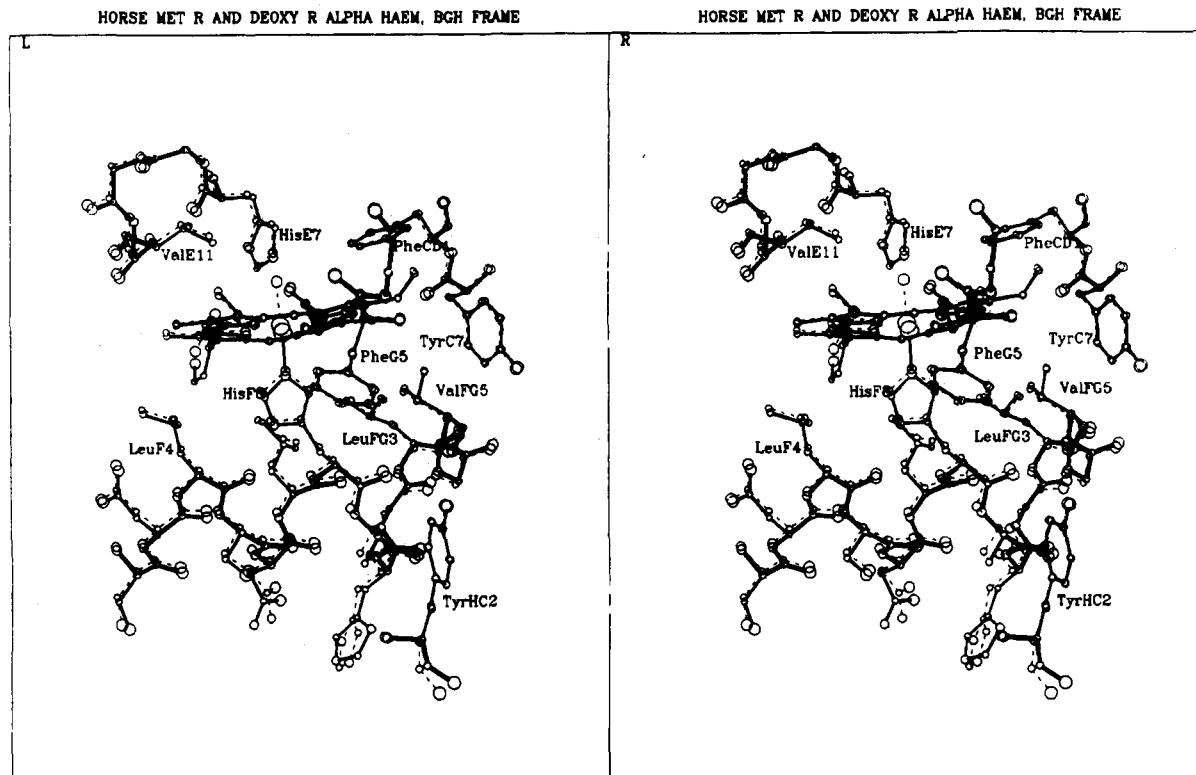


Figure 8. Same as Figure 6 but the derivatives superimposed are horse methemoglobin (broken lines) and BME-deoxyhemoglobin (full lines).<sup>10</sup>

even when fully reduced.<sup>25</sup> The structure of BME-DHb was determined at 1.8-Å resolution and compared with Ladner et al.'s structure of horse Hb<sup>+</sup>H<sub>2</sub>O at 2.0-Å resolution<sup>12</sup> after both structures had been subjected to combined least squares and energy refinement. With helices B, G, and the first 11 residues of H as a reference frame, rms differences between main-chain atoms were found to be greatest for helices E and F $\alpha$  and for D, E, and F $\beta$ . The  $\alpha$ -hemes stay put, while the  $\beta$ -hemes tilt very slightly in the same direction as on transition from R to T. The Fe's move away from the plane of the N<sub>porph</sub>'s by 0.2 Å and from the mean plane of the porphyrin by 0.3 Å; the porphyrins become domed as in DHb in the T structure (Figures 4 and 8). Table I shows the Fe-N distances to be the same within error as in human DHb in the T structure, while the displacement of the Fe's from the plane of the N<sub>porph</sub>'s is less by about 0.1 Å. This corresponds exactly to the difference between the displacements of the Fe's in synthetic Fe porphyrins with the hindered "T-state" 2-methylimidazole (0.40 Å) and the unhindered "R-state" 1-methylimidazole (0.31 Å) in the fifth coordination position.<sup>19,79</sup> The structure of the latter was solved *after* that of deoxyhemoglobin in the R structure and without knowledge of it. The close similarity between the 5-coordinated hemes in the T and R structures found in crystals has been corroborated by studies of the X-ray absorption near edge structures (XANES) of solutions of carp DHb in the two quaternary structures. Their XANES curves were identical, implying that the Fe-N distances remain the same within 0.01 Å and the displacements of the Fe's from the plane of the N<sub>porph</sub>'s differ by no more than 0.1 Å.<sup>71</sup> There is a concerted movement of the two subunits relative to

each other at the  $\alpha_1\beta_2$  contact, as if the molecule were trying to make the R  $\rightarrow$  T switch but could not muster enough energy to go more than a small part of the way. For example, during the R  $\rightarrow$  T switch the distance between the C $\beta$ 's of Thr C3(38) $\alpha_1$  and His FG4(97) $\beta_2$  increases by 6.5 Å, while on going from met (R) to deoxy (R) that distance increases by only 0.5 Å. There is no significant difference density at the C termini of the four chains.<sup>10</sup>

$(\alpha\text{-Fe}^{\text{III}}\text{H}_2\text{O})_2(\beta\text{-Fe}^{\text{III}}\text{H}_2\text{O})_2$  and  $(\alpha\text{-Fe}^{\text{III}}\text{F}^-)_2(\beta\text{-Fe}^{\text{III}}\text{F}^-)_2$  in the T Structure.<sup>8,41</sup> These two liganded T structures are isomorphous with the unliganded T structure and exhibit smaller changes in tertiary structure around the  $\beta$ -hemes than  $(\alpha\text{-Ni}^{\text{II}})_2(\beta\text{-FeCO})_2$ , because the smaller heme ligands experience less steric hindrance by the distal residues than CO or O<sub>2</sub>.

### The Heterotropic Ligands

All the heterotropic ligands lower the oxygen affinity by forming hydrogen bonds that specifically stabilize and constrain the T structure. For each mole of O<sub>2</sub> taken up at pH 7.4 and 25 °C, human Hb liberates 0.2 mol of H<sup>+</sup> in a deionized solution, 0.5 mol of H<sup>+</sup> in 0.1 M Cl<sup>-</sup>, and 0.7 mol of H<sup>+</sup> in the presence of a molar excess of DPG.<sup>26,27</sup> This is known as the alkaline Bohr effect. In deionized solutions all the protons come from His HC3(146) $\beta$  which donates a hydrogen bond to Asp FG1(94) $\beta$  in the T structure and accepts a hydrogen bond from its own main-chain NH in the R structure (Figure 5b).<sup>28,29</sup> In consequence, its pK<sub>a</sub> drops from 8.0 in DHb to 6.5 or less in HbO<sub>2</sub>.<sup>30</sup> The binding of Cl<sup>-</sup>

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by the T structure raises the  $pK_a$ 's of Val NA1(1) $\alpha$  and Lys EF6(82) $\beta$  which contribute an additional 0.28 mol of  $H^+$  to the Bohr effect. DPG enters a cleft flanked by the N termini and helices H of the  $\beta$ -chains and forms hydrogen bonds with Val NA1(1), His NA2(2), Lys EF6(82), and His H21(143) (Figure 10 of ref 7). The rise in  $pK_a$ 's of their cationic groups contributes 0.33 mol of  $H^+$  to the Bohr effect.<sup>27</sup>  $CO_2$  forms carbamino groups with Val NA1(1) $\alpha$  and  $\beta$ , and these in turn make hydrogen bonds with cationic groups of the globin.

In 0.1 M Tris HCl + 0.1 M NaCl at pH 7.4 and 21.5 °C, the first mole of  $O_2$  taken up releases 0.64 ( $\pm 0.07$ ) mol of  $H^+$ , the second and third mole of  $O_2$  combined release 1.62 ( $\pm 0.27$ ) mol, and the fourth mole of  $O_2$  releases only 0.05 ( $\pm 0.06$ ) mol of  $H^+$ .<sup>31</sup> How is their release related to the allosteric transition from T to R? Allosteric theory allows the equilibrium constant  $L_i = [T]/[R]$  at the  $i$ th step of oxygenation to be calculated from  $L_i = L_0(K_R/K_T)^i$ . Under the above nonphysiological conditions  $L_1 = 8.7 \times 10^4 \times 0.0073 = 633$ .<sup>34</sup> Thus, more than a quarter of the Bohr protons are discharged before 1/600 of the Hb molecules have switched from T to R, which implies that the hydrogen bonds responsible for  $H^+$  discharge must break in the T structure. The bulk of the protons are released in the T → R transition which takes place mostly at the second and third oxygenation steps. After the third oxygenation step  $L_3 = 0.034$ , leaving a little more than 1/30 of the Hb molecules in the T structure, which is roughly equivalent to the fraction of 1/20 of the protons to be discharged at the fourth oxygenation step.

### Stereochemical Mechanism

In 1970 Perutz proposed that "the oxygenation of hemoglobin is accompanied by structural changes in the subunits triggered by shifts of the iron atoms relative to the porphyrin and, in the  $\beta$ -subunits, also by the steric effect of oxygen itself. The oxygen-free form is constrained by salt-bridges (hydrogen bonds between oppositely charged ions) which are broken by the energy of heme–heme interaction with the release of  $H^+$ . 2,3-Diphosphoglycerate may add to the constraints by being stereochemically complementary to a site between the  $\beta$ -chains; this complementarity is lost on oxygenation".<sup>32</sup>

**Role of the Hemes.**<sup>61</sup> In 1970 the resolution of the best electron density maps was 2.8 Å, which was insufficient to resolve the atoms of the porphyrin; methods for refining atomic coordinates in protein structures did not yet exist. Bolton and Perutz measured the displacements of the Fe's from the porphyrins, assumed to be planar, with a ruler in a Richards box and obtained values of 0.75 Å for horse DHb and 0.3 Å for horse Hb<sup>+</sup>H<sub>2</sub>O, the only derivatives then available.<sup>33</sup> The uncertainties of these measurements aroused much controversy about the proposal that the T → R equilibrium is governed by the displacement of the Fe's from the plane of the porphyrin. Recent X-ray analyses at high resolution have confirmed that the iron atoms do shift and show that the conformation of the por-

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phyrins also changes. As a result, on transition from DHb in the T structure to HbO<sub>2</sub> in the R structure, the N<sub>ε</sub>'s of the proximal histidines move relative to the mean porphyrin plane by 0.6 Å in the  $\alpha$  and 0.5 Å in the  $\beta$  subunits (Figure 4). Do those movements trigger the allosteric transitions between the R and T structures, and if so, how are these transitions initiated?

Our semiliganded derivatives in the T structure show that on combination of  $O_2$  or CO with the  $\alpha$ -hemes the Fe's move by 0.15 Å toward the plane of the N<sub>porph</sub>'s while the doming of the porphyrins is preserved. The movements of the Fe's are transmitted to the proximal histidines and their adjoining residues, while the bulk of the protein remains unperturbed. Thus, perturbations are confined to what Gelin *et al.* have called the "allosteric core".<sup>16</sup> On loss of Fe-linked H<sub>2</sub>O and reduction of the Fe's in BME–Hb in the R structure, the Fe's move away from the plane of the N<sub>porph</sub>'s by 0.2 Å; the movements are transmitted not just to the proximal histidines and their adjoining residues, but also to the  $\alpha_1\beta_2$  and  $\alpha_2\beta_1$  contacts which shift a short way toward their positions in the T structure.

There have been suggestions that the hydrogen bonds between N<sub>δ</sub> of His F8 and the carbonyl of Leu F4 play a part in the allosteric mechanism.<sup>34,35</sup> The length of these bonds may change in transition states, but it remains the same in DHb and HbO<sub>2</sub>.<sup>9</sup> There has also been a suggestion that changes in charge-transfer interactions between the porphyrin and Phe CD1 contribute to the free energy of cooperativity,<sup>36</sup> but the distance between the phenylalanine side chain and the porphyrin is too large (3.8–4.1 Å) for such interactions to occur. *We are thus left with the distances of the Fe's and the proximal histidines from the porphyrin as the only determinants of the allosteric equilibrium visible in the  $\alpha$ -subunits.*

**Role of Distal Residues.** In the  $\alpha$ -subunits the replacement of the hydrogen-bonded H<sub>2</sub>O by O<sub>2</sub> causes no perceptible perturbation of the distal residues.<sup>9</sup> In the  $\beta$ -subunits, on the other hand, displacement of the distal His E7 and Val E11 is necessary for the binding of O<sub>2</sub> or CO to the ferrous hemes, though not for the binding of H<sub>2</sub>O or F<sup>−</sup> to ferric hemes.<sup>32,41</sup> Since a valine side chain attached to an  $\alpha$ -helix cannot rotate about the C<sub>α</sub>–C<sub>β</sub> bond, Val E11 cannot move relative to the heme without a displacement of helix E. What part does that displacement play in the mechanism of cooperativity?

Since crystals of human DHb grown in PEG and exposed to air show full substitution with O<sub>2</sub> at the  $\alpha$ -hemes, but none at the  $\beta$ -hemes, the O<sub>2</sub> affinity of the  $\beta$ -hemes in the crystalline T structure must be close to zero.<sup>7,8</sup> In solution, on the other hand, that affinity is lower than that of the  $\alpha$ -hemes by only a factor of 1.5–3.0, and the  $\beta$ -hemes react with O<sub>2</sub> 4 times faster than the  $\alpha$ -hemes.<sup>37</sup> The structure of  $(\alpha\text{-Ni}^{II})_2(\beta\text{-Fe}^{II}\text{CO})_2$  suggests that this is due to a transition in the tertiary structure of the  $\beta$ -subunits from t<sub>β</sub> to t<sub>β'</sub>, which takes place in solution, but is inhibited by the lattice constraints in DHb crystals.<sup>72</sup>

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Replacement of the distal histidine E7 and valine E11 of the  $\beta$ -chains by directed mutagenesis has helped to clarify their roles.<sup>38</sup> Replacement of Val E7 by Ile blocks the oxygen site in both the T and R structures but nevertheless fails to inhibit oxygen binding: it merely shifts the entire equilibrium curve to the right, roughly halving the oxygen affinity and implying that the oxygen affinity of the  $\beta$ -subunits may be zero in the T structure and may be reduced to about one-fifth of its normal value in the R structure. This shows that the R structure has enough flexibility to adjust the relative positions of the heme and the distal isoleucine sufficiently for ligands to bind, at a cost of only about 1 kcal per  $\beta$ -heme. Replacement of Val E11 by Ala removes the steric hindrance at the  $\beta$ -hemes in the T structure; it thereby raises  $K_T$  by an order of magnitude but leaves  $K_R$  unchanged, thus confirming the absence of steric hindrance in the normal R structure. Replacement of the distal histidine by valine raises  $K_T$ , probably by changing the tilt of the heme, and lowers  $K_R$ , presumably due to the loss of the hydrogen bond between  $O_2$  and His E7. Replacement of the distal histidine by glycine also lowers  $K_R$ , probably for the same reason, but has no significant effect on  $K_T$ , which confirms that steric hindrance to ligand binding in the T structure comes from Val E11 alone, as originally proposed.<sup>32</sup> Other replacements of the distal residues fit in with this general pattern.

Neither the  $\alpha$  nor the  $\beta$  heme pockets have an opening through which  $O_2$  can pass unless the distal histidines swing out of the way, which requires changes in tertiary structure.<sup>78</sup> These changes affect the quantum yield of  $O_2$  dissociation after a laser flash. In the R structure the apparent quantum yield as a function of the light intensity at 540 nm drops with decreasing temperature and increasing solvent viscosity, apparently because the changes in tertiary structure needed to allow  $O_2$  to escape are activated by heat and resisted by viscous solvents. In the T structure a plot of light intensity vs. oxygen dissociation shows two different quantum yields. The lower yield is the same as in the R structure and is equally dependent on temperature and viscosity. The other, 10 times larger, yield is only slightly influenced by temperature and viscosity which implies that oxygen can escape with minimal changes in tertiary structure.<sup>39</sup> With Fe-Co hybrid Hb's, that larger quantum yield has been assigned to the  $\beta$ -subunits,<sup>40</sup> which exhibit a rate of dissociation of CO 7.5 times faster than that of the  $\alpha$ -subunits.<sup>39</sup> These findings suggest that in the T structure combination of the  $\beta$ -hemes with  $O_2$  causes helix E to be displaced from the hemes sufficiently far to leave the heme pockets wide open so that  $O_2$  can escape unhindered. On the other hand, in  $(\alpha\text{-Ni}^{II})_2(\beta\text{-Fe}^{II}\text{CO})_2$  the  $\beta$ -heme pockets are closed, and CO could not escape without changes in tertiary structure. Thus, the X-ray data offer no explanation for the temperature- and viscosity-independent quantum yield.

#### Role of Hydrogen Bonds (Salt Bridges) between Subunits in the T Structure.

According to allosteric

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theory, the low  $O_2$  affinity of the T as compared to that of the R structure arises from increased energy and/or number of bonds between the subunits.<sup>42</sup> The contact areas and the number of bonds between segments  $C\alpha_1$  and  $FG\beta_2$  and between segments  $C\beta_2$  and  $FG\alpha_1$  are about equal in the R and T structures;<sup>5</sup> the C-terminal residues and DPG, on the other hand, form 14 salt bridges between the subunits which are absent in the R structure (Figure 4). The bond energies of the two pairs of salt bridges made by the C-terminal residues have been measured. They amount to about 8 kcal mol<sup>-1</sup>, leaving only 600 cal mol<sup>-1</sup> per salt bridge to be contributed by the remaining 10 salt bridges, amply sufficient to account for the total free energy of cooperativity of 14.4 kcal per tetramer, because a salt bridge contributes usually at least 1 kcal mol<sup>-1</sup>. Absence of any of the bridges raises  $K_T$  and lowers  $L$ . Chothia et al. calculated the accessible surface area buried when two  $\alpha\beta$  dimers associate to form either the T or the R tetramer. They found that area to be greater by 700 Å in the T structure and concluded that this contributed 20 kcal per tetramer to the free energy of cooperativity.<sup>73</sup> Lesk and others have recalculated the buried surface area,<sup>74</sup> based on the recent atomic coordinates of Fermi et al. and of Shaanan and found the difference between the T and R structures to have shrunk to 140 Å; as this is less than 2% of the total buried surface area, and in view of the uncertainties involved in calculating the energies associated with the burial of many of the side chains, this figure may not be significant. Lesk and others argue that the arginines HC3 (141) $\beta$  form salt bridges also in  $\text{HbO}_2$ , making their contribution to the stability of the tetramer similar to that in the T structure, but in fact these salt bridges are made loosely with an ion of inorganic phosphate present only in the crystal which would be absent in vivo.

**Location of Free Energy of Cooperativity. (a) Ferrous Hemoglobins.** Where is the free energy of 3.6 kcal per mole of heme stored when ligands combine with the T structure? The X-ray analyses described here show that part of the strain is stored in the heme and part in the globin. For 6-coordinated low-spin hemes, the conformation of lowest free energy is flat, with the iron in the porphyrin plane, but in  $(\alpha\text{-Fe}^{II}\text{-O}_2)_2(\beta\text{-Fe}^{II})_2$  the  $\alpha$ -hemes remain domed as in DHb, and the Fe's lie halfway between their positions in DHb and  $\text{HbO}_2$ . The remainder of the strain is differently distributed in the two  $\alpha$ -subunits: in one it takes the form of a lengthening of the Fe-N<sub>e</sub> bond and in the other of a dislocation of His F8(87) and its adjoining residues toward the porphyrins. The difference arises through the different lattice contacts made by the two  $\alpha$ -subunits in the crystal, but it is not possible to say which of the two perturbations is representative of conditions in solution. In  $(\alpha\text{-Fe}^{II}\text{-CO})_2(\beta\text{-Co}^{II})$  and  $(\alpha\text{-Fe}^{II}\text{-CO})_2(\beta\text{-Mn}^{II})$ , dislocations of His F8(87) and its adjoining residues are seen in both subunits, but the resolution was insufficient to measure the lengths of the Fe-N<sub>e</sub> bonds.

Since contraction of the Fe-N bonds accompanies ligand binding and triggers the T  $\rightarrow$  R transition, the T structure is likely to oppose ligand binding by

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stretching these bonds. In the absence of sufficiently accurate X-ray data, this proposition has been tested by spectroscopic and magnetic methods. The R  $\rightarrow$  T transition of DHb produces characteristic changes in the adsorption spectra in the near-UV, the visible, and the near-IR regions. They come mainly from the  $\alpha$ -subunits and have been interpreted in terms of a lengthening of the Fe–N bonds.<sup>45,46,68</sup> This has been confirmed by resonance Raman spectroscopy (RR).  $\nu(\text{Fe}-\text{N}_\epsilon)$  in free deoxy  $\alpha$ -subunits or in  $\alpha$ -subunits in the R structure is  $223 \text{ cm}^{-1}$ , while in deoxy  $\alpha$ -subunits in the T structure it drops to  $203 \text{ cm}^{-1}$ . In free deoxy  $\beta$ -subunits it is  $224 \text{ cm}^{-1}$ , and in  $\beta$ -subunits in the T structure it drops to  $220 \text{ cm}^{-1}$ . These data show that the lengthening of the Fe–N $\epsilon$  bonds at the  $\alpha$ -hemes is greater than at the  $\beta$ -hemes, but the strain energies calculated from the Raman shifts amount to no more than a few calories.<sup>47</sup> At one stage it was thought that  $\nu(\text{Fe}-\text{N}_\epsilon)$  decreases with decreasing oxygen affinity of the T structure,<sup>48</sup> but evidence published in support of that idea has proved to be an artifact. RR spectra of Fe–Ni hybrids have shown  $\nu(\text{Fe}-\text{N}_\epsilon)$  to remain constant and independent of changes in  $K_T$ .<sup>49</sup> Thus, the strength of the Fe–N $\epsilon$  bond in the *unliganded* T structure is independent of the constraints that decrease its oxygen affinity. The same is true of the hydrogen bond that links N $\delta$  of the proximal histidine to the main-chain carbonyl in the preceding turn of the F helix: Nagai *et al.* found the chemical shift of the N $\delta$  proton to be independent of  $K_T$ .<sup>50</sup> There are as yet no spectroscopic data on  $\nu(\text{Fe}-\text{N}_\epsilon)$  or on the chemical shift of N $\delta$ H–O in the *liganded* T structure. Paradoxically,  $\nu(\text{Fe}-\text{O})$  and  $\nu(\text{Fe}-\text{CO})$  are the same in the liganded R and T structures, implying that the strengths of the Fe–O<sub>2</sub> and Fe–CO bonds are independent of the several 100-fold differences between the equilibrium constants of O<sub>2</sub> and CO with the two quaternary structures.<sup>51,52</sup> The X-ray structure of  $(\alpha\text{-Fe}^{\text{II}}-\text{O}_2)_2(\beta\text{-Fe}^{\text{II}})_2$  described above does indicate that at least one of the Fe–N $\epsilon$  and both N $\delta$ H–O bonds in the oxygenated  $\alpha$ -subunits are longer than in DHb, but the degree of stretching (0.2–0.3 Å) is within experimental error, thus leaving the question of the location of the strain in that semi-*liganded* T structure undecided. Striking evidence in favor of tension at the heme in one liganded T structure comes from optical, IR, RR, and ESR experiments with HbNO, which proved that transition from the R to the T structure cleaves the Fe–N $\epsilon$  bonds at the  $\alpha$ -hemes, because the Fe–N $\epsilon$  bonds are weakened by transfer of unpaired electron density from the NO to the d<sub>2</sub> orbital of the Fe.<sup>53–55</sup>

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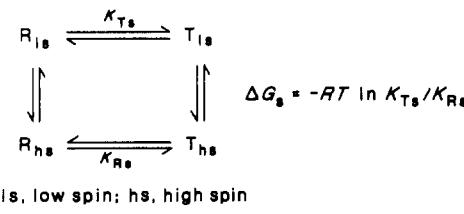
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$\nu(\text{Fe}-\text{N}_\epsilon)$  is also observable by RR spectroscopy of DHb produced by photolysis of HbCO or HbNO. Immediately after a flash of 25 ps or 10 ns the Hb molecules are still in the R structure, because the R  $\rightarrow$  T transition is relatively slow, with a rate constant of the order of  $5 \times 10^4 \text{ s}^{-1}$ . The transient species observed after these short time intervals have  $\nu(\text{Fe}-\text{N}_\epsilon)$  frequencies that are between 1 and  $17 \text{ cm}^{-1}$  higher than in the ground deoxy T state, indicative of a shortening of the Fe–N $\epsilon$  bonds. That rise in frequency is greatest in Hb's with high O<sub>2</sub> affinity and smallest in those with low O<sub>2</sub> affinity. The frequency shift between T state deoxy and transient is about twice as large as between T and R state deoxy ( $\sim 15 \text{ cm}^{-1}$  vs.  $\sim 7 \text{ cm}^{-1}$ ).<sup>56</sup>

Photolysis of HbCO by a 10-ns laser flash produces a transient photoproduct (DHb\*) with a visible difference absorption spectrum relative to DHb that is similar to the R–T difference spectrum of DHb but smaller in amplitude. That transient DHb\* has a lifetime of  $1/e \sim 40 \text{ ns}$ .<sup>57</sup> Photodissociation with a 250-fs flash produces a DHb\* after 300 fs that persists for the period of measurement of 100 ps and may be the same as the one found after 10 ns.<sup>58</sup> This suggests that the time taken by the Fe to snap back from the porphyrin plane is only 300 fs; it is not clear whether it snaps back all the way to the structure seen in BME–DHb or whether the full movement requires another step that is completed only after 40 ns. Finally, after some microseconds the globin switches from R to T.

**(b) The Evidence of Changes in the Spin State.** Ferric Hb derivatives (Hb<sup>+</sup>) range from pure high-spin Hb<sup>+</sup>F<sup>–</sup> through mixed-spin compounds to low-spin Hb<sup>+</sup>CN<sup>–</sup>. All ferric Hb's normally have the R structure, but high-spin human Hb<sup>+</sup>'s and all carp Hb<sup>+</sup>'s can be switched to the T structure at low pH by addition of inositol hexaphosphate. Fe–N stretching frequencies have not been assigned. Clear evidence for an extension of the Fe–N bonds in the T structure comes from measurements of magnetic susceptibilities and IR and RR spectra of certain Hb<sup>+</sup> derivatives which are in a thermal equilibrium between two alternative spin states. In carp Hb<sup>+</sup>N<sub>3</sub><sup>–</sup> the R  $\rightarrow$  T transition biased that equilibrium toward higher spin by the equivalent of 1 kcal mol<sup>–1</sup>.<sup>59,60</sup> An even larger rise was found in carp Hb<sup>+</sup>NO<sub>2</sub><sup>–</sup>.<sup>58</sup> These spin equilibria can be represented by the scheme



X-ray studies of Fe chelates have shown that changes to higher spin are invariably accompanied by length-

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ening of the Fe-ligand bonds.<sup>61</sup> Recently, Philo and Dreyer reported that they found no significant rise in paramagnetic susceptibility on transition from R to T of the human mutant  $\text{Hb}^+\text{N}_3^-$  Kansas and only small increases in several other human mixed-spin  $\text{Hb}^+$  derivatives.<sup>62</sup> Perutz and Cowan have confirmed Philo and Dryer's result with  $\text{Hb}^+\text{N}_3^-$  Kansas but found that human  $\text{Hb}^+\text{NO}_2^-$ , which Philo and Dryer had not tested, behaves much like that of carp. On transition from R to T its paramagnetic susceptibility rises by 45%.<sup>63</sup> The absence of a rise in human  $\text{Hb}^+\text{N}_3^-$  may be related to the  $\text{O}_2$  affinity of human Hb in the T state being much higher than that of carp, so that the human T structure fails to stretch the Fe-N bonds in  $\text{Hb}^+\text{N}_3^-$ , and most of the strain energy is stored in the globin. In  $\text{Hb}^+\text{NO}_2^-$ , on the other hand, the Fe-N bonds appear to be weaker, so that the T structure of both carp and human Hb stretches them.

### Conclusions

Changes in quaternary structure are initiated by changes in the allosteric core of the protein.<sup>16</sup> In deoxyhemoglobin the porphyrins are domed, and the iron atoms are displaced by 0.4 Å from the plane of the

porphyrin nitrogens regardless of the quaternary structure of the globin. On binding  $\text{O}_2$  or CO, the iron atoms move toward the porphyrin. These remain domed in the T structure but flatten on transition to the R structure. As a result, on transition from deoxyhemoglobin in the quaternary T structure to oxyhemoglobin in the quaternary R structure, the iron atoms and the proximal histidines move toward the mean planes of the porphyrins by 0.5–0.6 Å. In deoxyhemoglobin in the quaternary T structure, combination of the  $\beta$ -hemes with  $\text{O}_2$  or CO is blocked by the distal valine; in the R structure this block is removed by a shift of the  $\beta$ -hemes relative to helix E.

In reaction intermediates, combination with  $\text{O}_2$  or CO of the  $\alpha$ -hemes in the T structure causes the iron atoms, the proximal histidines, and their adjoining residues to move toward the porphyrin by 0.15–0.3 Å, while the porphyrins remain domed, showing that their conformation is constrained by the globin. On combination with CO of the  $\beta$ -hemes a transition in tertiary structure from  $t_\beta$  to  $t'_\beta$  changes the tilt of the heme, shifts the iron and proximal histidine toward the porphyrin, and moves the distal residues away from the ligand site. On loss of heme ligands from the R structure, the transition from R to T is initiated by movements of the iron, the proximal histidines, and their adjoining residues away from the porphyrin by 0.3 Å and by doming of the porphyrins (Figure 4). *The doming and undoming of the porphyrins, the movements of the irons and of the proximal histidines, and, in the  $\beta$ -subunits, the movement of the distal residues relative to the heme are seen as the only perturbations that could set the changes in quaternary structure in motion.*

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There is uncertainty concerning the location of the strain responsible for the low oxygen affinity of the T structure. The location of the strain appears to vary from derivative to derivative and in hemoglobins of different species, depending on the relative strengths of the restraints of the globin and of the Fe-N bonds,<sup>64</sup> but this does not imply, as is sometimes claimed, that the mechanism of cooperativity differs in different species. The majority of the residues in contact with the hemes and those essential for the allosteric switch between the R and T structures are the same in all bony vertebrates. If the components of the machine have remained the same, its mechanism cannot have changed. Only the response to heterotropic ligands has evolved differently in different phyla.<sup>67</sup>