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MECHANISMS REGULATING THE REACTIONS OF HUMAN HEMOGLOBIN WITH OXYGEN AND CARBON MONOXIDE

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PERSPECTIVES AND SUMMARY

The reactions of hemoglobin with oxygen and carbon monoxide are subject to regulation by the heme and the residues surrounding it and by the effectors, also known as heterotropic ligands (H^+, Cl^-, CO_2 and 2,3-diphosphoglycerate), that regulate the equilibrium between its two forms, the oxy or R-structure with high, and the deoxy or T-structure with low oxygen affinity. The stereochemical mechanisms of regulation have been studied for many years by a variety of methods. What is new since the subject had last been surveyed in *Annual Review of Biochemistry* (38) is determination of the crystal structures at resolutions sufficient to resolve individual atoms of the heme and its surroundings; such structures have now been determined for deoxy, oxy, and carbonmonoxyhemoglobin, and for several analogues of

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transition states in the reaction with oxygen and carbon monoxide. In the past much useful information about the stereochemical mechanism of hemoglobin has come from the study of abnormal human hemoglobins. Now that the genes for the α and β chains of hemoglobin and for the single chain of myoglobin have been cloned in *E. coli*, directed mutagenesis has provided new tools for probing the reactions of these proteins with ligands. Finally, an (unsuccessful) search for possible antisickling drugs has led to the discovery of a family of compounds that are more powerful allosteric effectors than the natural one, 2,3-diphosphoglycerate (DPG), and that combine with sites that are far removed from the diphosphoglycerate binding site.

In this review I shall describe the new insights into the allosteric mechanism provided by X-ray analysis at high resolution, or perhaps I should say, the refinement of the mechanism I proposed in 1970 (36). I then suggested that combination of the heme iron with oxygen or carbon monoxide is accompanied by a shift of the iron atom and its attached histidine relative to the porphyrin. That shift causes changes in the tertiary structure of the subunits that lead to a rearrangement of the subunits from the quaternary deoxy or T, to the oxy or R-structure. I proposed that the low oxygen affinity of the T-structure is due to additional bonds between the four subunits that take the form of hydrogen bonds between groups of opposite charge, also known as salt bridges, and that these bonds oppose the movement of the iron that is needed for the binding of oxygen. On oxygenation these salt bridges are broken, with the release of the hydrogen ions that are linked to the Bohr effect.

All these proposals have been confirmed, but certain aspects of my mechanism have remained unresolved. The structure of deoxyhemoglobin showed that the ligand sites in the β-subunits are obstructed by the distal valines, which suggests that no ligands can bind, but chemical studies showed that the oxygen affinity of the β-hemes in the T-structure is only slightly less than that of the α-hemes. X-ray analysis of a transition state analogue has now resolved this apparent contradiction. Another puzzle concerned the transmission of stereochemical effects from the hemes to the salt bridges. I proposed a system of levers that turned out to be wrong, but none of the studies of transition state analogues have suggested what the right mechanism could be. The partition coefficients of hemoglobin, and especially of myoglobin between oxygen and carbon monoxide, are much smaller than that of free heme. This difference is vital because one mole of carbon monoxide is produced endogenously for each mole of porphyrin broken down and would otherwise block the transport of oxygen. Directed mutagenesis has shown how myoglobin and the α-subunit discriminate between oxygen and carbon monoxide, but has left the mechanism of discrimination by the β-subunits obscure.

INTRODUCTION

Common Features of Hemoglobins

All hemoglobins have similar structures. The globin chain has a characteristic fold that envelops the heme in a deep pocket with its hydrophobic edges inside and its propionates facing the solvent. The chain is made up of seven or eight α-helical segments and an equal number of nonhelical ones placed at the corners between them and at the ends of the chain (Figure 1). According to a notation introduced by Watson & Kendrew (49), the helices are named A to

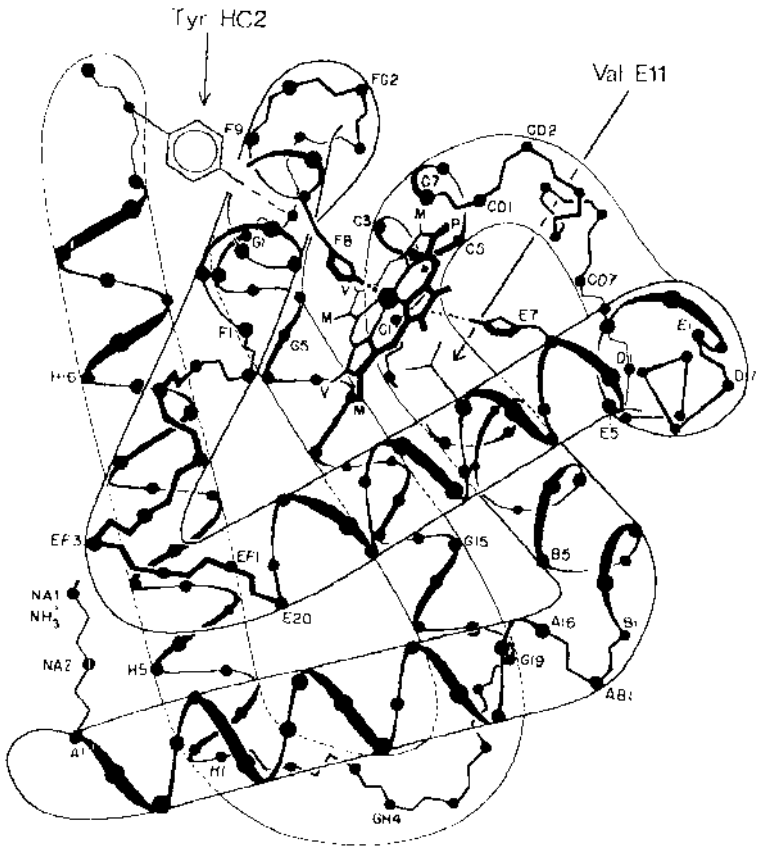


Figure 1 Tertiary structure of β-chains of human hemoglobin, typical of hemoglobins and myoglobins of all other species. The figure also shows the proximal and distal histidines, marked F8 and E7, the distal valine E11, and the tyrosine HC2 that ties down the C-terminus by its hydrogen bond to the main chain carbonyl of valine FG5.

H, starting from the amino end; the nonhelical segments that lie between helices are named AB, BC, CD, and so on. The nonhelical segments at the ends of the chain are called NA and HC. Residues within each segment are numbered from the amino end. A1, A2, CD1, CD2, and so on. Evolution has conserved this fold of the chain despite great divergence of the sequence: the only residues common to all hemoglobins are the proximal histidine F8 and the phenylalanine CD1, which wedges the heme into its pocket. Most, but not all, globins also have a histidine on the distal (oxygen) side of the heme. Ionized residues are excluded from the interior of the globin chains, which is filled largely by hydrocarbon side chains, but some serines and threonines also occur there. The proximal and distal histidines (also called the heme-linked histidines) are potentially polar, but the proximal histidine does not ionize, and the pK_a of the distal one is so low ($\sim$5.5) that the fraction ionized in vivo is negligible.

REACTION WITH OXYGEN AND CHANGE OF QUATERNARY STRUCTURE

Hemoglobin combines with oxygen and carbon monoxide cooperatively. This cooperativity arises not primarily by any direct interaction between the active sites, but mainly by a change in equilibrium between the two alternative structures, T and R, at successive steps of ligand binding. The degree of cooperativity is expressed as the slope n at the midpoint of the a plot of log $p(O_2)$ against log $y/1-y$, where y is the fractional saturation with oxygen or carbon monoxide. Cooperativity ensures that most of the molecules are either fully oxygenated or fully deoxygenated. This has first been demonstrated directly by Perella & his collaborators who devised a method of trapping the intermediates in the reaction of hemoglobin with carbon monoxide (33, 34) (Figure 2).

The oxygen affinity of the R-structure is slightly larger than the average one of free α- and β-subunits; that of the T-structure is lower by the equivalent of the free energy of cooperativity. The oxygen equilibrium can be described by the oxygen association constants K_T and K_R, usually expressed in (mm Hg)$^{-1}$, and by the equilibrium constant L_o = [T]/[R] in the absence of oxygen. Imai has shown empirically that log K_T/K_R = A $-$ 0.25 log L_o, 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 pH7, but is little affected by the other ligands. (1, 3, 5, 10, 13, 17, 38, 41).

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

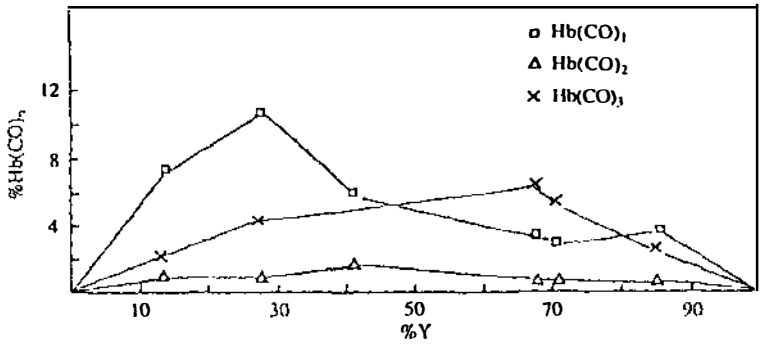


Figure 2 Observed distribution of intermediates in the reaction of hemoglobin with CO as a function of percentage saturation with CO. The reaction showed a Hill's coefficient of 3.0. The concentration of intermediates was obtained after quenching the reaction of two solutions expelled into a mixing chamber from two separate syringes. Either one syringe was filled with deoxy and the other with carbonylhemoglobin, or one was filled with partially oxidized carbonylhemoglobin and the other with a solution of dithionite. After mixing the solution was expelled into a cryochamber at −25°C containing a tenfold molar excess of ferricyanide in an equal mixture of phosphate buffer and ethyleneglycol. Salt and ferricyanide were removed, and the different species separated by isoelectric focusing at −25°C. Contrary to the claims of Gill et al (16), the triligated species is significantly populated. The diligated species are nearly all of the type $\alpha(CO)\beta(CO)\alpha\beta$ (from Perrella et al, 33, 34).

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 o.8Å. 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 (Figure 3).

The key questions for the understanding of hemoglobin function are these: how does the reaction with oxygen 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 oxygen affinity? By what mechanisms do the heterotropic ligands influence the oxygen affinity? Single crystal X-ray analyses of deoxy and oxyhemoglobin, and of analogues of intermediates in the reactions with oxygen or carbon monoxide, together with chemical, spectroscopic, and magnetic studies have furnished some of the answers.

STRUCTURAL MECHANISM

Changes in the Allosteric Core

Figure 4 summarizes the stereochemistry of the hemes in deoxyhemoglobin, oxyhemoglobin, and in two intermediates. In deoxyhemoglobin the iron is high spin ferrous (S=2) and five-coordinated. The iron atoms are displaced

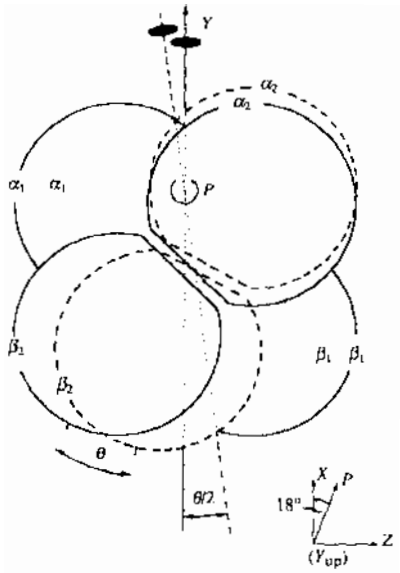
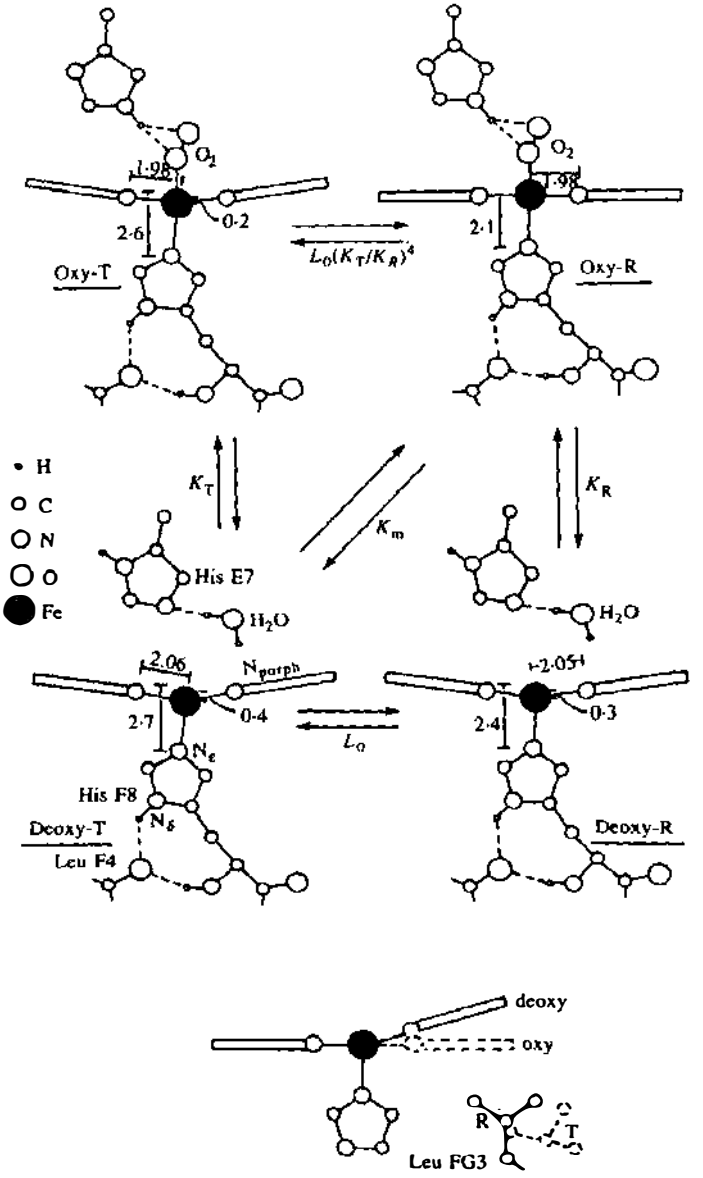


Figure 3 Change of quaternary structure of mammalian hemoglobin on transition from deoxy or T *(full lines)* to oxy or R *(broken lines)*. The dimer $\alpha_2\beta_2$ turns by $\theta = 13°$ about the P axis; this entails a rotation of the dyad symmetry axis Y by $\theta/2 = 6.5°$. If the four subunits were identical, P would have to coincide with X (from Baldwin & Chothia, 4).

Figure 4 Change of allosteric core on-going from deoxy-T via oxy-T to oxy-R and from there via deoxy-R back to deoxy-T. The vertical bars indicate the distance of N_ϵ of the proximal histidine F8 from the mean plane of the porphyrin nitrogens and carbons (excluding β and γ carbons of the side chains). The horizontal bar gives the Fe-$N_{porphyrin}$ distance, and the figure to the right of the iron atoms in the lower two diagrams the displacement of the iron from the plane of the porphyrin nitrogens. L_o is the allosteric constant in the absence of oxygen, K_T and K_R are the association constants with oxygen of the T- and R-structures, and K_m is the mean oxygen association constant. Note that the porphyrin is flat only in oxy-R and that the proximal histidine tilts relative to the heme normal in the T-structures. Note also the water molecule attached to the distal histidine in deoxy. The bottom diagram illustrates how the flattening of the porphyrin on-going from deoxy to oxy exerts a leverage on leucine FG3 and valine FG5, which lie at the switching contact between the two structures. (from Perutz et al, 41). The differences in heme geometry between deoxyhemoglobins in the T- and R-structures shown here are closely similar to those found between sterically hindered 2-methyl- and unhindered 1-methylimidazole iron porphyrin complexes (Momenteau et al, 29).


O2
1·98
0·2
2·6
Oxy-T
L0(KT/KR)4
2·1
Oxy-R
H
C
N
O
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Km
KR
His E7
H2O
2·06
Nporph
0·4
2·7
Ne
His F8
Nδ
Deoxy-T
Leu F4
L0
2·05
0·3
2·4
Deoxy-R
deoxy
oxy
R
T
Leu FG3

from the planes of the porphyrin nitrogens, and the porphyrins are domed. On oxygenation the iron becomes low spin ferrous (S=0) and six-coordinated. The porphyrins flatten, and the Fe-$N_{porphyrin}$ bond lengths contract from 2.06 to 1.98Å, thus moving the iron atoms towards the porphyrin planes (36, 38). As a result the proximal histidines come 0.5–0.6Å closer to the porphyrin planes in oxy than in deoxyhemoglobin. Do these movements trigger the allosteric transitions between the R- and T-structures, and if so, how are the transitions initiated?

Semi-liganded derivatives in the T-structure show that on combination of oxygen or carbon monoxide with the α-hemes the irons move by 0.15Å toward the plane of the porphyrin nitrogens, while the doming of the pyrroles is preserved. The movements of the irons 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 (15) have called the "allosteric core". On loss of iron-linked H_2O and reduction of the irons of a ferric hemoglobin in the R-structure, the iron atoms move away from the plane of the porphyrin nitrogens by 0.2Å, and the porphyrins become domed; 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 that shift a short way towards their positions in the T-structure (25, 41).

There have been suggestions that the hydrogen bonds between N_δ of His F8 and the carbonyl of Leu F4 play a part in the allosteric mechanism. The lengths of these bonds may change in transition states, but they remain the same in deoxyhemoglobin and oxyhemoglobin. 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, but the distance between the phenylalanine side chain and the porphyrin is too large (3.8–4.1Å) for such interactions to occur. We are thus left with the distances of the Fes and the proximal histidines from the porphyrin as the only determinants of the allosteric equilibrium visible in the α-subunits. In the β-subunits, displacement of the distal valine relative to the heme is necessary in the T-structure before oxygen can bind. In the R-structure this steric hindrance is absent.

Changes in the Globin Chains

THE α-SUBUNITS Since the $\alpha_1\beta_1$ contact undergoes no significant changes during the R → T transition, the atoms at this contact can serve as a reference frame for changes in tertiary structure elsewhere; except for residues G1–4 and H18–21, the B, G, and H helices were also found to be static. The largest movements relative to either of these frames occur in helix F, in segment FG, and in residues G1–4, H18–21, and HC1–3. Figure 5 shows the heme environment of deoxyhemoglobin superimposed on that of oxyhemoglobin. It

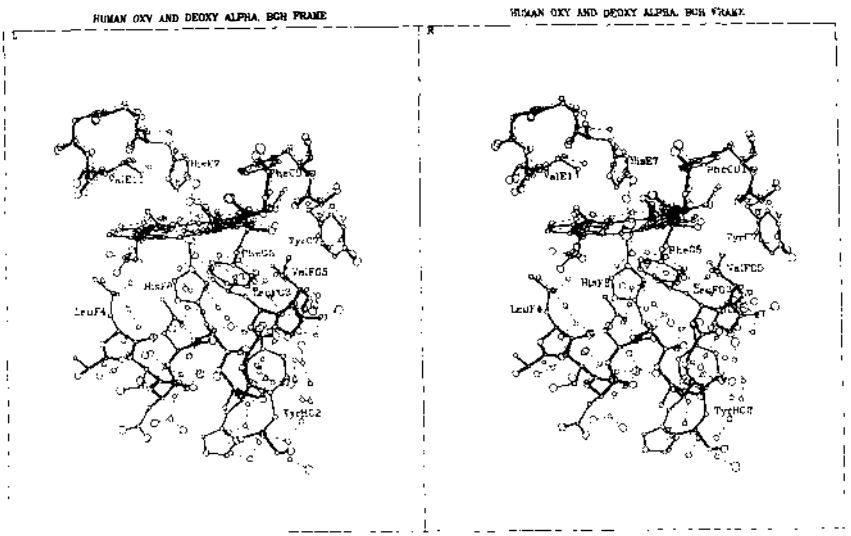

Figure 5 Change of tertiary structure near the α-heme on-going from deoxy-T *(full lines)* to oxy-R *(broken lines)*. Note how the proximal histidine straightens and moves closer to the porphyrin, carrying the residues in helix F with it (from Perutz et al, 41).

can be seen that on oxygenation helix Fα shifts towards the heme and to the right and carries the FG segment with it. In deoxyhemoglobin the imidazole of His F8 is tilted relative to the heme normal; in oxyhemoglobin the shift of helix F relative to deoxyhemoglobin aligns it with the heme normal. Taking as a reference frame residues F1–8 to which the heme is attached, the heme flattens and turns clockwise by 10°; the motion of its right-hand edge pushes down Leu FG3(91)α and Val FG5(93)α, which form part of the $\alpha_1\beta_2$ contact where the quaternary switch occurs (Figure 4). In the T-structure the N- and C- termini form the hydrogen bonds shown in Figures 6–8. In the R-structure these hydrogen bonds are broken, and the terminal residues are seen only at a low level of electron density, which implies that they are mobile.

THE β-SUBUNITS Figure 9 shows that on oxygenation helix F moves towards 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 farther into its pocket along a line linking porphyrin N_1 to N_3, and the heme rotates about an axis close to the line linking N_2 to N_4. Referred to residues F1 to F6, the iron stays still and the porphyrin becomes coplanar with it. In the T-structure, CγH$_3$ of Val E11(67) obstructs the ligand site at the iron; in the oxygenated R-structure that obstruction is cleared by a

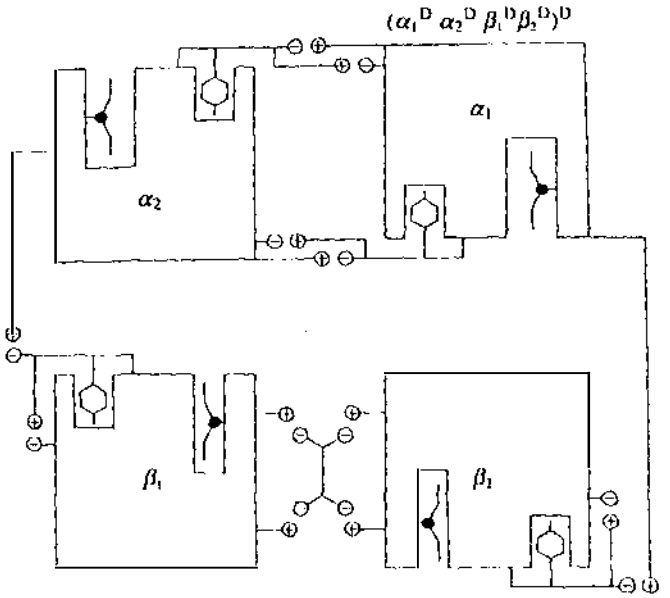

Figure 6 Diagrammatic representation of salt bridges in the T-structure. Those at the top link the C-terminal Arg HC3(141)α_2 to Asp H9(126)α_1 and Lys H10(127)α_1. The others link the C-terminal His HC3 (146)β_1 to Asp FG1 (94)β_1 and Lys C5(40)α_2. The bridge between the β-subunits represents 2,3-diphosphoglycerate (from Perutz, 36).

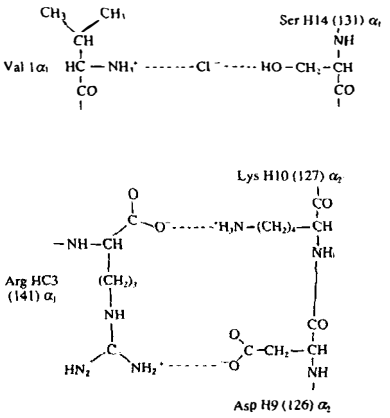

Figure 7 Salt bridges between the α-chains in the T-structure.

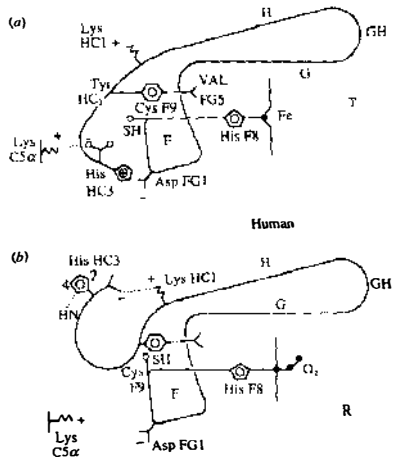

Figure 8 Change in conformation of histidine HC3 (146)β and cysteine F9 (93)β on-going from the T- to the R-structure. In the T-structure the imidazole of the histidine donates a hydrogen bond to Asp FG1 and is positively charged ($pK_a = 8.0$). Its carboxylate accepts a hydrogen bond from Lys C5α. The SH is *cis* to CO and points away from the heme. In the R-structure the imidazole accepts a hydrogen bond from the histidine's main chain NH and has a pK_a of 7.1 or below; and the C-terminal carboxylate accepts a weak hydrogen bond from Lys HC1. The SH group is *cis* to NH and in contact with Tyr HC2.

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, and as a result their pK_as drop on oxygenation. The conformation of the reactive sulfhydryl groups of Cys F9(93)β also changes (figure 8) (41).

In the T-structure the two β-subunits form a binding site for 2,3-diphosphoglycerate (Figure 10). In the R-structure the gap between the two β-chains becomes too narrow to accommodate it.

THE HETEROTROPIC LIGANDS

According to allosteric theory the low oxygen affinity of the T- as compared to that of the R-structure arises from increased energy and/or number of bonds between the subunits (31). The contact areas and the number of bonds between segments Cα_1 and FGβ_2 and between segments Cβ_2 and FGα_1 are about equal in the R- and T-structures (4); the C-terminal residues and DPG, on the other hand, form 14 salt bridges between the subunits that are absent in the R-structure (Figures 6–9). The bond energies of the four pairs of salt bridges made by the C-terminal residues have been measured. Those formed by the C-terminal histidines and histidines H21 (143) of the β-chains together contribute 7.6 kcal mol^{-1} (26), and those formed by arginine HC3 (141)α

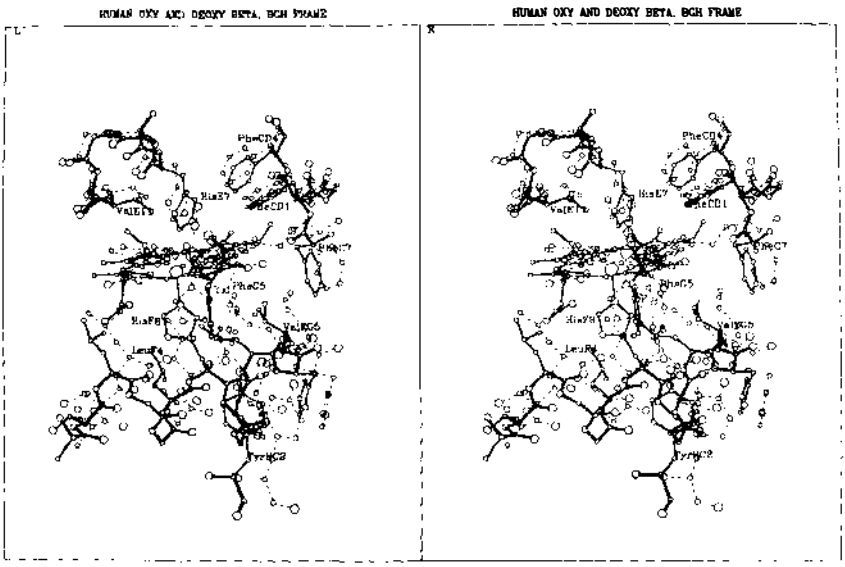

Figure 9 Change of tertiary structure near the β-heme on-going from deoxy-T *(full lines)* to oxy-R *(broken lines)*. Note how the heme moves to the right into the heme pocket, and the distal valine and histidine make way for the bound oxygen (from Perutz et al, 41).

contribute at least 4 kcal mol^{-1}, which leaves only 300 cal mol^{-1} per salt bridge to be contributed by the remaining eight salt bridges, sufficient to account for the total free energy of cooperativity of 14.4 kcal/tetramer, because a salt bridge contributes usually at least 1 kcal mol^{-1} (14). Absence of any of the bridges raises K_T and lowers L. The salt bridges keep the subunits rigidly in the tertiary deoxy structure and hinder the movement of the iron atoms into the planes of the porphyrin nitrogens and the flattening of the porphyrins themselves. This hindrance manifests itself in spectroscopic and magnetic differences between liganded hemoglobins in the two quaternary structures; these have recently been reviewed (41).

All the heterotropic ligands lower the oxygen affinity by forming additional hydrogen bonds that specifically stabilize and constrain the T-structure. The most important heterotropic ligands are protons. The linkage of proton uptake to oxygen release, and vice versa, is known as the Bohr effect. For each mole of oxygen released at pH7.4 and 25°C, human Hb takes up 0.2 moles H^+ in a deionized solution, 0.5 moles H^+ in 0.1 M Cl^-, and 0.7 moles H^+ in the presence of a molar excess of DPG (19, 39). The identity of the residues that take up protons has been determined by X-ray crystallographic and chemical studies of normal and mutant hemoglobins. In deionized solutions all the protons are taken up by His HC3(146)β, which donates a hydrogen bond to Asp FG1(94)β in the T-structure and accepts a hydrogen bond from its own

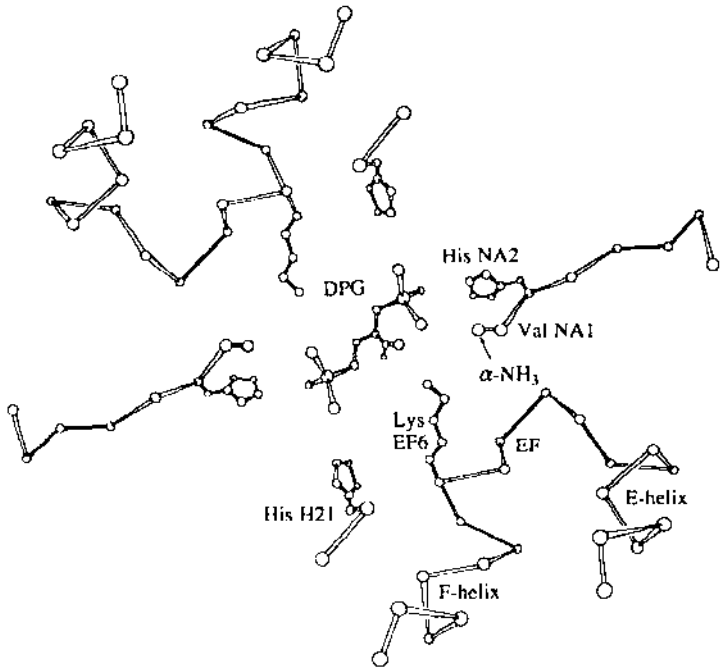

Figure 10 Hydrogen bonds between 2,3-diphosphoglycerate; and cationic groups of the β-chains in the T-structure. In the R-structure the gap between the EF corners closes and the N-termini move apart (from Arnone, 2).

main chain NH in the R-structure (Figure 8). In consequence its pK_a rises from 7.1 or less in oxyhemoglobin to 8.0 in deoxyhemoglobin (20, 27). The binding of Cl^- by the T-structure raises the pK_as of Val NA1(1)α (Figure 7) and Lys EF6(82)β, which contribute an additional 0.28 moles H^+ to the Bohr effect. DPG enters a cleft flanked by the N-termini and helices H of the β-chains and forms hydrogen bonds with Val NA1(1), His NA2(2), Lys EF6(82), and His H21(143) (Figure 10). The rise in pK_as of their cationic groups contributes 0.33 moles H^+ to the Bohr effect (19). Carbon dioxide forms carbamino groups with Val NA1(1)α and β, and these in turn make hydrogen bonds with cationic groups of the globin. All the groups that bind heterotropic ligands are at some distance from the hemes, consistent with Monod et al's prediction (30) that "no direct interaction need occur between the substrate of the (allosteric) protein and the regulatory metabolite which controls its activity".

In 0.1 M Tris HCl + 0.1 M NaCl at pH 7.4 and 21.5°C, the first mole of oxygen taken up releases 0.64(±7) moles H^+, the second and third mole of oxygen combined release 1.62(±27) moles H^+, and the fourth mole of oxygen releases only 0.05(±6) moles H^+ (8). 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 ith step of oxygenation to be calculated from $L_i = L_o (K_R/K_T)^i$. Under the above non-physiologic conditions $L_1 = 8.7 \times 10^4 \times 0.0073 = 635$ (3, 17). 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 that takes place mostly at the second and third oxygenation steps. After the third oxygenation step $L_3 = 0.034$, which leaves a little more than 1/30 of the Hb molecules in the T-structure, roughly equivalent to the fraction of 1/20 of the protons discharged at the fourth oxygenation step.

EFFECTS OF ABNORMAL HEMOGLOBINS ON RESPIRATORY FUNCTION

Study of the abnormal human hemoglobins has taught us a great deal about the respiratory function. The $\alpha_1 \beta_2$ contact acts as a two-way switch between the T- and R-structures. Each position of the switch is stabilized by a different set of hydrogen bonds. Disruption of any bond that specifically stabilizes the R-structure lowers the oxygen affinity and raises the allosteric constant L, and disruption of any bond that stabilizes the T-structure does the reverse. For example, hemoglobin Kansas [Asn G4(102)β→Thr] has a low oxygen affinity and low Hill's coefficient because the R-structure is destabilized (Figure 11), while hemoglobin Kempsey [Asp G1(99)→Asn] has a high oxygen

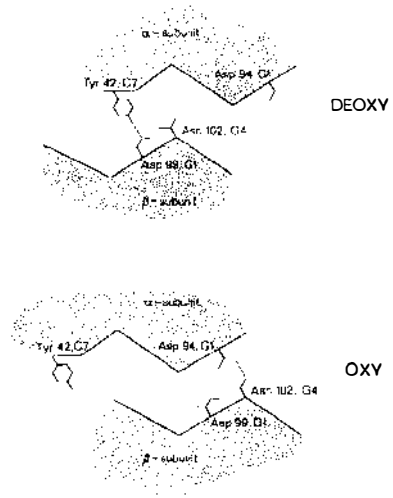

Figure 11 The $\alpha_1\beta_2$ contact as a two-way switch, showing alternative hydrogen bonds stabilizing the deoxy-T and oxy-R structures.

affinity and low Hill's coefficient because the T-structure is destabilized. Hill's coefficient is low in both hemoglobins because of the bell-shaped curve that relates it to the allosteric constant L. Mutations that disrupt the C-terminal salt bridges in the T-strucure also have high oxygen affinities and low Hill's coefficients. The surprise came when a mutation that disrupts hydrogen bonds common to both the R- and T-structures produced the same effect. This happens in hemoglobin Philly [Tyr C1(35)$\beta \rightarrow$ Phe] where the loss of the phenolic hydroxyl disrupts a network of hydrogen bonds at the $\alpha_1\beta_1$ contact that does not change in the T$\rightarrow$R transition. It has since become clear that the loss of any bond, either between or within the subunits, even the creation of a cavity, relaxes the T-structure. This relaxation raises K_T and lowers L. K_R is unaffected, since it is almost the same as that of free α- and β-subunits or $\alpha\beta$ dimers. In all instances changes in L are linked to changes in K_T; it is not possible to alter these parameters independently (13).

DIRECTED MUTAGENESIS OF RESIDUES IN THE HEME POCKETS

Despite the enormous amount of research done on the structure and function of hemoglobin, some of its most vital properties have remained ill understood. Free ferrous porphyrins are rapidly oxidized by oxygen, and their affinity for oxygen is several thousand times smaller than that for carbon monoxide. Globin keeps the iron ferrous, which is necessary because only ferrous iron combines reversibly with molecular oxygen; globin also discriminates in favor of oxygen and against carbon monoxide. A combination of directed mutagenesis and functional studies has recently clarified these problems.

In myoglobin and in the α-subunits of hemoglobin, N_ϵ of the distal histidine forms a hydrogen bond with the bound oxygen, but not with carbon monoxide; in the β-subunits that bond is either weak or absent (Figure 12) (42, 45; X. Cheng & B. Schoenborn, private communication). X-ray analysis shows that the distal histidine blocks access to the heme pocket (35). Neither oxygen nor carbon monoxide can enter or leave unless the side chain of the distal histidine swings out of the way, which it can do only by elbowing the helix E to which it is attached, away from the heme. Thus oxygen transport relies on the dynamics of the globin.

Olson, Nagai, Sligar & their colleagues (32) have replaced the distal histidines in myoglobin and in the α- and β-subunits of hemoglobin by glycines, which opened access to the heme pockets, and they have measured the resulting changes in the rates of association with and dissociation from oxygen, carbon monoxide, and a more bulky ligand, methylisocyanide. Their results are best analysed in terms of transition state theory (48). If the

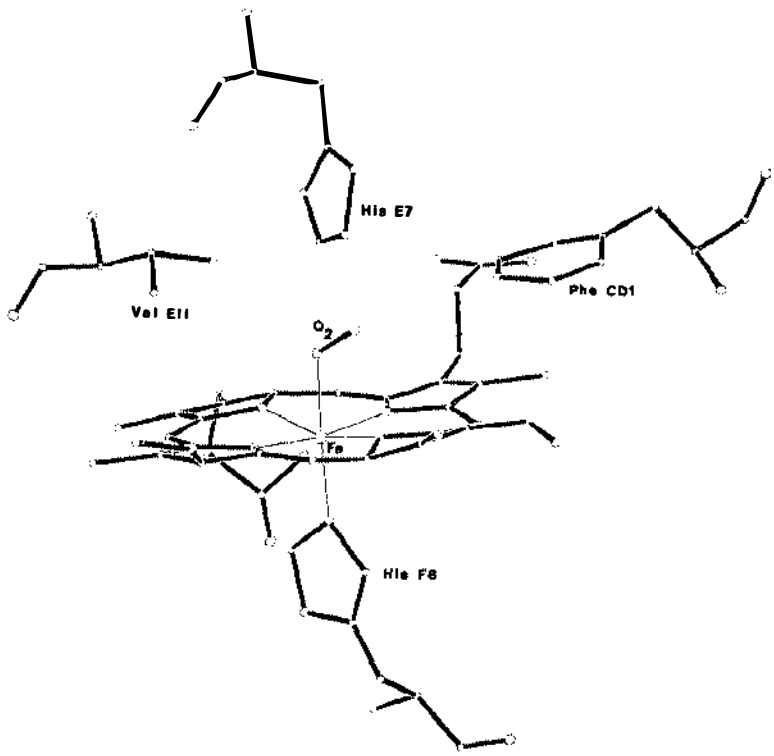

Figure 12 Proximal and distal residues of the heme in myoglobin. They are the same in hemoglobin, but the oxygen is less inclined to the heme axis.

transition state is product-like, any rise in affinity can be brought about mainly by a rise in the rate of association. If it is reactant-like, any rise in affinity can be achieved mainly by a drop in the rate of dissociation. These rules generally hold, even though neither rate gives directly the rate of formation or dissociation of the transition state.

The replacement of the distal histidine by glycine leaves the oxygen affinity and kinetic constants of the β-subunits unchanged within error; it diminishes the oxygen affinity of the α-subunits eightfold, equivalent to stabilization of the bound oxygen by hydrogen bonds with the histidines by the equivalent of 1.0 kcal mol^{-1}. The reduction in affinity is brought about by a sixtyfold increase in the dissociation (off) rate that more than compensates the tenfold increase in association (on) rate due to the opening of the heme pocket. The acceleration brings the on rate to a value of $15(\pm 5) \times 10^7 s^{-1} M^{-1}$, which is close to Szabo's estimate (48) of $50 \times 10^7 s^{-1} M^{-1}$ for a hypothetical globin in which that rate is limited only by diffusion into the heme pocket through a

hole of 2.6Å radius. The absence of any acceleration by the His→Gly replacement in the β-subunit implies that in native hemoglobin histidine E7β must be swinging in and out at least 10^9 times per second, while in myoglobin and in the α-subunits that rate appears to be about a hundred times slower. All the off rates are several orders of magnitude slower than the on rates, which indicates that the rate-limiting step for the off rates is rupture of the Fe-O bond rather than opening of the heme pocket.

Replacement of the distal histidine by glycine increases the affinity for carbon monoxide fourfold in the α-subunits, largely because of a rise in the on rates, and decreases it threefold in the β-subunits. In the native proteins, the on rates for carbon monoxide are slower by an order of magnitude than those for oxygen, whence the acceleration is likely to be due to the removal of static steric hindrance by the distal histidines within the heme pocket rather than its function as a gate. If we multiply the decrease in oxygen affinity by the increase in affinity for carbon monoxide, we find that the distal histidine discriminates against carbon monoxide by the equivalent of about 2 kcal mol^{-1} in the α-subunits, but does not discriminate in the β-subunits.

The switch in quaternary structure from R to T reduces the oxygen affinity of hemoglobin by the equivalent of over 3 kcal per mole heme. Like the reduction in oxygen affinity due to the His→Gly replacement, it is brought about mainly by acceleration of the off rates. Hence all the evidence points to the transition state with oxygen being mainly reactant-like. By contrast, the decrease in carbon monoxide affinity in the R → T transition is due to a drop in the on rates, consistent with the present evidence that the transition state is mainly product-like.

What role does the distal valine play in the discrimination between oxygen and carbon monoxide? Its replacement by alanine increases both the on and off rates of oxygen with the α-subunits sevenfold and leaves those with the β-subunits unchanged. It increases the on rate of carbon monoxide with the α-subunits tenfold and leaves the off rates unchanged. Hence the distal valine in the α-subunits discriminates against carbon monoxide by the equivalent of 1.3 kcal mol^{-1}, apparently by steric hindrance, but is ineffective in the β-subunits. However, this is only part of the story because the results of Olson et al (32) are confined to the R-structure. In the T-structure steric hindrance by the distal valine E11β plays a key role, which future experiments may quantify. Nevertheless the mechanism of discrimination in the β-subunits remains a mystery.

Other evidence on the mechanism of discrimination between oxygen and carbon monoxide has come from recent crystal structure determinations. In synthetic model compounds that offer no steric hindrance to the ligands, oxygen binds with an Fe-O-O angle of 120°, while carbon monoxide lies on the heme axis. The heme pockets of myoglobin and hemoglobin seemed to be tailored to accommodate the bent oxygen and force the carbon monoxide off

the heme axis, which might have accounted for their low carbon monoxide affinity. This is true in myoglobin, where carbon monoxide is seen in two orientations, with Fe-C-O inclined at either 120° or 140° to the heme axis (22). On the other hand, recent X-ray analyses of human carbonmonoxy-hemoglobins at 2.2–2.3Å resolution have shown inclinations of less than 10°, too little to account for the observed energy of discrimination. There are no significant displacements of the distal residues, but the porphyrins are ruffled (Derewenda et al 9a). X-ray analysis of a synthetic "hindered pocket" iron porphyrin that has a carbon monoxide affinity lower than that of the unhindered "picket fence" iron porphyrin by the equivalent of 1.2 kcal/mole shows similar geometry. Fe-C-O is inclined to the heme axis by only 7.5° and the porphyrin is markedly ruffled (21). It appears that in both the hindered pocket porphyrin and in hemoglobin a major part of the strain energy responsible for the low carbon monoxide affinity may be stored in the porphyrin.

According to the atomic models, replacement of valine E11β by isoleucine should block the oxygen site in both the T- and R-structures, but in fact isoleucine at E11 fails to inhibit oxygen binding: it merely shifts the entire equilibrium curve to the right, roughly halving the oxygen affinity, which implies a fourfold reduction of the affinity of the β-hemes if the α-hemes remain unaffected. It appears that both the T- and R-structures have 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 1.6 kcal/mole β-heme.

Computer simulations of the molecular dynamics of the exit of carbon monoxide from the interior of myoglobin suggest that there may be several alternative pathways in addition to that via the distal histidine (11), even though the latter is the most direct. Experimental evidence in support of the histidine as the door to the heme pocket comes from the structure of imidazole and phenylhydrazine myoglobin in which the side-chain of the distal histidine has been turned out of the heme pocket by the bulk of the ligand (6, 44) and from a recent crystal structure determination of ethylisocyanide myoglobin that shows the side-chain of the distal histidine in two alternative positions, either in or out of the heme pocket, exactly as it would have to move to admit or release ligands (18). The dynamic movements of the heme pocket are attested by NMR studies showing that phenylalanines CD1 and CD4, which wedge the heme into its pocket and are packed tightly between the heme and the distal helix E, flip over at rates faster than $10^4 s^{-1}$ (9). They can do so only if the entire heme pocket breathes fast. The fast exchange of most main chain imino hydrogens with tritium also attests to the dynamic state of the hemoglobin molecule (12). Oxygen has been found to quench the fluorescence of buried tryptophans in other close-packed proteins at velocities near the diffusion limit, which could not have happened unless the proteins' dynamic motion had opened gaps wide enough to let the oxygen pass (7, 23).

Springer et al (46, 47) have studied the protection of the heme iron from oxidation by replacing the distal histidine in sperm whale myoglobin by ten different amino acid residues and shaking the deoxygenated myoglobin solutions in air in 75 mM potassium phosphate + 25 mM EDTA pH 7.0 at 37°C. All replacements reduced the oxygen affinity and accelerated autoxidation. Phenylalanine, methionine, and arginine produced the smallest accelerations (~50-fold); aspartate the largest (350-fold).

How can these results be interpreted? Paradoxically, combination with oxygen protects the heme iron from oxidation, as can be shown by performing the same experiments at several atmospheres of pure oxygen. Apparently oxidation occurs in that fraction of molecules that is deoxygenated at any one moment. The larger that fraction is at atmospheric oxygen pressure, the faster myoglobin autoxidizes. For example, replacement of the distal histidine by phenylalanine reduced the oxygen affinity 170-fold, so that a larger fraction of myoglobin molecules will have remained deoxygenated at atmospheric oxygen pressure and therefore have become autoxidized. However, this can be only part of the explanation, because the replacement of histidine by glycine reduces the oxygen affinity merely elevenfold, yet accelerates autoxidation over a hundredfold.

Autoxidation is catalyzed by protons, hence the 350-fold acceleration by aspartate. I suggest that the distal histidine protects the ferrous heme iron by acting as a proton trap. The distal histidine has a pK_a of about 5.5; at neutral pH it is protonated only at N_δ, which faces the solvent. Any proton entering the heme pocket of deoxymyoglobin would be bound by N_ϵ, and simultaneously N_δ would release its proton to the solvent. When the histidine side chain swings out of the heme pocket, the protons would interchange, restoring the previous state. No other amino acid side chain could function in this way. Evolution is a brilliant chemist.

HEMOGLOBIN AS A DRUG RECEPTOR

In a search for compounds that might prevent the aggregation of deoxyhemoglobin S in patients with sickle cell anemia, two anti-lipidemic drugs, clofibric acid and its analogue bezafibrate, were found to lower the oxygen affinity of hemoglobin. X-ray analysis of crystals grown in the presence of these compounds showed that they combine with deoxy, but not with oxyhemoglobin. They stabilize the T-structure by combining with sites in the central cavity that are about 20Å away from the DPG binding sites; their effects and that of DPG on the allosteric equilibrium are additive (40).

This discovery led Lalezari to synthesize a family of new compounds related to, but more active than bezafibrate (24, 50); one of these, which has the formula shown in Figure 13, has turned out to be the most powerful

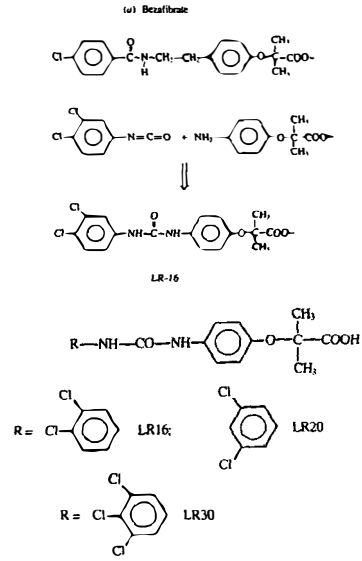

Figure 13 Novel allosteric effectors of hemoglobin: bezafibrate, an anti-lipidemic drug, and compounds derived from it by Dr. I. Lalezari (50).

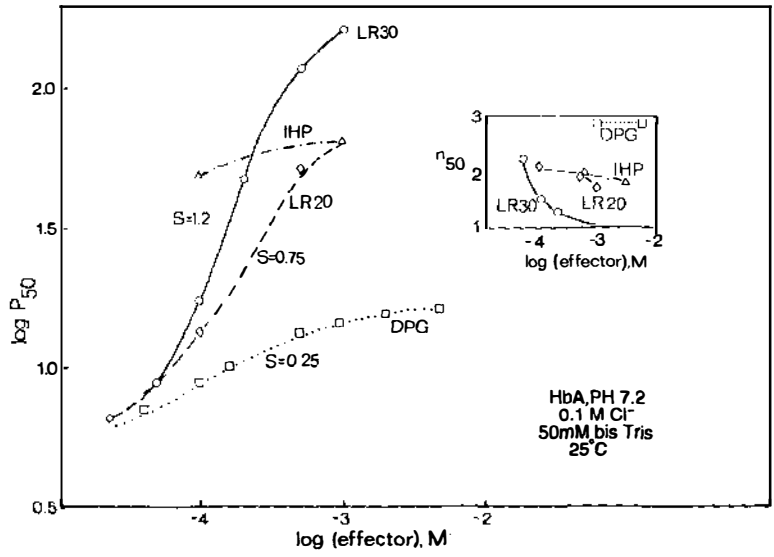

Figure 14 Effect on partial pressure of oxygen at half-saturation (P_{50}) of 2,3-diphosphoglycerate (DPG), inositol hexaphosphate (IHP) and two of Dr. Lalezari's synthetic effectors LR20 and LR30. The inset shows their effect on Hill's coefficient at half-saturation. At 1mM LR30 the hemoglobin is only half-saturated at 2 × atmospheric oxygen pressure and its Hill's coefficient is near unity because the allosteric constant L is very large. (50).

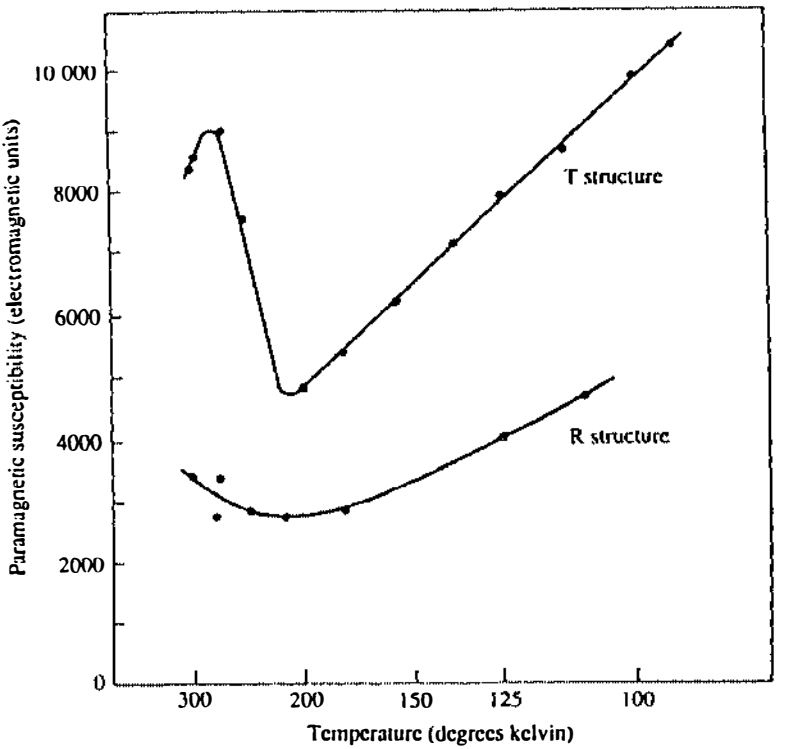

Figure 15 Temperature dependence of paramagnetic susceptibility of carp azidemethemoglobin in the R- and T-structures. Below about 200°K the susceptibility rises with falling temperature in accordance with Curie's law. Above that temperature a thermal equilibrium between a high and a low spin form masks that behavior, since the high spin form gains stability with rising temperature. It has longer Fe-N bond distances than the low spin form. Tension at the heme in the T-structure therefore shifts the equilibrium towards higher spin (from Messana et al 28).

allosteric effector yet found. At an effector concentration equimolar to heme and at pH6, it lowers the oxygen affinity of solutions of human hemoglobin to a level found until now only in fish hemoglobins that exhibit a Root effect i.e. a drastic drop in oxygen affinity below pH7 (Figure 14). In such fish hemoglobins, the tension on the heme in the T-structure that is associated with the low oxygen affinity has been demonstrated directly. In azidemethemoglobin the iron is in a thermal equilibrium between two different spin states that are characterized by different lengths of the iron nitrogen bonds; they are longer in the high spin than in the low spin state. In azidemethemoglobin of trout, transition from the R- to the T-structure induces a transition to higher spin, equivalent to a stretching of the Fe-N bonds (28, 37). This is manifested by an increase in paramagnetic susceptibility equivalent to a change in free

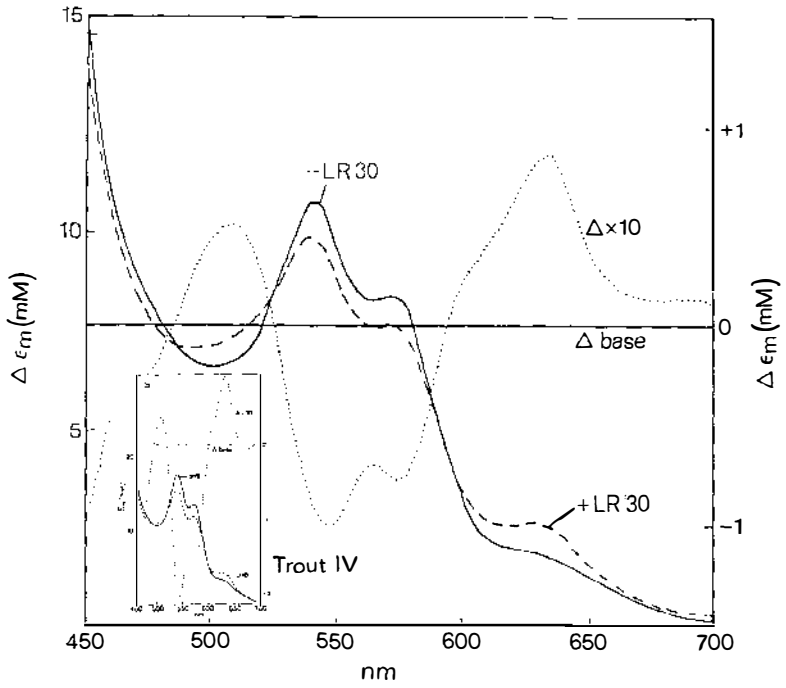

Figure 16 Optical absorption spectra of human and trout IV azidemethemoglobin in the R- and T-structures, showing the rise in intensity of the high spin bands at 500 and 630 nm and the fall of the low spin bands at 540 and 570 nm. *Full lines:* T-structure; *broken lines:* R-structure; *dotted lines:* difference spectra 10 × enlarged (50).

energy of 1 kcal/mol (Figure 15) and also by a change in optical spectra that show a rise in intensity of the high spin bands at 500 and 630 nm and a drop in intensity of the low spin bands at 540 and 570 nm. Until recently it was not possible to induce this transition in human azidemethemoglobin (43), but combination with the powerful new synthetic effector induced a difference spectrum identical to that of trout hemoglobin, which demonstrates that the hemes are under tension also in human hemoglobin in the liganded T-state (Figure 16).

X-ray analysis and oxygen equilibria show that four molecules of the effector combine with one molecule of human deoxyhemoglobin both in the crystal and in solution. One pair of symmetry-related binding sites is close to those of bezafibrate; the other pair is nearly at right angles to the first (Figure 17). The four trichlorobenzene moieties form a parallel stack. Their close packing seems to dominate their mode of binding. The combination of human

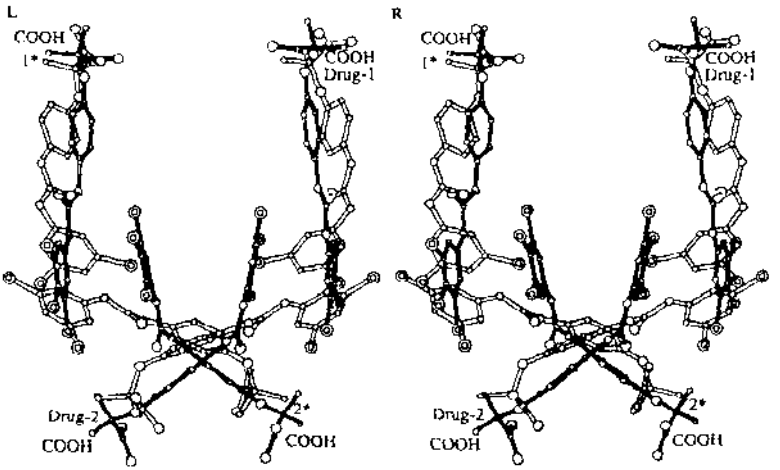

Figure 17 Arrangement of the two effectors LR20 and LR 30 in the central cavity of hemoglobin. The vertical pairs lie mainly between the two α-chains in sites that overlap those of bezafibrate. The other pairs occupy positions not taken up by bezafibrate, and the sites of the 3,5-dichloro derivative (LR20, open bonds) differ from those occupied by the 3,4,5-trichloro derivative (LR30, shaded bonds). The four trichlorobenzene moieties form a close-packed parallel stack right in the center of the hemoglobin molecule, while the dichlorobenzene moieties stack in separate pairs. (50).

deoxyhemoglobin with the effectors induced no stereochemical changes at the allosteric core that were visible at 2.5Å resolution, and they produced a decrease of the Fe-N_ϵ stretching frequency by only 1–2cm^{-1} (T. Kitagawa, private communication). Only in the liganded T-structure do the effectors make themselves felt strongly at the hemes.

The work on drug binding by hemoglobin has led to generalizations that are relevant to the binding of effectors, transmitters, and drugs to other proteins. The stereochemistry of binding is determined by the available van der Waals space and, within that space, by interactions of a wide range of polarity, from strong hydrogen bonds between ionized groups of opposite charge to weak interactions between aromatic quadrupoles, and non-polar interactions between aliphatic hydrocarbons. The detailed stereochemistry is governed by a tendency to maximize the sum of the energy of electrostatic interactions, for example by aligning effectors relative to the protein so that the mutual polarizabilities are maximized. Drugs may influence the allosteric equilibrium of a protein receptor in the same direction as the natural effector even though they are chemically unrelated to it because proteins may offer a variety of binding sites not used in nature (40).

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