THE TIES THAT BIND

The hemoglobin protein ties four structures called hemes into one system. Researchers have only begun to detail how that protein, globin, influences the oxygen-binding behavior of the hemes.

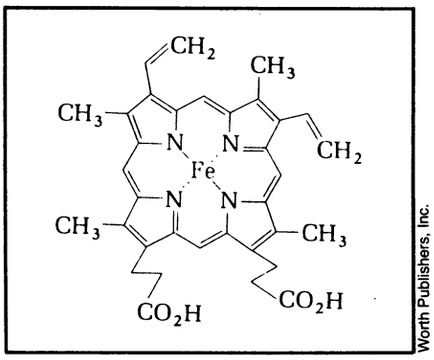
BY LINDA GARMON

Like spokes of a wheel without the rim, hemes are useless without the globin. Hemes, the components of hemoglobin that pick up oxygen in the lungs and unload it in body tissues, simply could not function without the globin part of hemoglobin. And while the experimental data for it exceed those available for any other protein, globin is still a chemical frontier. Using a variety of techniques, researchers now are beginning to define precisely its role in hemoglobin oxygen binding.

Understanding that role could open doors in related research. It could lead, for example, to new chemical therapies for sickle cell disease — a genetic malady in which hemoglobin molecules stick together, causing the red blood cells that enclose them to distort (see p. 379). In addition, the function of the hemoglobin protein could serve as a useful model for the behavior of complex enzymes — special proteins in plant and animal cells that catalyze, or speed up, chemical reactions.

Although hemoglobin is not really an enzyme, it holds an "honorary enzyme" title due to certain of its characteristics. Some enzymes have more than one site of catalytic activity — binding sites for the reactants in the process the enzyme is catalyzing. In certain of these enzymes, the binding of one reactant affects the catalytic activity of the other sites. Presumably that effect is transmitted through the protein backbone of the enzyme since the sites are too far away from each other to cause direct physical interaction. A similar phenomenon occurs in hemoglobin.

In hemoglobin, however, the centers of activity tied into one system by the protein structure are not catalytic sites, but rather four oxygen-binding sites — the hemes. A heme is a flat structure with four rings around a central iron atom that can irreversibly combine with oxygen. Like a coin in a leather pouch, each heme is enfolded in one of four chains of amino acids that collectively constitute the protein, or globin, portion of hemoglobin. There are two identical pairs of globin chains — denoted alpha$_1$ beta$_1$ ($\alpha_1\beta_1$) and alpha$_2$ beta$_2$ ($\alpha_2\beta_2$) — containing a total of 574 amino acid units. An imidazole group (a three-carbon, two-nitrogen ring) from a histidine amino acid on each chain reaches out to hold a heme iron.



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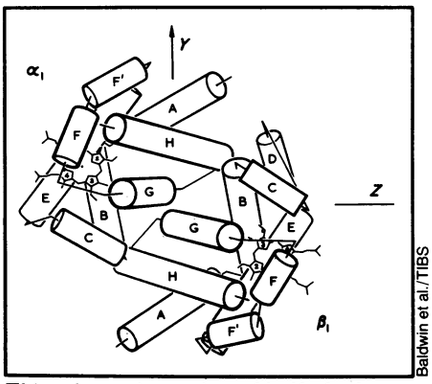
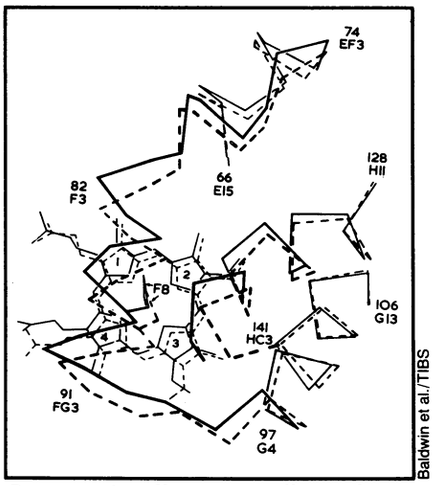
Heme, with oxygen-binding iron in center.



Baldwin et al./TIBS

This schematic version of half of a hemoglobin molecule depicts the helical regions of the globin as rods.



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Representing X-ray crystallography results, the drawing depicts a section of α chain with its heme group shown in the deoxy (continuous lines) and liganded (dashed lines) states. The rings of the heme group are numbered, and the letter-number combinations code for specific amino acid units.

This article is the first of a two-part series on hemoglobin. The second article will describe current research on the families of genes that code for globin proteins.

It is extremely difficult for any one of those heme irons to nab oxygen until at least one of the hemes gets the binding ball rolling. While it may sound like a chemical Catch-22, this phenomenon, known as a cooperative effect, actually ensures an efficient all-or-nothing oxygen transport. For when the iron of one of the globin chains somehow takes up oxygen, it affects the other oxygen-binding sites in an enzyme-like fashion. The oxygen-combining activity of the other three iron atoms is enhanced, and by the time three of the iron atoms have taken on oxygen, it is a cinch for the fourth iron to follow suit. Similarly, once one oxygen molecule is released from hemoglobin, unloading becomes easier.

It now is generally believed that this cooperative effect is possible because of the globin's ability to change between two alternative structures that for historical reasons are referred to as the T (the deoxy, or without oxygen) and R (oxy) states. Apparently, the hemoglobin molecule is in equilibrium between the two states — R having a higher attraction for oxygen than T — and a heme combining with oxygen swings the equilibrium toward the high-affinity R structure.

This proposed two-state mechanism for cooperative oxygen binding in hemoglobin is strongly backed by the work of X-ray crystallographers. These researchers form crystals of a compound and then irradiate a single crystal with an X-ray beam to determine its structure. When the beam hits the crystal, it diffracts in thousands of different directions, depending on the arrangement of the atoms in the crystal. By collecting these scattered X-rays on a photographic plate, researchers obtain a diffraction pattern from which the location of the crystal atoms can be determined.

Using this analytical technique, Joyce Baldwin and colleagues at the MRC Laboratory of Molecular Biology in Cambridge, England, recently compared the deoxy- and fully-liganded (all the heme binding sites occupied) hemoglobin structures. Such a comparison is a journey on a Lilliputian scale where changes of mere angstroms (10^{-8} centimeters) are "striking differences." Baldwin describes these differences in the August TRENDS IN BIOCHEMICAL SCIENCES.

One major difference she discusses could prove significant in assessing the globin role in binding ligands such as oxygen. In the deoxy structure of hemoglobin, there are salt bridges — interactions between certain positively and negatively charged groups — between the two globin halves, $\alpha_1\beta_1$ and $\alpha_2\beta_2$. X-ray crystallographs indicate that when the hemoglobin structure moves to its liganded state, the gaps for these salt bridges become too

Sickle Cell Disease-Chemical Warfare

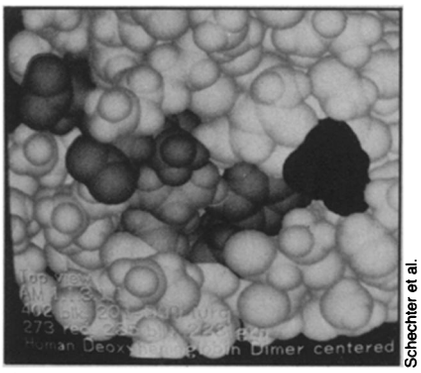
Alan N. Schechter can stand smack dab on the mutant portion of a sickle cell hemoglobin and look at the molecular surface to which it binds; he does this with computer molecular graphics. It's all simulation, of course, but the computer's-eye view is helping Schechter and other National Institutes of Health researchers select chemicals that will interfere with the hemoglobin aggregation associated with sickle cell disease.

Hemoglobin — which transports oxygen from the lungs to the tissues — is the major protein of red blood cells. Although they are very concentrated in the red cells, normal hemoglobin molecules behave as individual entities, moving about freely. The red blood cells are therefore flexible and can easily squeeze through narrow capillaries. In sickle cell disease, however, the red blood cells lose their flexibility when the hemoglobin deoxygenates, or loses its oxygen.

This loss of flexibility can be traced back to a single point mutation on the gene that codes for two of the four polypeptide (linked amino acids) subunits of hemoglobin. As a result of this mutation, the negatively charged amino acid glutamate is replaced by the neutral amino acid valine on the peptide subunits referred to as the β chains. The structural change causes the abnormal hemoglobin molecules in sickle cell disease to stick together upon deoxygenation: The valine portion of one β chain binds to a normal portion of another β chain, forming long, helical polymers (giant molecules composed of smaller identical molecules). The red blood cells that contain these polymerized, or gelling, hemoglobin molecules toughen and distort into sickle-shaped cells that periodically block the capillaries in a painful sickle cell crisis.

But a crisis is usually averted. Between hemoglobin deoxygenation and polymerization, there is a peculiar delay time that depends on surrounding physiological conditions, such as the red blood cell concentration of hemoglobin. Usually that delay is time enough for the red blood cells to escape into larger venous vessels before hemoglobin polymerization takes off; occasionally the delay is too short. So in searching for a way to interfere chemically with hemoglobin polymerization, the name of the game is finding ways to increase the peculiar delay time.

Schechter's use of computer molecular graphics offers such an approach. After studying the computer-simulated surfaces of the sickle hemoglobin molecules at the points of intermolecular contact in the aggregates, Schechter and colleagues attempt to design competitive inhibitors — small molecules that will run interference for hemoglobin, blocking the mutant, aggregating portions of other hemoglobin molecules in the red blood cell. The competitive inhibitors that the NIH researchers have zeroed in on are short



A computer version of a globin surface to which an abnormal β chain binds.

peptide strands containing two to 15 amino acid units.

This NIH peptide approach to interfering with sickle cell disease now is pursued in several laboratories worldwide. In studies at the Weizmann Institute of Science in Rehovot, Israel, for example, researchers have found that the peptides most active in preventing gelling are those containing two hydrophobic — or water-repelling — rings in their structures. After some structural tinkering with such peptides, the Israel researchers — collaborating with R. Votano and Alexander Rich of the Massachusetts Institute of Technology in Boston — designed drugs that not only prevent hemoglobin gelation, but also soften the red cell membrane to prevent sickling. Although the effectiveness of these compounds has thus far been demonstrated only outside the body using the blood of donors, initial trials in rats indicate that no ill effects follow dosages dozens of times greater than those that would be required to prevent sickling.

Another approach to sickle prevention involves putting a leash on hemoglobin deoxygenation. Arthur Arnone and colleagues at the University of Iowa in Iowa City have found a chemical agent that binds to hemoglobin and keeps it from unloading all of its oxygen. Hemoglobin gelation, and therefore red cell sickling, is inhibited.

A third approach to increasing the crucial delay time of polymerization focuses on the red blood cells rather than the hemoglobin. Because one factor triggering the formation of sickle cells is the intracellular concentration of deoxyhemoglobin, Robert M. Rosa of the Beth Israel Hospital in Boston and colleagues reasoned that sickling may be inhibited by swelling red cells, thereby dispersing the hemoglobin inside and increasing the time required for gelation.

In studies reported in the Nov. 13 NEW ENGLAND JOURNAL OF MEDICINE, Rosa and co-workers swelled red cells in three patients with severe sickle cell disease by reducing the sodium level in their blood — a state called hyponatremia. The patients' sodium intake was reduced, they were encouraged to drink 3 to 4 liters of fluid each day and they ingested an antidiuretic hormone, 1-desamino-8-d-arginine vasopressin to help the kidneys retain the fluids.

While all three patients had a history of severe crises about every 30 days, such crises were fewer and shorter during the study. In fact, one patient was free of crises during the entire 190 days of hyponatremia. Still, cautions Rosa, three apparent successes do not constitute conclusive results, and further studies are needed. The long-term effects of hyponatremia, for example, must be investigated.

Taking another approach to battling the effects of sickle cell disease, George J. Brewer and colleagues at the University of Michigan at Ann Arbor are attempting not to increase the polymerization delay time, but rather to return deformed sickle cells to their normal shape by inhibiting the membrane-stiffening molecule calmodulin (SN: 8/23/80, p. 119). The researchers are checking the effects of the strong tranquilizer thioridazine and zinc acetate — two calmodulin inhibitors.

The research on calmodulin inhibition and the three approaches aimed at increasing polymerization delay time are still in their preliminary stages, says Schechter. "There are problems with every one of the approaches," he explains. Some of the drugs have trouble permeating red cells; others impart to hemoglobin much too high an oxygen affinity. Still, "We are on base one," says Schechter. "An era of rational approaches to interfering chemically with sickle hemoglobin polymerization is now underway."

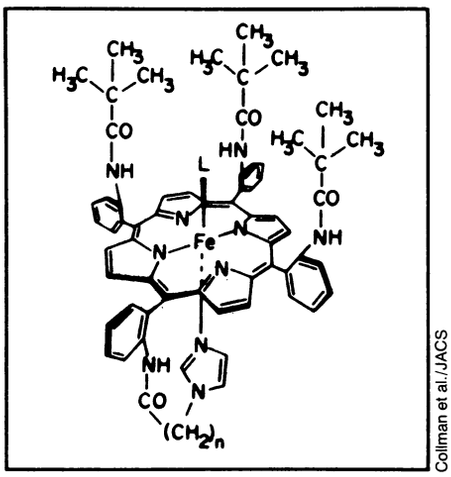
narrow in some cases and too wide in others; so the bridges are destroyed. The salt bridges may serve to stabilize and oppose the change to the oxy (R) state.

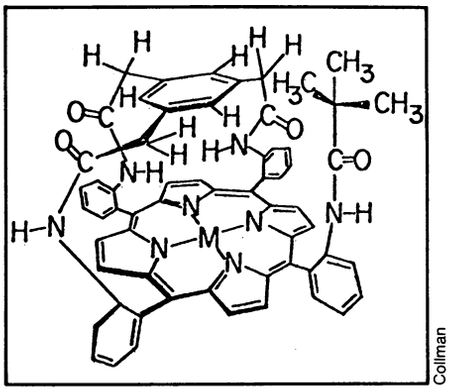
The other structural changes Baldwin describes can best be understood by dividing into separate regions the amino acid helices that constitute the four globin subunits. There are seven helical regions (A, B, C, E, F-F′, G and H) on the α subunits and eight (A, B, C, D, E, F-F′, G and H) on the βs. "The regions where there are significant structural changes [between the deoxy- and fully-liganded states] include the heme, the F helix and the FG corner region of each subunit and the E helix of β," Baldwin reports. In addition, the persnickety protein globin demands some inter-subunit changes: At the contact between the C helix on the α chain and the FG region on the β chain, for example, a "large" 6-angstrom jump is evident on going from deoxy- to liganded-hemoglobin. As a result of these changes, the hemes move deeper into their protein pockets — the α hemes by 0.5 angstroms and the β hemes by 1.5 angstroms — and there is a marked change in the position of the imidazole ring (of the amino acid histidine at the F8 position) that hangs on to the heme iron.

Although X-ray crystallography has painted a fairly precise picture of the deoxy- and fully-liganded globin structures, to stop there would be like writing only the first and last chapters of a molecular epic. In other words, to understand the precise role the globin plays in binding oxygen, hemoglobin devotees need more than the static pictures of the "end" (T and R) states; they need a protein panorama.

One way to determine the detailed mechanism of the dynamic transitions between the T and R states is to slow down that mechanism. Hans Frauenfelder and colleagues of the University of Illinois at Urbana are doing just that. The Illinois researchers are slowing the ligand-binding process of hemoglobin by lowering the reaction temperatures in an attempt to identify various stages of the ligand approach. Frauenfelder's work eventually may identify some of the supposed barriers the globin sets up to resist its own change from the T to the R state. In addition, his "low temperature kinetics" studies may help to solve one of the great globin mysteries: What change occurs after one heme combines with oxygen to set in motion the globin transition from the T to the R state?

Some hemoglobin researchers believe the answer lies in the imidazole-iron-oxygen bond. The heme iron is always bound to the four nitrogens of the flat heme structure and to the globin via a nitrogen bond of an imidazole ring. Iron's sixth binding site — on the side of the heme plane opposite the imidazole bond — is reserved for oxygen. According to the imidazole-iron-oxygen concept — called the proximal pull theory — the T-state imidazole pulls the heme iron slightly out of its heme plane. It is extremely difficult for an oxygen molecule to bind to a displaced iron, because this forces it to bump against the nitrogens of the heme plane. To avoid such nitrogen-bumping, the first oxygen molecule that dares to brave the T (low-affinity-for-oxygen) state engages in a sort of tug-of-war with the imidazole, each group trying to pull the iron in its direction. The oxygen molecule eventually is victorious over the imidazole and pulls the iron back to the heme plane. "That's the triggering device for cooperativity," says University of California at San Diego chemist Teddy G. Traylor, a firm believer in the proximal pull effect. The iron returning to its plane pulls the imidazole, which in turn pulls on the rest of that globin subunit. Because each globin interacts, the effect "dominoes" from one chain to another, making it easier for oxygen molecules to bind to the other sites.


Collman et al./JACS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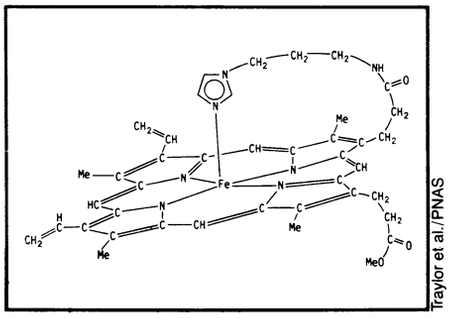

Traylor et al./PNAS

Recent hemoglobin models: the "tailed picket fence" (top), the "picket pocket" (center) and the "chelated protoheme."

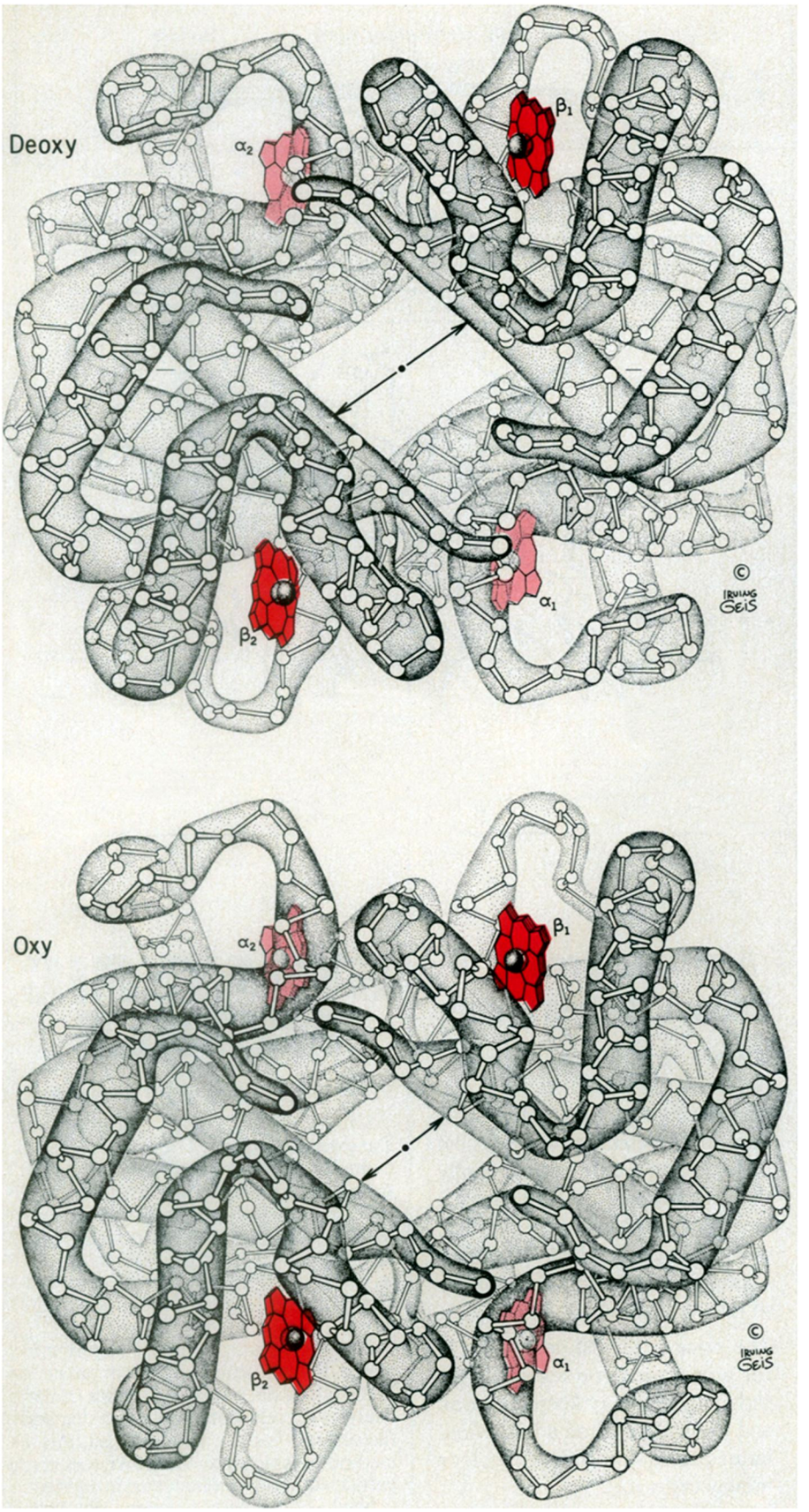
The proximal pull theory — first proposed by M.F. Perutz of the MRC Laboratory in England — is not new to the hemoglobin field. What is new, however, is that the globin-theory pendulum may be swinging back to the pre-proximal pull days. Then the emphasis in detailing the globin's role in oxygen binding was not on the imidazole-iron-oxygen system, but rather on a series of globin side chain rearrangements — a focus now advocated primarily by Martin Karplus and colleagues at Harvard University in Cambridge, Mass.

The separate Karplus-theory and proximal-pull groups in the field of hemoglobin research are testimony to the field's diversity. Karplus and colleagues, for example, take the theoretical approach, using sophisticated computer programs to study the changing globin structure. Traylor and other proximal pull enthusiasts prefer to build model molecules that mimic the activity of hemoglobin.

There is a trick to hemoglobin model building, says James P. Collman of Stanford University. An iron heme with no surrounding protein undergoes an irreversible reaction with oxygen — one that destroys the oxygen molecule. The pathway to molecular oxygen destruction involves two different hemes binding to the same oxygen molecule, Collman explains. So hemoglobin model builders must design "synthetic hemoglobins" with protective enclosures around their heme analogs to prevent such heme-to-heme interactions. "Capped heme" models include a loose shield over one face of the heme analog to mimic the globin's role in preventing heme-to-heme contact. A bit more complicated in structure is Collman's "picket fence" model. In these hemoglobin analogs, four separate chains of atoms jut out from the heme plane like pickets on a fence, inhibiting large molecules — such as other heme analogs — from encountering the binding site for oxygen.

Recently, Collman replaced one of the pickets from his fence with a "tail." In his "tailed picket fence" model — described in the June 4 JOURNAL OF THE AMERICAN CHEMICAL SOCIETY — Collman replaces the fourth picket with an imidazole ring tail to "recreate" the imidazole iron bond found in hemoglobin. While Collman's "tailed picket fence" has an attraction for oxygen similar to that of the high affinity (R) state of hemoglobin, it fails to mimic another hemoglobin-like characteristic — lowered attraction for carbon monoxide.

When the body "junks some of its hemoglobin molecules," says Collman, trace amounts of carbon monoxide are produced so organisms must tolerate a constant low level of carbon monoxide. Although hemoglobin can bind to carbon monoxide instead of to oxygen, if its at-



On going from the deoxy (above) to the oxy (below) state, the β chains of the hemoglobin molecule move closer together. The colored structures represent the heme groups.

traction for CO were too strong, the supply of oxygen to the body might decrease. And this relates to the problem with Collman's "tailed picket fence" — it likes CO too much. So Collman has toyed with the structure by adding a cap to sit on top of the fence pickets. The cap and pickets create a "pocket" that inhibits CO binding, Collman says. At the recent International Union of Pure and Applied Chemistry Conference on Physical Organic Chemistry in Santa Cruz, Calif., and in a paper recently submitted to JACS for publication, Collman reports that the CO affinity of this "picket pocket" model approaches that found in nature.

Collman theorizes that the globin chains play an important role in keeping CO-binding to minimum. Each chain surrounds the sixth binding site of the heme iron, leaving a pocket that favors the binding of oxygen molecules — which bind at an angle to avoid bumping the pocket — over carbon monoxide — which tries to bind linearly and therefore bumps into the globin enclosure. This hypothesized role of the globin in ligand-binding is termed the distal side steric effect — a theory that is the center of "acrimony and controversy" in the field of hemoglobin research, Collman says.

Traylor opposes Collman in the distal side steric effect controversy. In the June PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Traylor reports that studies with his simpler chelated protoheme model indicate that while the distal side steric effect is present in hemoglobin molecules, it is not the reason that hemoglobin binds more oxygen than carbon monoxide. Traylor says, "Some people believe the distal side steric effect protects the body from carbon monoxide poisoning, but I say 'phooey.'"

If one group of modelers continues to say "yes" and another "no" to a specific steric effect, then resolution of the controversy may depend on approaches other than model building. Keith Moffat and colleagues at Cornell University in Ithaca, N.Y., for example, are attempting to tie together the three-dimensional structure of hemoglobin — as determined by X-ray crystallography — and data on the kinetics, or rates, of ligand binding.

The data obtained solely from models are limited, says Moffat, because "most of the processes studied in one model or another prove capable of affecting ligand affinity or rates of binding. We have not been able to rule out any of them [the processes]."

Which of these processes represent the most important roles the globin plays in binding oxygen? Answering the question will be a central theme of globin research in the coming years. While Moffat suspects that researchers eventually will discover that different processes are more important for different ligands or states (R and T) of the hemoglobin molecule, for now, he says, "We are left with the question." □