Allosteric proteins, such as hemoglobin, are assemblies of functional units, which undergo quaternary structural transitions in response to concentration changes of a specific ligand. Functional properties of hemoglobin ligation intermediates indicate that the tertiary structural changes induced by the ligand do not promote an equilibrium of quaternary structures.

The term allostery means “other sites.” Allosteric proteins, such as hemoglobin, are “intelligent” molecules that vary their activity in response to environmental stimuli in the form of concentration changes of ligands, such as ions, metabolites, and macromolecules. The property of assuming different conformations provides the common mechanism underlying this behavior.

Allosteric proteins can be part of very large molecular assemblies or membrane receptors, but the paradigms for allostery are soluble proteins, ranging in structural complexity from a single polypeptide chain to oligomers, which are quaternary assemblies of a finite, small number of equal or different functional chains, or protomers. Each protomer has a binding site for the “homotropic” ligand, e.g., a transported molecule or a substrate, and other sites for binding “heterotropic” ligands, which modulate the protein interactions with the homotropic ligand. Alternatively, the homotropic and heterotropic binding sites may reside on different chains. Hemoglobin, a tetramer made up of two \( \alpha \) and two \( \beta \)-chains, is a paradigm for allosteric oligomers partly due to its physiological importance and partly because the change in quaternary structure brought about by ligation was first observed in the crystals of unliganded (or deoxy-) hemoglobin (Hb) and methemoglobin (Hb\(^+\)), which has the same structure as the fully liganded protein (4).

To explain the mechanism of the quaternary structural transition, it is of fundamental importance to know the functional/structural properties not only of the end states, Hb and fully liganded hemoglobin, but also of the intermediate ligation states. This review focuses on the information on the allosteric mechanisms provided by the study of the hemoglobin intermediates.

Models of allostery

The concept of the equilibrium between different structures is the tenet of the concerted model for allostery introduced by Monod, Wyman, and Changeaux, called the MWC model (8). The MWC model assumes that the two structures, one named T, for tense, and the other R, for relaxed, are in equilibrium at whatever ligation state due to the concerted transition of the protomers from one structure to the other. In the absence of ligand, the equilibrium is governed by the allosteric constant, \( L = [T_0]/[R_0] \), where the subscript indicates zero ligand. A homotropic ligand binds the molecules in the T structure with low affinity \( (K_T) \) and binds to those in the R structure with high affinity \( (K_R) \). The model is translated into an equation relating \( Y \), the ratio between the bound sites and the total number of sites, with the ligand concentration, which takes the form of a sigmoid curve. Such a curve indicates that the sites of the homotropic ligand cooperate positively in binding, since the affinity for the ligand increases progressively with \( Y \).

The alternative sequential model by Koshland, Némethy, and Filmer, called the KNF model (7), does not assume an equilibrium of quaternary structures. The switch from the T to the R structure occurs progressively through intermediate structures induced by the homotropic ligand binding. The structural pathways for the transition depend on the specific protein case. This model can describe positive and negative cooperative interactions.

The MWC model has had a major impact on the understanding of cooperativity because of the universality of the equilibrium concept and the simplicity of the formulation, which is capable of describing the behavior of many allosteric proteins with good accuracy by using only three parameters. In contrast, to formulate the mechanism of the allosteric protein the KNF model requires a detailed knowledge of the functional/structural properties of all of the intermediates, in addition to the end states, a prerequisite that in most cases is too difficult to fulfill. Both models are a simplification of a phenomenon involving a multitude of interactions. The perturbation of these interactions may have localized effects or propagate through the molecule to produce global effects, which are difficult to decipher at a molecular level. Nevertheless, these models maintain a great didactic value since they allow researchers to test and confront the basic principles on which the models are built and gradually gain insight into the phenomenon.

Hemoglobin properties

In 1925, G. S. Adair discovered that hemoglobin is made up of four functional units (14), each capable of reversible oxygen binding

\( \text{Hb} + \text{O}_2 \leftrightarrow \text{Hb(O}_2^+) \).
and

\[ A_i = \frac{[\text{Hb(O}_2)_i]}{[\text{Hb}_i][\text{O}_2]^i} \]

The Adair constant \( A_i \) where \( A_0 = 1 \) and \( i = 0-4 \) can be used to describe the curve of \( Y \) vs. \( O_2 \) concentration \([O_2]_i\) and to calculate the fractional concentrations \( f_i \) of the ligation intermediates

\[ Y = \sum_{i=0}^{4} f_i \sum_{i=0}^{4} A_i [O_2]^i \]

and

\[ f_i = \frac{A_i [O_2]^i}{\sum_{i=0}^{4} A_i [O_2]^i} \]

The structural studies then revealed that the homotropic ligands, \( O_2 \) and \( CO \), bind the heme moiety of each chain, yielding the eight intermediates shown in Fig. 1. Other sites on the chains are available for binding allosteric modulators, such as \( H^+ \), 2,3-biphosphoglycerate, \( CO_2 \), and \( Cl^- \), which have greater affinity for \( Hb \) and decrease the affinity for the homotropic ligands.

The macroscopic \( A_i \) constants enclose the molecular code of the allosteric mechanism. Decades of a host of researchers’ efforts have been devoted to deciphering the code through the analyses of the \( O_2 \) binding curves. Such an approach has severe theoretical and experimental limitations. The measurement of the ligand binding equilibria allows the determination of the free energy changes for the binding of \( i \) ligands \( \Delta G_i \), where \( i = 1-4 \), which can be expressed as

\[ \Delta G_i = \Delta G_a + \Delta G_c^i \]

\[
\begin{align*}
(\alpha\beta)(\alpha\beta) & \quad (\alpha^1\beta)(\alpha\beta) & \quad (\alpha\beta^1)(\alpha\beta) \\
(\alpha^1\beta)(\beta\alpha) & \quad (\alpha\beta^1)(\beta\alpha) & \quad (\alpha^1\beta^1)(\alpha\beta) \\
(\alpha\beta^1)(\alpha\beta) & \quad (\alpha\beta)(\alpha\beta^1) & \quad (\alpha^1\beta^1)(\alpha\beta^1) \\
(\alpha^1\beta)(\beta^1\alpha) & \quad (\alpha^1\beta^1)(\beta^1\alpha) & \quad (\alpha\beta^1)(\beta^1\alpha) \\
(\alpha\beta^1)(\alpha^1\beta) & \quad (\alpha^1\beta)(\alpha^1\beta) & \quad (\alpha\beta^1)(\alpha^1\beta) \\
(\alpha^1\beta^1)(\beta\alpha) & \quad (\alpha^1\beta^1)(\alpha\beta) & \quad (\alpha^1\beta)(\beta\alpha) \\
(\alpha\beta)(\beta\alpha) & \quad (\alpha\beta^1)(\beta\alpha) & \quad (\alpha\beta)(\alpha\beta) \\
(\alpha\beta^1)(\alpha\beta) & \quad (\alpha\beta)(\alpha\beta^1) & \quad (\alpha\beta)(\alpha\beta) \\
(\alpha^1\beta^1)(\beta\alpha) & \quad (\alpha^1\beta)(\beta^1\alpha) & \quad (\alpha^1\beta^1)(\beta^1\alpha) \\
(\alpha\beta^1)(\alpha^1\beta) & \quad (\alpha^1\beta)(\alpha\beta^1) & \quad (\alpha\beta^1)(\alpha^1\beta) \\
(\alpha^1\beta)(\beta\alpha) & \quad (\alpha\beta)(\beta\alpha) & \quad (\alpha\beta^1)(\beta\alpha) \\
(\alpha\beta)(\alpha^1\beta) & \quad (\alpha\beta)(\alpha^1\beta) & \quad (\alpha^1\beta)(\alpha\beta) \\
(\alpha\beta)(\beta\alpha) & \quad (\alpha\beta)(\beta\alpha) & \quad (\alpha\beta)(\alpha\beta) \\
(\alpha\beta^1)(\alpha^1\beta) & \quad (\alpha\beta^1)(\alpha^1\beta) & \quad (\alpha\beta^1)(\alpha^1\beta) \\
(\alpha\beta)(\alpha\beta) & \quad (\alpha\beta)(\alpha\beta) & \quad (\alpha\beta)(\alpha\beta) \\
\end{align*}
\]

\textbf{FIGURE 1.} The 10 hemoglobin ligation states. The protein is made up of two \( \alpha \)- and two \( \beta \)-chains of approximately similar molecular weight and tertiary structure with a twofold symmetry axis due to the different \( \alpha \)-chain contacts. Superscript \( L \) indicates the homotropic ligand. The \( \alpha^1\beta^1 \) and \( \alpha^1\beta^1 \) contacts of the dimeric units, shown in parentheses, do not dissociate under physiological conditions. The weaker \( \alpha^1\beta \) and \( \alpha^1\beta \) interdimeric contacts allow a reversible tetramer-dimer dissociation, as shown in the inset. Symmetrical tetramers made up of identical dimers, e.g., dimers in the same ligation state, can exist as single species, since the dimers reassociate into the original tetrameric molecule. Asymmetrical tetramers disproportionate into a ternary mixture of the asymmetrical species and two symmetrical parental species.

where \( \Delta G_a \) is the part of the energy due to noncooperative binding and \( \Delta G_c^i \), the cooperative free energy, is the energy contribution of the allosteric interactions among the chains relative to a particular binding step \( i \). Since the Adair constants are macroscopic quantities, they allow only the calculation of the \( \Delta G_c^i \) values relative to \( i = 1-4 \) but do not provide the information on each of the eight binding steps in Fig. 1. Such information is necessary for assessing the mechanism. For example, the MWC model does not predict functional differences between the diliganded species \((\alpha\beta)(\alpha^1\beta^1)\) and \((\alpha^1\beta)(\alpha\beta^1)\), which are not equivalent, since the \( \alpha \)- and \( \beta \)-liganded chains have different configurations in the tetramer.

The first \( A_1 \) and last \( A_4 \) Adair constants are accessible from experiments under limiting conditions. The determination of the second and third constants requires highly precise measurements and a sophisticated statistical analysis of the data. The curve shape is critically influenced by the presence of \( H^+ \), which is often unavoidable, and noncooperative dimers, which form in dilute protein solution due to the tetramer-dimer equilibrium (Fig. 1). This last effect is illustrated in Fig. 2, which compares the \( O_2 \) equilibrium curves obtained under similar conditions by methods differing in the experimental and data analysis approach.

In the panorama of the cooperative proteins, hemoglobin is a unique case because experimental and theoretical methods have been developed to study the functional properties of the eight intermediates (1, 9). The information summarized below has been selected partly because of its significance with regard to the partial clarification of the allosteric mechanism and partly to exemplify the unprecedented level of detail in the knowledge of the ligand-hemoglobin interactions that was needed for reaching such an aim.

\section*{The CO intermediates}

By using cryogenic quenching and electrophoresis methods, the intermediates in the reaction with CO have been isolated, identified, and quantified both at equilibrium and in the association reaction (9). The experimental data on the equilibrium distributions of intermediates are shown in Fig. 3 (11). From such data the Adair constants for a simplified equilibrium, ignoring the chain heterogeneity, can be calculated, and henceforth the equilibrium curve shown in Fig. 2 (solid line) can be constructed. The analysis of the intermediates’ distributions has pinpointed the pivotal role of the diliganded intermediates in the allosteric mechanism. However, a precise determination of the concentrations of each diliganded intermediate, especially species \((\alpha\beta)(\alpha^1\beta^1CO) \) and \((\alpha\beta^1)(\alpha\beta^1CO) \), to distinguish the MWC and KNF models is not possible with the present state of the technique. Furthermore, the Adair constants calculated according to the MWC and KNF models from the data in Fig. 3 yield equilibrium curves that cannot be correlated with the experimental data. The curve shape is critically influenced by the presence of \( H^+ \), which is often unavoidable, and noncooperative dimers, which form in dilute protein solution due to the tetramer-dimer equilibrium (Fig. 1). This last effect is illustrated in Fig. 2, which compares the \( O_2 \) equilibrium curves obtained under similar conditions by methods differing in the experimental and data analysis approach.
This property has been usefully exploited for obtaining the cooperative free energies of the ligation analogs (1). The ligation analogs

The most detailed studies on the properties of the intermediates have been carried out by using ligation analogs and, in particular, the deoxy/cyanomet intermediates in which the ligation state is mimicked by the complex of cyanide with the chains oxidized to the ferric state (1). Part of this work is now being questioned due to the recent discovery that mixtures of cyanomet hemoglobin (HbCN) and Hb undergo exchange reactions of whole heme groups or electrons, called valency exchange reactions, that make the cyanomet analog unstable (15). Nevertheless, a new insight into the allosteric mechanism has been provided by the study of the ΔG_C values (1) and of the Bohr effect of the cyanomet intermediates and some of their chemically modified derivatives under controlled valency exchange conditions (10, 13).

Bohr effect of the intermediates. The Bohr effect is the release of hydrogen ions due to the rupture of specific salt bridges brought about by ligation. Curves of the Bohr effect vs. pH of Hb and some intermediates are shown in Fig. 4, A and B (10). In the pH range from ~6.3 to 9, the Bohr effect of Hb reaches a maximum value at physiological pH. At pH < 6.3 the Bohr effect is reversed, i.e., hemoglobin takes up hydrogen ions on oxygenation, and at pH > 9 hydrogen ions are not released on oxygenation because the salt bridges responsible for the Bohr effect are ruptured by the titration of the groups involved in the bridges. Since there is evidence that Hb maintains the T conformation in the entire pH range (1), the bell-shaped curve could be assumed to characterize the Bohr effect of a molecule in the T quaternary structure. The Bohr effect curves have been determined for all of the intermediates (10) and confirmed under conditions of insignificant valency exchange for all species except (αε)(α+CN−β+CN−) (13).

With respect to the shape, these curves are of two types. The curves of the monoliganded intermediates are bell shaped, suggesting that these species retain the Hb T quaternary structure. The ΔG_C values measured for the cyanomet analogs, and those yielded by the analyses of the CO intermediates at equilibrium, have also been attributed to molecules in the T conformation (1, 9). At a sufficiently alkaline pH, the Bohr effect due to monoligation vanishes, but the unliganded chains can potentially release the amount of H+ corresponding to the difference between the curves of the Bohr effect of Hb and of the monoliganded species at the same pH, i.e., the Bohr effect of

\[
\Delta G_C \text{values}
\]
the three unliganded chains. The di- and triliganded intermediates yield sigmoidal curves. In particular, the sigmoidal curve of each symmetrical diliganded intermediate, \((\alpha^+CN-\beta)(\alpha^-CN-\beta)\) and \((\alpha^-CN-\beta)(\alpha^+CN-\beta)\), is not twice the curve of the corresponding monoliganded intermediate. At sufficiently alkaline pH, the Bohr effect curves of these species merge with the Bohr effect curve of Hb, indicating that all of the Bohr hydrogen ions have been released despite the presence of two still unliganded chains. This functional effect is expected in a molecule that has no Bohr effect due to a complete change in quaternary structure from T to R. At acidic pH values, the Bohr effect is less than the Bohr effect of the monoliganded species. Thus the sigmoidal Bohr effect curves of the symmetrical diliganded intermediates cannot be explained as the functional effect of an equilibrium between T and R structures. Instead, they are more likely the functional effect of molecules in the R structure in which the tertiary structure of the chains is modulated by pH. The Bohr effect curve of the asymmetrical intermediate \((\alpha^-CN-\beta)(\alpha^-CN-\beta)\), not shown, is the mean of the curves of the two symmetrical diliganded species. A similar interpretation applies to the sigmoidal curves of the triliganded intermediates.

Bohr effect and role of the salt bridges. A stereochemical mechanism proposed by M. F. Perutz (12) explains how the hemoglobin molecules overcome the energy barrier for the T/R transition and equilibrate between two quaternary structures. Ligation induces structural changes transmitted from the heme to a network of hydrogen bonds and salt bridges, which constrain the molecule in the T structure, forcing them to break and release hydrogen ions. The constraint release allows part of the hemoglobin molecules to switch from the T to the R conformation, which lacks the Bohr effect.

Such an explanation is not consistent with the data shown in Fig. 4. C and D. The ribbon curves in Fig. 4C represent the loss of a large fraction of the total hemoglobin Bohr effect due to the chemical modification of both \(\beta\)-chain Bohr groups by N-ethylmaleimide (NEM), which reacts with the \(93^b\) thiol groups. This protein is named NESHb. Curve 2 shows hydrogen ions lost because of the chemical modification by NEM of one \(\beta\)-chain Bohr group, \((\alpha^-CN-\beta)(\alpha^-CN-\beta)\) NEM. Black line shows one-half of curve 1. D. Bohr effect of the \(\beta\)-monoliganded intermediate chemically modified at the second \(\beta\)-chains, \((\alpha^-CN-\beta)_{\beta\beta\beta}\) NEM. Black line is the theoretical line calculated by summing the curve of the \(\beta\)-monoliganded intermediate in B and curve 2 in C, omitting the error. Adapted from Ref. 13.
In contrast, the Bohr effect of the monoliganded intermediates (Fig. 4, A and B) is not additive, meaning that the changes in tertiary structure induced by ligation of one chain cause the release of Bohr hydrogen ions and at the same time signal the ligation state to the chains in the adjacent dimer. Thus binding a second ligand to another chain in the adjacent dimer promotes a quaternary T-to-R switch. The ribbon-like curve in Fig. 4D represents the Bohr effect of β-monoliganded hemoglobin singly modified at the Bohr group of the other β-chain in the adjacent dimer (13). Such a Bohr effect is equal, within error, to the sum of the Bohr effect of the β-monoliganded intermediate and the Bohr hydrogen ions lost because of the chemical modification of a single β-chain Bohr group, shown as a line in the figure. This finding indicates that the effects of the β-chain ligation in a dimer and of the chemical modification of the β-chain Bohr group in the adjacent dimer are independent. This would not be the case if ligation, or chemical modification, promoted a change in the quaternary conformation of the molecule. Thus the additivity of the two effects is not consistent with the hypothesis that monoliganded hemoglobin exists as an equilibrium between two quaternary structures and that salt bridge breaking facilitates such an equilibration.

**Controversies**

By using the ligation analogs, and particularly the cyanomet analog, detailed studies of the ΔGᵢ.values for each ligation step have been carried out and a new model of hemoglobin allosteroy has been proposed (1) called the Symmetry Rule (SR). This model assumes that 1) Hb, the monoliganded intermediate, and the asymmetrical diliganded (αβ)(α¹β¹) intermediate are in the T quaternary structure and 2) all other diliganded and triliganded intermediates are in the R quaternary structure as fully liganded hemoglobin. The SR implies that part of the cooperative free energy is accounted for by the intradimer cooperativity within the T structure in the ligand-binding pathway from Hb to intermediate (αβ)(α¹β¹) and in part by the T-to-R quaternary structural change in the course of ligand binding to this intermediate. The SR model has been the object of debate for the past 10 years due to the controversial estimate of the cooperative free energy of intermediate (αβ)(α¹β¹).

**Conclusions**

The MWC and KNF models are limiting cases of a general theory described by Eigen (5), which allows for both conformational equilibria of quaternary structures and tertiary structural changes induced by ligand binding. Rapid protein conformational equilibria have been demonstrated, e.g., by the NMR analysis of monomeric proteins in solution. A combination of such equilibria and tertiary structural changes induced by the ligand could optimize the protein-ligand interaction (2). In a multimeric protein such as hemoglobin, which binds oxygen very rapidly, rapid tertiary structural transitions have been observed (6), but rapid quaternary structure equilibria at all ligation stages have yet to be proved. In contrast, there is clear evidence that the allosteric equilibrium, \( L = [T^\beta]/[R^\alpha] \), is attained very slowly, due to the high activation energy required for the concerted conformational switch. The data on the intermediates, particularly the cyanomet analogs, that have been accessible to study and partly reported in this review indicate that the intermediates exist in either the T or the R quaternary structure with no evidence of a quaternary conformational equilibrium. Tertiary structural changes induced by ligation, signaled to the other chains through the interdimeric and/or intradimeric contacts, contribute to the change in ligand affinity in the course of ligation and provide the mechanisms for overcoming the energy barrier for the quaternary transition. Thus the study of the intermediates suggests that protein allosteroy is the result of a partial combination of the concepts underlying the two-state MWC model, i.e., concerted transition of all of the subunits from one to the other quaternary structure, and the KNF model, i.e., a progressive transition brought about by the sequential changes in tertiary structures induced by ligation.

This review is dedicated to the memory of Eraldo Antonini, a pioneer in hemoglobin functional studies.

We are grateful to H. R. Bosshard and P. Cerretelli for their useful comments on the manuscript.

Part of the work described in this paper was supported by a grant from the Ministero Universita Ricerca Scientifica Tecnologica.

**References**