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Critical Review

Cooperativity and Allostery in Haemoglobin Function

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Summary

Tetrameric haemoglobins display a cooperative ligand binding behaviour, which has been attributed to the functional interrelationship between multiple ligand binding sites. The quantitative description of this feature was initially carried out with a phenomenological approach, which was limited to the functional effect of the occupancy by a ligand molecule of a binding site on further binding steps. However, subsequent development of structural–functional models for the description of the cooperativity in haemoglobin brought about a much deeper information on the interrelationships between ligand binding at the heme and structural variations occurring in the surrounding free subunits. This approach opened the way to the evolution of the concept of allostery, which is intended as the structural–functional effect exerted by the presence of a ligand in a binding site on other binding sites present in the same molecule. This concept can be applied to either sites for the same ligand (homotropic allostery) and for sites of different ligands (heterotropic allostery). Several models trying to take into account the continuous building up of structural and functional information on the physicochemical properties of haemoglobin have been developed along this line.

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Abbreviations Hb, haemoglobin

INTRODUCTION

Cooperativity for O_2 binding is expressed in haemoglobin (Hb) because the interaction of the O_2 molecule with one heme facilitates the binding of additional O_2 molecules to the other heme sites. This feature, which was first described by Bohr (*1*) and then further investigated by others (*2, 3*), elicited over the last century the interest for the formulation of a model that could account for this behaviour. This elicited a large number of structural and functional studies (*4, 5*), which have allowed a wealth of information, never attained before for any other biological macromolecule, part of which are reported in this review, trying to get an overall view of the actual knowledge of its reaction mechanism with ligands, mainly O_2.

COOPERATIVITY OF HEMOGLOBIN AND MODELS

A phenomenological description of the cooperative effect, which implies an equilibrium binding constant for each sequential ligand binding step, is inadequate for the determination of all equilibrium binding constants when the number of binding steps exceeds two, which indeed is the case for most of the investigated macromolecules. Thus, the knowledge of the binding mechanism requires the determination of the concentration of all different ligand-bound species (Fig. 1) that is the binding polynomial. For Hb with four binding sites, this is represented by the five populations at different ligation stage:

$$P = [M] + [MX] + [MX_2] + [MX_3] + [MX_4]. \quad (1a)$$

Since

$$[MX_i] = K_i \cdot [MX_{i-1}] \cdot [X]. \quad (1b)$$

where K_i is the equilibrium constant for the ith binding step, P becomes

$$P = 1 + 4 \cdot K_1 \cdot X + 6 \cdot K_1 \cdot K_2 \cdot X^2 + 4 \cdot K_1 \cdot K_2 \cdot K_3 \cdot X^3 + K_1 \cdot K_2 \cdot K_3 \cdot K_4 \cdot X^4, \quad (1c)$$

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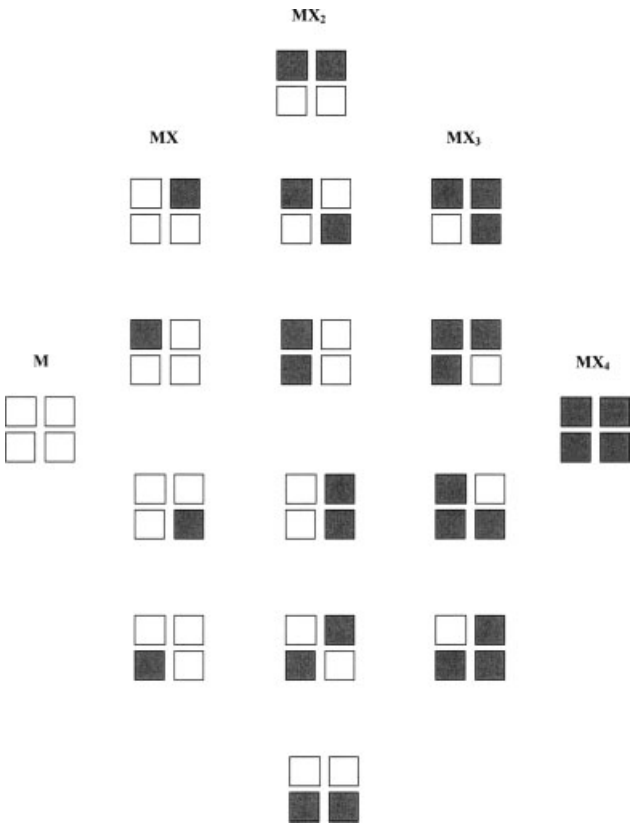

Figure 1. Schematic representation of the statistical ligand distribution among different binding sites of the different ligand-bound species (filled squares).

where coefficients refer to the statistical factors for a molecule with four equivalents binding sites accounting for the various potential populations existing at different ligation stages (Fig. 1). Since the coupling among different binding constants impairs the accurate determination of individual macroscopic binding constants except for K_1, a great effort has been dedicated in the second half of the century to formulate thermodynamic models, which allowed to describe the cooperativity with a reduced number of parameters, which have physical meaning. In this review, it is not possible to deal with all these efforts because some of them differ each other only for few details; therefore, we will describe only those that show a conceptual diversity, mentioning all variations on the theme in the framework of the general description of a specific model.

Two-State Allosteric Model

This model was formulated in its original version (*6*), referring to multisubunit cooperative proteins as oligomers made of identical monomers or (at least) as oligomers that display a symmetry axis. In this way, the model could be applied to O_2 binding to Hb, accounting for the two types of subunits assembled in a symmetric fashion (*7*). In this first version, the eventual functional difference between α- and β-chains was disregarded and it was postulated that no direct intersubunit functional influence was occurring during ligand binding; in other words, ligand binding to any one of the subunits does not directly affect the ligand affinity for the other unliganded subunits of the oligomer. The central stage for binding cooperativity was instead identified in the quaternary conformation of the whole protein, since the model implied the existence of an equilibrium between two quaternary structures (characterized by different intersubunit contacts), one of which (called T state) was predominant in the fully unliganded species and the other one (called R state) was predominant in the fully liganded form. Ligand binding to the active site of a subunit is characterized by a low-affinity constant K_T when the oligomer is in the T state whereas the transition to the R state of the whole oligomer brings about a high ligand binding affinity corresponding to K_R. Therefore, the observed equilibrium constant K_{obs} is related to the relative percentage of oligomers in either one of the two quaternary states

$$K_{obs} = \frac{K_T \cdot [T] + K_R \cdot [R]}{[T] + [R]} \tag{2a}$$

and cooperativity (represented by the increase of K_{obs} as more subunits bind the ligand) is then associated to the fact that the quaternary equilibrium is displaced in favour of the R state as more subunits bind the ligand, so that a progressively increasing percentage of oligomers switches to R state, displaying a higher affinity constant. For tetrameric Hb, this system turns out to be much simpler than the phenomenological approach [see Eqs. (1b) and (1c)] since in the binding polynomial

$$\begin{aligned} P &= P_T + P_R \\ &= M_T + M_T X + M_T X_2 + M_T X_3 + M_T X_4 + M_R \\ &\quad + M_R X + M_R X_2 + M_R X_3 + M_R X_4 \end{aligned} \tag{2b}$$

for a specific quaternary state all i binding steps are characterized by the same equilibrium binding constant:

$$K_T = \frac{[M_T X_i]}{[M_T X_{i-1}] \cdot [X]} \tag{2c}$$

$$K_R = \frac{[M_R X_i]}{[M_R X_{i-1}] \cdot [X]}. \tag{2d}$$

A third parameter is required for determining the quantitative relationship between the relative amount of oligomers in the T and R states, as requested by Eq. (2a), and this is defined as the quaternary equilibrium constant as

$$L_0 = \frac{[M_R]}{[M_T]}, \tag{2e}$$

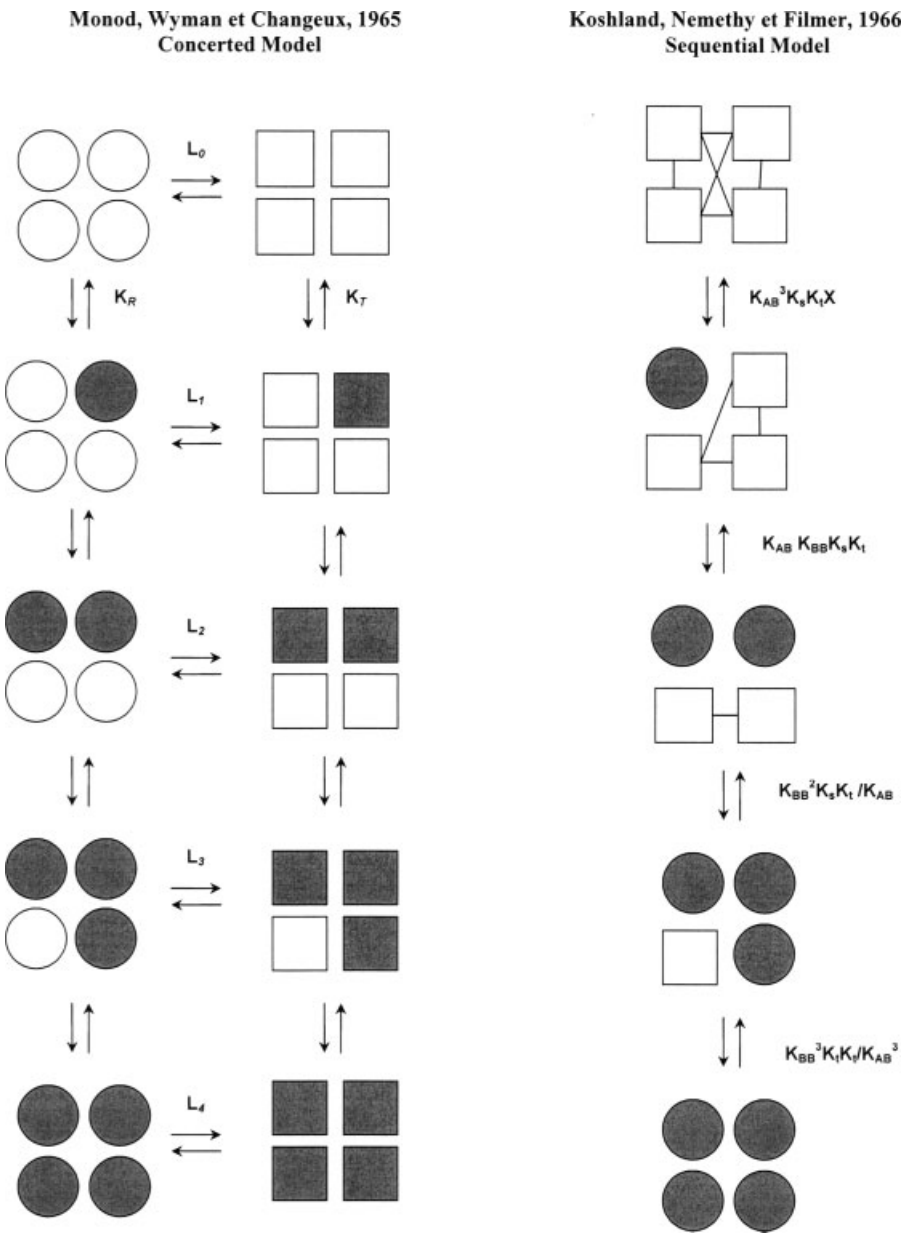

Figure 2. Allosteric and induced-fit models for oxygen binding to haemoglobin. (Left scheme) Circles and squares represent, respectively, the relaxed form (R), displaying a very high affinity for the gaseous ligand, and the tense form (T) whose affinity for the ligand is very low (filled symbols represents ligand-bound subunits) according to the allosteric concerted MWC model. (Right scheme) Circles and squares represent the ligand-bound and ligand-free subunits, respectively, according to the induced-fit KNF model. Solid lines indicate salt bridges interactions among ligand-free subunits.

and the equilibrium constants for binding are related to the allosteric constants at different ligation steps by the following relationship:

$$L_i = L_0 \cdot \frac{K_{\mathrm{R}}^i}{K_{\mathrm{T}}^i}, \tag{2f}$$

rendering possible to rewrite the binding polynomial of Eq. (2b) with only three parameters instead of four [see Eq. (1b)]:

$$P = \frac{1}{1+L_0} \cdot \sum_{i=0}^{i=4} (K_{\mathrm{T}} \cdot X)^i + L_0 \cdot (K_{\mathrm{R}} \cdot X)^i, \tag{2g}$$

where the factor $1/(1 + L_0)$ is simply a factor to scale for the molar fraction of the two quaternary conformations (Fig. 2). Further, these three parameters have a clear-cut physical meaning, referring to the ligand binding affinity in the two quaternary states and to the allosteric equilibrium between the two

conformations. In the original formulation, no functional heterogeneity for the two subunits was taken into account, but its introduction (*8*) does not modify the essential features of the model. The formulation of a three-state model (*9*), where a third quaternary conformation S is postulated to account for the effect of organic polyphosphates, can only be considered a simple extension of this model, even though its introduction complicates significantly the model and reduces the physical meaning of individual parameters.

Sequential Induced-Fit Model

This model (*10*) implies that the individual subunit of a multisubunit macromolecule is in equilibrium between two tertiary states, one of which (called A) in unable to bind the ligand and the other one (called B) can bind a ligand molecule. There is a conceptual difference with respect to the two-state allosteric model since in one case (i.e., the sequential induced-fit model) the overall quaternary state of the whole molecule changes during the ligand binding process, being progressively altered by the mixture of tertiary conformations of individual subunits according to whether they are liganded or not (Fig. 2) while in the other case (i.e., the two-state allosteric model) the oligomer is in equilibrium between two alternative quaternary conformations corresponding to either the fully unliganded and/or the fully liganded oligomer (see Fig. 2). Therefore, in the sequential induced-fit model, the binding affinity for a given subunit is given by

$$K_{\text{obs}} = K_{\text{s}} \cdot \frac{[B]}{[B] + [A]} = K_{\text{s}} \cdot \frac{K_{\text{t}}}{K_{\text{t}} + 1}, \tag{3a}$$

where K_{s} is the intrinsic affinity of the ligand for the subunit in state B and K_{t} represents the equilibrium constant between the two states in the unbound subunit:

$$K_{\text{s}} = \frac{[BL]}{[B] \cdot [L]} \tag{3b}$$

$$K_{\text{t}} = \frac{[B]}{[A]}. \tag{3c}$$

The cooperativity arises because ligand binding to a subunit alters the constant K_{t} in the neighbouring still unliganded subunits, thus changing the value of K_{obs} in Eq. (3a) for the subunits that bind subsequent ligand molecules. This effect is represented by two additional equilibrium constants, which consider for a pair of interacting subunits in the A state the different probability (and thus the energetics) for the A $\rightarrow$ B transition of only one of them [Eq. (3d)] or of both [Eq. (3e)]:

$$K_{\text{AB}} = \frac{[AB] \cdot [A]}{[AA] \cdot [B]} \tag{3d}$$

$$K_{\text{BB}} = \frac{[BB] \cdot [A] \cdot [A]}{[AA] \cdot [B] \cdot [B]}. \tag{3e}$$

It is obvious that intersubunit positive cooperativity arises if $K_{\text{BB}} > K_{\text{AB}}^2$, no interaction if $K_{\text{BB}} = K_{\text{AB}}^2$ and negative intersubunit cooperativity if $K_{\text{BB}} < K_{\text{AB}}^2$.

All these parameters are present in defining the equilibrium constant for ligand binding to a subunit but their relative influence is different according to (i) the interaction network and (ii) the binding step. Therefore, in the case of a tetrameric Hb, the simplest case can be represented by the existence of three interactions for each subunit with the other three ones (for a total number of six interactions, see Fig. 2, where unliganded subunits in the A state are represented by rhombs and liganded subunits in the B state by squares). In the unliganded form (see upper species in Fig. 2), we have all six AA interactions, marked by solid lines, which disappear as ligand binding takes place and form different number of AB and BB pairs. The resulting binding polynomial for a tetrameric Hb [see Eq. (1a)] is

$$P = 1 + 4K_{\text{AB}}^3 K_{\text{s}} K_{\text{t}} X + 6K_{\text{AB}}^4 K_{\text{BB}} K_{\text{s}}^2 K_{\text{t}}^2 X^2 + 4K_{\text{AB}}^3 K_{\text{BB}}^3 K_{\text{s}}^3 K_{\text{t}}^3 X^3 + K_{\text{BB}}^6 K_{\text{t}}^4 K_{\text{t}}^4 X^4, \tag{3f}$$

where K_{AB} and K_{BB} appear in the equilibrium binding constants raised to the power corresponding to their number. This formalism allows to introduce a much larger variety of modulatory mechanisms since it describes direct intersubunit interactions, which can differ for the different subunits of a given macromolecule. As a matter of fact, it is able to account for positive and negative cooperativity (*11*) (while the two-state model can only describe positive cooperativity) and, implying different intersubunit interactions, it can reproduce asymmetric binding curves, appearing more appropriate for nonsymmetric multisubunit cooperative systems. However, this model, although able to account for a large variety of network interactions, is much less stringent in the description of the functional behaviour of a macromolecule, allowing a large number of degrees of freedom, which render very difficult to assign a physical significance to the resulting parameters; thus, the coupling between the different types of equilibrium constants [see Eq. (3f)] allows to modify the different parameters in a large variety of modes to fit the experimental data, coming out with a wide range of physical explanations equally valid. Therefore, although it may turn out very useful for describing systems with only two or three interacting binding sites, it may become exceedingly complex (and physically useless) for the functional description of more complex macromolecules.

Szabo–Karplus Model

This model (*12*), which is a sort of synthesis between the two previous models, represents an approach to the statistical mechanics of cooperativity, which stems from the structural information on the differences between unliganded and liganded Hb (*13*); therefore, it is often referred as the Perutz–Szabo–Karplus model. Thus, according to the X-ray three-dimensional structures of Hb, a change in the quaternary structure is observed upon ligand binding (mainly affecting the interface

relationships between different types of subunits along one of the axis, namely, $\alpha_1\beta_2$), which is accompanied by variations of the tertiary structure of individual subunits. Obviously, since the original structural information (*13*) concerns only the initial (i.e., the fully deoxygenated Hb) and the final form (i.e., the fully liganded Hb), no distinction can be made on the role of tertiary and quaternary structural changes in characterizing the cooperative behaviour of ligand binding to Hb. However, formulating a stereochemical mechanism, Perutz seems to attribute a predominant role to the ligand-linked tertiary structural changes taking place in individual subunits, implying that the intrasubunit structural changes are spread over the intersubunit contacts, triggering the quaternary structural transition. In particular, one of the ligand-linked tertiary structural changes concerns the intrachain salt bridge between the carboxy-terminal residue His146β and the Asp94β, which is present in the unliganded subunit but not in the liganded form (*13, 14*), likely accounting from most of the pH-dependence observed on ligand binding (i.e., Bohr effect) (*15–17*). However, since the rupture of the intrachain salt bridge is accompanied by a structural change of Tyr145β, which alters the quaternary structural relationships, a close connection between tertiary and quaternary structural changes is required for the Bohr effect.

The Szabo–Karplus model tries to account for all these structural information, assuming the existence of two quaternary structures in equilibrium, as in the two-state model (*6*), but inserting a modulatory role played by the salt bridges. As a result, the different ligand affinity between the two quaternary states is due to the presence (only in the T quaternary conformation) of a set of salt bridges (distributed among the different subunits), which are released when the subunit binds a ligand molecule. This effect is represented by an allosteric constant Q, which reflects the stabilization factor between the T structure and the R structure in the absence of salt bridges. This stabilization factor is then scaled by the parameter S^h, where h is the number of intact salt bridges in T unliganded form and S refers to the energy associated to the presence of a single salt bridge, such that, by comparing with the two-state model, $L_0 \cong Q \cdot S^h$. Therefore, this model encloses this mixture of tertiary and quaternary effects, connecting them to the number of salt bridges, and thus to the energy related to their ligand-linked rupture, as well as to the proton concentration. It immediately comes out that, unlike the two-state model, the ligand affinity varies within a given quaternary structure according to the number of liganded subunits and cleaved salt bridges. A complication arises because the distribution of salt bridges is not uniform in the tetramer, which introduces the possibility of different functional properties for the two types of subunits. As an example of this, the binding constant for the first step in the T quaternary state is

$$K_{T1} = 2 \cdot \frac{K^\alpha}{S^n} \cdot \frac{(1 + \mu \cdot H^\alpha)^r}{(1 + \mu \cdot \frac{H^\alpha}{S})^{n-r}} + 2 \cdot \frac{K^\beta}{S^m} \cdot \frac{(1 + \mu \cdot H^\beta)^s}{(1 + \mu \cdot \frac{H^\beta}{S})^{m-s}}, \quad (4a)$$

where K^α and K^β are the affinity constants of the ligand for the two chains in the R quaternary structure, S is the decreasing factor due to the presence of a ligand-linked salt bridge (see earlier), raised to the power corresponding to the number of salt bridges affecting that type of subunit, μ is the proton concentration (pH = log $(1/\mu)$), H^α and H^β are the protonation constant for the salt bridge residues [pK = log (H)], which identify the pH range over which these groups are functionally relevant.

It must be outlined that this model represents a very interesting and elegant synthesis of previous models since it is able to introduce only physically meaningful parameters (like the protonation constant H), rendering the model more appropriate to account for different functional modulatory aspects. On the other hand, some weakness originates because it is exceedingly detailed in identifying the factors responsible for the affinity change; thus, a variety of additional factors could be postulated, immediately increasing the complexity of the model and reducing the physical meaning of individual parameters.

Cooperon Model

This model (*18*) has not been conceived specifically for the cooperative binding of Hb but it originates from the idea of multiple constellations to account for the cooperative oxygen binding displayed by giant oxygen carriers, such as erythrocruorins and hemocyanins (*19*). The idea underlying this model is that within an oligomeric macromolecule, which behaves as a whole according to the two-state model (*6*), there is a smaller cooperative unit, made by n active sites (or subunits), that interact according to an induced-fit mechanism. Ligand binding brings about a tertiary structural change of the bound subunit (from t_0 to t_1 if the macromolecule is in the T quaternary state or from r_0 to r_1 if the macromolecule is in the R quaternary state). Therefore, ligand binding to this cooperative unit, called "cooperon", may be cooperative, though keeping the macromolecule in the same quaternary structure; each cooperon behaves as a subunit in the allosteric two-state model, not interacting with the other cooperons.

In the original formulation (*18*), a dimeric cooperon was considered the basic cooperative unit, following from the original idea of Antonini (*20*) that the α-β dimer was the important unit for the Hb action. The ligand binding within the cooperon was then described according to the same conceptual formalism of the induced-fit model (see earlier), with three stabilizing factors, which are τ_{00}, τ_{01} and τ_{11} (or ρ_{00}, ρ_{01} and ρ_{11}) for the unliganded, monoliganded and diliganded dimer, respectively, in the T (or R) overall quaternary structure. These factors represent the energetic levels within the dimeric cooperon of the three possible intersubunit relationships [i.e., τ_{00} (or ρ_{00}) when they are both unliganded, τ_{01} (or ρ_{01}) when one is liganded and the other is unliganded and τ_{11} (or ρ_{11}) when they are both liganded]. Therefore, the binding polyno-

mial for a tetrameric Hb (made of two dimeric cooperons) is as follows:

$$P = (\tau_{00} + 2 \cdot \tau_{01} \cdot K_t \cdot X + \tau_{11} \cdot K_t^2 \cdot X^2)^2 + (\rho_{00} + 2 \cdot \rho_{01} \cdot K_r \cdot X + \rho_{11} \cdot K_r^2 \cdot X^2)^2, \quad (5a)$$

which can be written as

$$P = \frac{1}{1+L_0} \cdot \left[L_0 \cdot \left(1 + 2 \cdot K_T \cdot X + \delta_T \cdot K_T^2 \cdot X^2\right)^2 + \left(1 + 2 \cdot K_R \cdot X + \delta_R \cdot K_R^2 \cdot X^2\right)^2 \right], \quad (5b)$$

where

$$L_0 = \frac{\tau_{oo}^2}{\rho_{00}^2};\ K_T = \frac{\tau_{01}}{\tau_{00}} \cdot K_t;\ K_R = \frac{\rho_{01}}{\rho_{00}} \cdot K_r;\ \delta_T = \frac{\tau_{00} \cdot \tau_{11}}{\tau_{01}^2};\ \delta_R = \frac{\rho_{00} \cdot \rho_{11}}{\rho_{01}^2}, \quad (5c)$$

where L_0, K_T and K_R are the same parameters as reported in Eq. (2c)–2(e) and δ_T and δ_R are the intersubunit interaction constants within the dimeric cooperon in the T and R quaternary conformation, respectively, meaning positive cooperativity for $\delta > 1$, no cooperativity for $\delta = 1$ [as for the original two-state model, see ref. (*6*)] and negative cooperativity for $\delta < 1$.

This model allows the existence of a distribution of different tertiary structures among the subunits of an oligomer in a given quaternary state, thus accounting for a functional modulation (e.g., by protons and/or by organic polyphosphates) that is not only of quaternary but also of tertiary origin. On the other hand, in spite of its flexibility, this approach can be considered only an extension of the two-state allosteric model, in the attempt to realize a synthesis between the features of the two main models. It may be interesting to underline that the dimeric cooperon may refer to any combination of dimers within the tetramer (Fig. 3), and not only to the dimer resulting from the physical dissociation (i.e., the $\alpha_1\beta_1$, see Fig. 3), as originally postulated by Antonini (*20*), which turned out to be noncooperative in the dissociated form (*21*). As a matter of fact, an other likely dimeric cooperon could be the $\alpha_1\beta_2$ (see Fig. 3), which has been proposed to be the interface more affected during the quaternary conformational change (*13*) and turns out to be the most effective in modulating the cooperativity, as observed in mutants (*22*).

APPLICATION OF MODELS TO EXPERIMENTS: CHALLENGES, SUCCESSES AND FAILURES

The description of the functional behaviour of Hb concerns (i) the interrelationships between ligand (i.e., O_2, CO, etc.) binding to the heme of different subunits (*homotropic interactions*), which represents the main aspect of the cooperativity mechanism and/or (ii) the interrelationships between ligand binding to the heme (i.e., O_2, CO, etc.) and binding of other ligands (e.g., H^+, organic polyphosphates, etc.) to topologically different sites (*heterotropic interactions*), which represents the main aspect of the functional modulation of cooperativity.

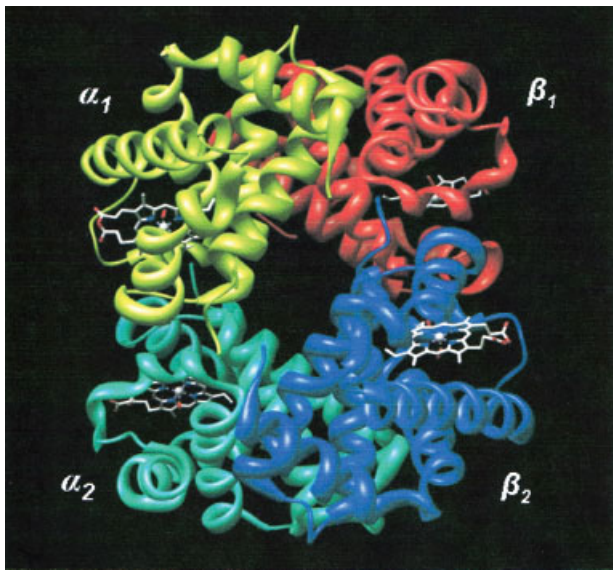

Figure 3. Three-dimensional structure of the haemoglobin tetramer. The haemoglobin molecule is a symmetric assembly of four globular subunits arranged in a dimer of dimers, each dimer being made up of one α and one β chain. Each individual chain carries a noncovalently bound heme group that binds oxygen reversibly. (Protein Data Bank code: 1GZX.)

Homotropic Interactions

The quantitative description of homotropic interactions of Hb is indeed the primary goal of every theoretical model illustrated earlier, which aims to give a detailed prediction of the probability distribution of intermediate partially liganded species. Therefore, the applicability of either one of models is faced with studies on the prevailing population of intermediates along the ligand binding process and on their functional properties as compared to the initial (i.e., fully unliganded Hb) and the final species (i.e., fully liganded Hb).

This investigation is complicated because in a cooperative ligand binding system, such as Hb, the concentration of intermediate species is always minor with respect to the extreme ones (i.e., fully unliganded and fully liganded Hb) (Fig. 4) and usual spectroscopic probes are useless for the characterization of intermediate species, unless they are unique for them. In this respect, even careful statistical thermodynamic studies and data analysis of the O_2 binding isotherms (*23, 24*), although providing very useful information on the fundamental parameters that

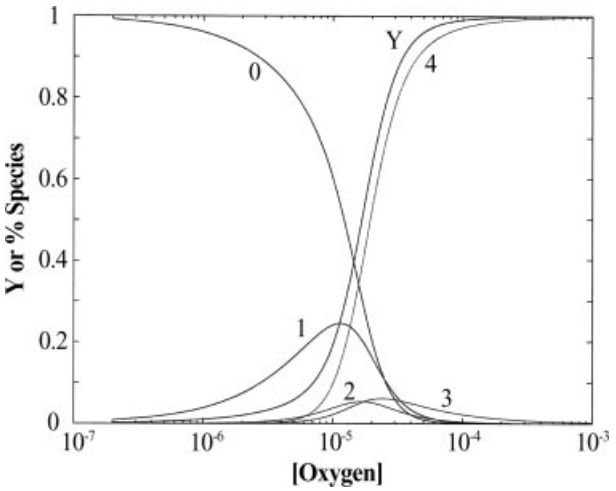

Figure 4. Distribution of liganded species as a function of oxygen pressure. Oxygen binding isotherm (Y) for a ligand binding process characterized by the following parameters (according to the two-state MWC formalism): $k_T = 1 \times 10^4\ M^{-1}$, $k_R = 2 \times 10^6\ M^{-1}$, $L_0 = 1 \times 10^{-6}$. Employing the same parameters, the relative percentage of unliganded (0), monoliganded (1), diliganded (2), three-liganded (3) and fully liganded (4) tetramers is reported along the binding process.

characterize the binding process, do not unravel any relevant direct clue observation concerning intermediate populations.

An important contribution arises from structural studies that indicate that ligand binding is accompanied by a movement of the Fe-His proximal bond of either one of two types of subunit, followed by a translation of the whole F helix across the face of the heme (*25*). The consequent changes in the tertiary structural changes of individual subunits are linked to quaternary structural changes; however, no significant structural change occurs in the central region of the $\alpha_1\beta_1$ dimer whereas a dramatic change can be observed for the interface between α_1 (or α_2) with β_2 (or β_1), underlying a crucial role for structural changes at the $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) interfaces (*25*). In particular, an asymmetric role seems to be played by different regions of this interface since (i) the contacts between the FG corner of the α_1 chain (or of the α_2) with the C helix of the β_2 (or of the β_1) act as a "flexible joint", allowing only small relative motions, while (ii) the contacts between the C helix of the α_1 chain (or of the α_2) with the FG corner of the β_2 (or of the β_1) act as a "switch" region, displaying two different stable positions (corresponding to the T and R quaternary structure) with different side-chains in contact (*25*) (Fig. 3). A third quaternary structure (called R_2) has been subsequently unravelled for liganded Hb (*26*), which may coexist with the T and R quaternary structures, and it is characterized by a looser $\alpha_1\beta_2$ interface, mainly at the "switch" region between His97β on one side and Thr38α and Thr41α on the other side (see figure 3 in ref. *26*). This additional quaternary structure, which in solution appears to be in equilibrium with the traditional R conformation (*27*), seems to be farther from the T structure than the R structure itself (*28*), and it can be observed even more populated in other mammalian Hbs, providing evidence that this dimer–dimer interface has in the liganded form a wider range of conformationally accessible structure related each other by a sliding motion (*29*).

An additional important information for the cooperative mechanism derives from the structural characterization of a liganded tetramer in the T quaternary state (*30*), where two main events can be detected, namely (i) a strain exerted at the level of the proximal Fe-His bond, where proximal His moves toward the heme plane upon ligand binding, though keeping a partially tilted position with respect to the heme (as in the deoxygenated species), and (ii) some alteration at the level of the $\alpha_1\beta_2$ interface intermediate between the two extreme quaternary structures (see earlier), mainly affecting the "flexible joint" (*25*). The observation (i) clearly shows the importance of the geometry of the proximal and the distal portion of the heme pocket upon ligand binding, which might be modulated in a different way for the two types of subunits (*31*). On the other hand, the observation (ii) is very important since it envisages the occurrence of a heme–heme interaction even without a quaternary structural change, creating tension at the "flexible joint" portion of the $\alpha_1\beta_2$ interface. This is a clear evidence that ligand binding brings about tertiary structural changes even in the absence of a switch of the quaternary conformation, but, more important, it shows a change in the tension, and thus in the energy, of the intersubunit contacts because of the ligand-linked tertiary structural changes. Therefore, it arises the question of the possibility of some cooperativity within the T-state.

However, in spite of these structural information, knowledge on the functional properties and relative population of intermediates can only be obtained by either (i) detailed analysis of the ligand binding kinetic behaviour or (ii) trapping artificial intermediates and studying their functional properties. However, both approaches suffer intrinsic weaknesses, which are mainly represented by that (i) kinetics of O_2 binding are very difficult to carry out (owing to the very fast binding rate constants, impairing the possibility of an investigation by stopped-flow, and the low photosensitivity for laser photolysis studies, see later) and (ii) artificial intermediates are intrinsically different (from the thermodynamic viewpoint) from the actual intermediates and they can only be "a close copy" of the species to be described. The kinetic problem is partially bypassed employing the ligand CO, which mimicks the cooperative features of the ligand O_2 quite well (*32, 33*); further, the photosensitivity of the HbCO allows to study the partially unliganded species after very short laser pulses, which allow to follow structural tertiary and quaternary changes together with ligand binding processes (*34–36*). However, it must be pointed out that coupling of tertiary and quaternary structural changes with populations of partially liganded species can be significantly different for CO and O_2 binding, thus requiring caution

in extrapolating data for one ligand to the behaviour of the other one.

A strong contribution to the plausibility of the application of the two-state allosteric model (*6*) to the O_2 binding properties stems from data obtained on single crystal deoxyHb (*37*), where, under conditions in which the T $\rightarrow$ R quaternary transition is impaired by crystal lattice, a noncooperative binding isotherm is observed characterized by a very low T-like affinity. These data represent a clear indication that ligand affinity does not depend on the number of ligand molecules bound to the four subunits of the tetramer, as predicted by the two-state allosteric model, ruling out the applicability of a pure induced-fit sequential model (*10*). Analogous indication comes from the observation that partial laser photolysis of HbCO is followed by a fast ligand recombination characterized by a rate constant independent on the extent of photolysis (*38*). Thus, since after photolysis of bound CO by a fast (<30 ns) laser pulse a rapid CO recombination (called geminate phase) occurs before a quaternary structural change takes place (*39*), this recombination process concerns binding in the R-state; the independence of the rate constant on the extent of photolysis is a clear demonstration that R-state binding is independent on the number of ligands already bound. An additional aspect in favour of the existence of only two main reacting species (i.e., T and R molecules) in rapid quaternary equilibrium is represented by the dependence of geminate recombination after laser photolysis as a function of the ligand binding process in a flow-flash experiment (*40*); thus, this experimental approach allows to follow the kinetics after laser photolysis in the course of ligand binding dynamics triggered by rapid mixing. The dependence can be accounted for by assuming that only molecules in the R-state display a significant amount of geminate recombination whereas T-state Hb has virtually no geminate recombination, as indeed subsequently demonstrated (*41*). This result, together with the observation that the proximal Fe-His bond is crucial in determining the rate constant difference for CO binding between T- and R-state molecules (*42*), clearly indicates that only two populations, characterized by different stereochemical constraint(s) of the proximal bond (and thus by different activation free energy for the CO rebinding), are enough to account for the CO binding dynamics. As a whole, the two-state allosteric model turns out to be fairly compatible with a detailed analysis of CO rebinding kinetics, where geminate and bimolecular processes have been taken into account together with spectroscopic variations associated to tertiary and quaternary structural transitions (*43*).

A serious challenge to the applicability of the original two-state allosteric model (*6*) to the functional behaviour of Hb is represented by studies on the isolation of intermediates [by mixing appropriate parent species to produce them, see ref. (*44*)] and their quantitation by either (i) measuring the rate of dimerization (*44*) or (ii) trapping the intermediate species by cryogenic methods (*45*). Thus, in this case, the percentage amount of each intermediate species can be calculated [in case (i)] or even visualized [in case (ii)], allowing to reconstruct the distribution of intermediate liganded forms along the binding isotherm. The intrinsic weakness of this approach is that you need to make intermediate species from different parent molecules, which must be distinguished and therefore they are not homogeneous intermediates but they are either valency hybrids intermediates (where some hemes have ferric and others have ferrous irons) or else metal hybrids intermediates (where hemes have different metals); therefore, an extrapolation is required and this is a very delicate aspect (*46*), since the different parent molecules should have exactly the same behaviour (which is never exactly true). In any event, different types of approaches have all come to a common conclusion that a nonbinomial distribution of ligand over the four hemes is present in the diliganded species (differently from what expected by a pure two-state allosteric model), displaying a preferential population of molecules with liganded $\alpha_1\beta_1$ hemes (Figs. 1 and 3) (*47, 48*) with essentially no liganded $\alpha_1\alpha_2$ or $\beta_1\beta_2$ species (*45*). Altogether, these observations have led to the formulation of a molecular code for cooperativity (*48*), where after the first binding event to anyone of the four subunits [no significant functional heterogeneity is observed, see ref. (*49*)] the second ligand binding occurs preferentially to the partner chain belonging to the same $\alpha_1\beta_1$ dimer; this underlies a positive cooperativity across the $\alpha_1\beta_1$ interface, even though the cooperative behaviour of the $\alpha_1\beta_1$ dimer has been questioned (*50*). Conversely, a negative cooperative interaction in the T-state is envisaged between the homologous chains (α_1-α_2 and β_1-β_2 interfaces), as suggested by the absence of these intermediate species (*45*). Since the behaviour of this intermediate (α_1-Lβ_1-L)($\alpha_2\beta_2$) suggests it to be still in a T-like quaternary state (*51*), its preferential formation underlies the existence of a cooperative ligand binding within the T quaternary state, which fully disagrees with the pure two-state allosteric model (*6*), but it can be predicted by the cooperon model (*18*), which in fact is able to fully account for this observation (*52*).

An additional challenge to the applicability of a pure two-state allosteric model to ligand binding to Hb is represented by data obtained for Hb trapped in wet porous silica gels, which, like polyethylenglycol crystals (*37*), fix Hb molecules in a quaternary state (*53*). Thus, data from Hb encapsulated in the deoxygenated form (and presumably locked in the T-state) indicate the existence of two distinct conformations (*54*), one characterized by the very low affinity observed for the crystal (*37*) and an other one with a higher O_2 affinity [much higher than K_1 in solution, see ref. (*23, 24*)]. Such a behaviour (which is observed only after a long incubation of the gel at 35 °C, a treatment claimed to be necessary for an equilibration between the different conformations) does not necessarily imply the existence of two tertiary functionally different T-states, as suggested by the authors (*54*), since a functional heterogeneity has not been convincingly ruled out, considering that the extinction coefficients for the two chains have been shown to be drastically different in the unliganded species (*55*). Furthermore, also a negative cooperativity can be taken into consideration for

interpreting this behaviour, in the light of the likely negative interaction for ligand binding in the T-state between homologous chains (i.e., α_1-α_2 and β_1-β_2) (*45, 52*).

Therefore, the possibility of multiple tertiary structures already for the unliganded T quaternary state can be considered still as a working hypothesis, whereas more convincingly evidence is available for the possibility of ligand-linked tertiary structural changes within the quaternary T conformation at intermediate ligation stages.

Heterotropic Interactions

In this chapter, we will limit ourselves to deal only with the pH-dependence of the affinity for O_2 binding (*1, 15*), which is called Bohr effect. As a consequence of this modulation, the O_2 saturation degree (Y) is related to the ligand-linked release (and/or uptake) of protons (H^+) according to the general linked function

$$\left(\frac{\partial \overline{Y}}{\partial \overline{H}^{+}_{ov}}\right)_{x,z} = \left(\frac{\partial \ln K_{ov}}{\partial pH}\right)_{\overline{H}^{+},z}, \qquad (6a)$$

where x and z are the concentrations of O_2 and H^+, respectively, and $K_{ov} = (K_1 \cdot K_2 \cdot K_3 \cdot K_4)^{1/4}$ is the overall O_2 affinity. As reported previously, the Bohr effect seems to be attributable to some residue(s), which undergo(es) a ligand-linked pK_a shift, which affects the O_2 affinity. Structural and functional evidence (*14, 56*) pointed out the role of intrachain salt bridges between His146β and Asp94β as well as of interchain salt bridges between (i) His146β_1 and Lys40α_2 (as well as His146β_2 and Lys40α_1) and (ii) Arg141α_1 with Asp126α_2 (as well as Arg141α_2 with Asp126α_1). However, the mechanism is more subtle since different experimental conditions may lead to an alteration of the role of these residues and other protonating groups might come into play in characterizing the Bohr effect (*57–59*). Going into further detail of the residues involved in the Bohr effect is outside the purpose of this review, which instead wants to point out the relationships between ligand-linked H^+ release (and/or uptake) and tertiary (and/or quaternary) structural changes. In this respect, an important information is represented by the observation that the ligand-linked proton release (and/or uptake) is not uniform for different oxygenation steps (*16*), clearly indicating that the first binding step is associated to the release (or uptake) of some O_2-linked protons, associated to the rupture of some salt bridge.

This is a very important finding, since in the original two-state MWC model effectors (such as H^+ in the Bohr effect) should only be allosteric ones, affecting the equilibrium between the two quaternary conformations; therefore, according to the simplest formulation of this model, ligand binding to one subunit (within a given quaternary structure, e.g., T state) should not bring about the rupture of the salt bridge associated to the Bohr effect. On the other hand, the sequential induced-fit model requires that tertiary structural changes, responsible for the cooperative behaviour, occur upon ligand binding to a specific subunit, thus fully accounting for the rupture of salt bridges when the subunit shifts from the unliganded A state to the liganded B state (see earlier). In this respect, the stereochemical mechanism formulated by Perutz (*13*) and mathematically developed by Szabo and Karplus (*12*) appears closer to the sequential induced-fit model, favouring a tertiary origin for the Bohr effect and associating the rupture of salt bridges to the oxygenation of the corresponding subunit (see earlier). However, this experimental evidence of a pH-dependence of the first oxygenation step is not *per se* representing a support to the sequential-fit model, since it could in principle be accounted for by the two-state model also by implying that after the first binding step a perturbation of the allosteric equilibrium occurs (with a partial T → R transition of some tetramers), which is then associated to some H^+ release (and/or uptake). However, the extent of proton exchange associated to this process [$\Delta H^+_1 = \partial \log K_1/\partial pH$, see ref. *16*] is much larger than what predicted on the basis of T ↔ R allosteric shift after the first binding step (*12, 60*), implying some tertiary contribution to the Bohr effect. Therefore, as a whole the pH-dependence for the first binding step indeed appears contradictory with respect to the simplest formulation of the two-state allosteric model (*6*), favouring slightly more elaborate models, such as the Perutz–Szabo–Karplus (*12*) or the cooperon model (*18*).

On the other hand, structural evidence has been building up in the last few years, supporting the idea that ligand binding without a quaternary structural change does not induce the rupture of the salt bridges (*61*), and this is confirmed by the observation that no pH-dependence can be detected for O_2 binding to Hb in the crystal, where no T → R quaternary transition takes place (*37*).

As a matter of fact, there is a clear contradiction between (i) the necessity of the quaternary conformational transition for the pK_a shift of residues involved in salt bridges (on one side) and (ii) the pH-dependence of the first O_2 binding step with the impossibility to attribute it to simply a shift in the allosteric equilibrium after the first binding step in solution. Thus, point (i) seems to rule out the applicability of the sequential induced-fit mechanism as well as the Perutz–Szabo–Karplus model whereas point (ii) clearly indicates the impossibility to describe the pH-dependence according to a simple form of the MWC two-state model. As a whole, the linkage between O_2- and H^+-binding demands the occurrence of multiple energy levels (corresponding to various functionally different tertiary states) within the T quaternary conformation, which can be populated to a different extent under various experimental conditions of pH (thus displaying a different functional behaviour). This hypothesis may reconcile these contradictory results, also accounting for observation on Hb locked in the T-state by encapsulation in wet porous silica gels, where a small Bohr effect can be observed (*62*), and in crystals, where the removal of salt bridges by mutation enhances the O_2 affinity (*63*). It must be pointed out that this interpretation, which seems to support the existence

of a third state (*9*), could also be compatible with a dimeric cooperon where in the T quaternary state a ligand-linked pK_a shift is associated to an intermediate ligation step (i.e., τ_{01}), triggering a tertiary structural change in the unliganded subunit of the dimeric unit (or cooperon) [see also ref. (*64*)].

The possible existence of multiple tertiary (and/or quaternary) structures can be also envisaged for the R liganded state, as indicated by the observation that the dimer–dimer interface of liganded Hb has a wide range of energetically accessible structures, which are related to each other by simple sliding motion (*29*). This possibility, which has been actually observed at moderately acid pH for human Hb (*26*) and acknowledged as the R_2 quaternary structure, might be responsible for the observed pH-dependence of the O_2 dissociation kinetics from the liganded form (*65*), also because in solution the quaternary structure of Hb appears to be an equilibrium between the two quaternary structures R and R_2 (*27*). This observation, which postulates the existence of a Bohr effect also for the R-state, could reflect a linkage between H^+ binding and the quaternary equilibrium between the two quaternary states mentioned earlier, as mediated by the protonation of a histidyl residue [likely His97β, which is involved in the $\alpha_1\beta_2$ interface, see Fig. 3 in ref. (*26*)], which facilitates the conformational transition. It may be interesting to observe that this transition can also be accounted for by the cooperon model, implying a pK_a shift only when the dimeric cooperon is fully saturated by the ligand.

CONCLUSIONS

The wealth of information reported in this review indicates that indeed the overall cooperative ligand binding behaviour of human Hb can be accounted for by a two-state allosteric model (*6*), even though a more detailed look at the structural–functional data unravels in a quite convincing way that some cooperativity is present within each quaternary conformation (in particular the T-state), envisaging the possibility of ligand-linked tertiary structural changes that modify the original affinity constants even without triggering a quaternary structural transition. The presence of manifold energy levels becomes even more evident in the presence of heterotropic effectors (such as H^+), leading to a wide variety of values for affinity constants. This behaviour has been tentatively synthesized in a "global allostery" model (*66*), which tries to describe the cooperative mechanism as a network of several ligand-linked tertiary structural changes within a general framework of two quaternary conformations. However, it emerges in a clear-cut fashion the existence of two main deep energy minima for the quaternary structure of human Hb, and this in spite of (i) the possibility of several shallow wells around each minimum, corresponding to different tertiary structural arrangements, and (ii) a splitting of the minimum of the R quaternary structure, corresponding to the R_2 conformation (*26*). Less clear is the energy diagram of the different energy levels within the T-state minimum, since at different ligation stages the ligand distribution among the four sites might correspond to somewhat different energy levels, underlying a nonuniform shape for the energy surfaces of these intermediate species. In this respect, the possibility of some intradimer cooperativity is very fascinating, since it could identify the dimer as the functional unit for cooperativity, as postulated by the observations on the ligand binding mechanism of the homodimeric Hb from *Scapharca inaequivalvis* (*67–70*) and of the homodimeric Mb from *Nassa mutabilis* (*71*). Thus, in these cases cooperativity occurs as a direct heme-heme interaction, following an induced-fit sequential mechanism (*10*) in a single dimeric cooperon (*18*), arising the question whether different thermodynamic modelling reported in this review simply refers to different levels of complexity of the interaction networks operative in an allosteric macromolecule (*72*).

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