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# A COOPERATIVE MODEL FOR LIGAND BINDING TO BIOLOGICAL MACROMOLECULES AS APPLIED TO OXYGEN CARRIERS

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This paper presents a model describing the thermodynamics of cooperative ligand binding to multimeric biological macromolecules and integrating some of the features of the two-state and induced-fit models. The protein is taken to be partitioned into a number of noninteracting functional constellations, each one existing in two possible quaternary conformations. Furthermore, the model postulates that a functional constellation is organized in several subsets of sites (called cooperons), in which subunits interact according to an induced-fit mechanism. In the present version the number of subunits forming a cooperon has been limited to two and the total number of parameters used for fitting experimental data is four, all having a precise physical meaning. Although the present application is limited to oxygen-carrying proteins (hemoglobins, hemocyanins, erythrocruorins), the model appears suitable to describe other biological macromolecules with functional interactions.

## 1. Introduction

Cooperative ligand binding to oligomeric proteins has elicited the formulation of models to account for the thermodynamic and kinetic properties of this phenomenon, which is basic to regulation in functional macromolecules. Among these, two theoretical descriptions of cooperativity have been more extensively employed and discussed, i.e., the allosteric two-state model (MWC) [1] and the sequential induced-fit model (KNF) [2]. They represent extreme cases of a more general mechanism in which all possible states of a functional macromolecule are considered [3,4].

The MWC model postulates the existence of an equilibrium between only two quaternary structures of the macromolecule at every degree of ligation, including the unliganded form; moreover, within each quaternary state ligand binding to one site does not affect the affinity of the remaining subunits. Given some initial conditions (i.e., relative stability of the two quaternary states and relative ligand affinity), cooperative ligand binding is related to the displacement of the quaternary equilibrium in favor of the higher affinity R state, which is stabilized by ligand binding.

The KNF model assumes that each subunit can exist in two different tertiary states but that the ligand-bound tertiary state is only induced by the ligand itself. Therefore, the observed binding constant reflects the energy necessary for the transition to the liganded tertiary structure of the subunit. Moreover, and differently from the MWC model, a liganded subunit affects the energy of the transition in the neighboring subunits, and thus the apparent binding constant, as more sites are present in the liganded form. Obviously, in such a description the geometrical relationships among subunits are important in defining how cooperativity is expressed and in their original paper Kosh-

land et al. [2] indeed evaluated several possible interaction networks.

Although both models can account to a first approximation for the thermodynamic and kinetic properties of ligand binding to several oligomeric proteins, for hemoglobins and hemocyanins the MWC model has been more widely used although a rigorous and quantitative description of the data has required modifications of the original formulation. Thus, the models developed by Szabo and Karplus [5] and by Minton and Imai [6] were proposed to account for the variation in the observed ligand affinity of either one of the two quaternary conformations of hemoglobin induced by effectors (like $H^+$ and/or 2,3-diphosphoglycerate), not otherwise explained by the original two-state MWC model. Likewise, the formulation of a model by Ogata and McConnell [7], taking into account a functional heterogeneity between the two types of hemoglobin subunits, was required to describe spin-label EPR studies.

Moreover, NMR studies on hemoglobin valency hybrids and on hemoglobins M (i.e., in which the hemes of either the $\alpha$- or the $\beta$-chains are in the ferrous form whereas the partners are in the ferric state) have provided experimental data which cannot be accounted for completely by either the MWC or the KNF models [8–11].

In spite of these limitations, the MWC model has been extended to the analysis of the functional properties of other multimeric oxygen carriers, such as hemocyanins and erythrocruorins; applications to these systems, carrying many $O_2$-binding sites, has led Colosimo et al. [12] to suggest modifications in view of the impossibility of describing ligand-binding isotherms assuming that the whole macromolecule (sometimes containing over 100 binding sites) is acting in a concerted cooperative fashion. Therefore, the concept of 'functional constellation' was introduced, postulating that in very large ('giant') macromolecules the concerted structural change is restricted to a group of subunits constituting the functional constellation [12]. Such a model, which divides arbitrarily the macromolecule into a number of functional constellations, physically interacting but functionally independent, has proven to be useful and stimulating, although it appears to suffer from difficulties when applied extensively; for example, the size of a functional constellation for the same molecule had to be arbitrarily varied in order to account for a complete set of data obtained under different experimental conditions (pH, anions or cations, temperature) [13,14].

A very general model, including interactions between tertiary and quaternary structural changes has been proposed previously (see ref. 15). In this paper we report on a model, characterized by some of the features of both the MWC and KNF models, which thus represents in some way a blend of the two original models. On the basis of appropriate assumptions, this model provides a reasonable and quantitative description of available experimental data on the binding properties of hemoglobin, hemocyanins and erythrocruorins, and allows one to circumvent arbitrary changes in the interaction geometry, which appeared necessary in the application of the original MWC extension to giant macromolecules [12]. At this preliminary stage, neither functional inequivalence of the subunits nor a ligand-linked subunit dissociation has been taken into account. In view of these simplifications, which may not be fully justified, we shall not attempt to formulate a very detailed model description of the ligand-binding features to a specific macromolecule, as recently proposed for human hemoglobin [16,17]. Therefore, in this paper we have focussed our attention on a limited set of already published ligand-binding data from different $O_2$ carriers, for which a quantitative analysis according to simpler models [1,2] required some arbitrary assumptions. To us, it appears that this model has a wider applicability in the description of the thermodynamic properties of different respiratory proteins, already in the simplest of its possible versions. Analysis of the experimental data was carried out on an HP 87 desk-top computer using a nonlinear least-squares fitting program which makes use of a Marquardt algorithm [18]. All parameters but $\delta_R$ and the number of cooperons in a functional constellation ($z$, see below) were allowed to float freely. $\delta_R = 1$ was imposed exclusively to speed up the fitting procedure, since, even when it was allowed to float freely, its value was not significantly different ($\pm 10\%$) from 1.0. The number of cooperons in a

functional constellation was fixed to the highest value which provided the best fit for a complete set of data, without imposing any structural constraint.

## 2. General features of the model

Functional interactions among ligand binding sites may be seen at different levels of complexity in a large macromolecule, which can be considered as being composed of one or more functional constellations [12], which may interact. In the absence of a ligand, any functional constellation can exist in only two quaternary states, $T_0$ and $R_0$, in equilibrium, $L_0 = [T_0]/[R_0]$; given that $K_R \neq K_T$, the equilibrium constant between the two quaternary conformations will be a function of the saturation degree. Within a functional constellation, the subunits are segregated into smaller functional units, called 'cooperons'; the number of subunits in a cooperon is called the 'valency' of the cooperon. Ligand binding to one subunit may affect the pairwise interactions only with the other subunits in the cooperon and different interaction geometries are possible when the valency of the cooperon is greater than 2. On the other hand, cooperons interact following a concerted mechanism depending only on the quaternary structure of the whole functional constellation.

In the present version the following simplifications have been applied:

(i) the macromolecule exists in only one state of aggregation and is formed by $m$ identical and noninteracting functional constellations;

(ii) functional equivalence of the subunits in the cooperon is assumed;

(iii) all the cooperons are dimeric (valency = 2), the simplest possible choice *;

(iv) two possible tertiary structures are postulated for the T state ($t_0$ and $t_1$) as well as the R state ($r_0$ and $r_1$);

(v) both the $t_0 \rightarrow t_1$ and $r_0 \rightarrow r_1$ transitions are ligand-linked, following the induced-fit mechanism [2].

From these assumptions the binding polynomial [19] of the functional constellation in the T state is

$$P_F^T(x) = \left(\tau_{00} + 2\tau_{01}K_t x + \tau_{11}K_t^2 x^2\right)^z \qquad (1)$$

where $x$ is the ligand activity, $z$ the number of cooperons which form the functional constellation, $K_t$ the intrinsic affinity constant of the T state and $\tau_{00}$, $\tau_{01}$ and $\tau_{11}$ the stabilization factors ** of the dimer configurations (0, empty site; 1, liganded site), within the quaternary T state.

Similarly, for the R conformation the binding polynomial of a functional constellation is

$$P_F^R(x) = \left(\rho_{00} + 2\rho_{01}K_r x + \rho_{11}K_r^2 x^2\right)^z \qquad (2)$$

where $K_r$ is the intrinsic constant of the R state and $\rho_{00}$, $\rho_{01}$ and $\rho_{11}$ the stabilization factors of the dimer configurations in the R quaternary state, the other symbols being as defined in eq. 1. Hence, the binding polynomial of the whole macromolecule can be written as

$$P_M(x) = \left\{ L_0\left(1 + 2K_T x + \delta_T K_T^2 x^2\right)^z + \left(1 + 2K_R x + \delta_R K_R^2 x^2\right)^z \right\}^m / \left(1 + L_0\right)^m \qquad (3)$$

where $m$ is the number of functional constellations in the macromolecule and the other five parameters in eq. 3 are related to the stabilization factors according to the following relationships:

$$L_0 = \left(\tau_{00}/\rho_{00}\right)^z;\ K_T = \left(\tau_{01}/\tau_{00}\right)K_t;$$

$$K_R = \left(\rho_{01}/\rho_{00}\right)K_r;$$

$$\delta_T = \tau_{11}\tau_{00}/\left(\tau_{01}\right)^2;\ \delta_R = \rho_{11}\rho_{00}/\left(\rho_{01}\right)^2$$

* It may be remarked that other cooperon valencies (e.g., trimeric, tetrameric, etc.) can be used if and when stringent structural and/or thermodynamic informations require it. In this paper we have refrained from doing so to avoid any unwarranted structural implication.

** Stabilization factors of the cooperon in a given quaternary conformation of the functional constellation (T or R) correspond to the Gibbs free energy levels of the different possible pairwise interactions among the subunits which form the cooperon. Thus, if $ij$ ( = 00, 01, 11) refers to the tertiary structures of two interacting subunits in a cooperon and $\alpha$ ( = $\tau$ or $\rho$) is related to the quaternary structure of the functional constellation (T or R, respectively):

$$\alpha_{ij} = \exp\left(-G_{ij}^{\alpha}/RT\right) \qquad \text{(eq. 1A)}$$

Table 1

Dependence of the parameters on the stabilization factors

+, increased; −, decreased; 0, unchanged.

| | | $L_0$ | $K_T$ | $\delta_T$ | $K_R$ | $\delta_R$ |
|---|---|---|---|---|---|---|
| $\tau_{00}$ | + | + | − | + | 0 | 0 |
| $\tau_{00}$ | − | − | + | − | 0 | 0 |
| $\tau_{01}$ | + | 0 | + | − | 0 | 0 |
| $\tau_{01}$ | − | 0 | − | + | 0 | 0 |
| $\tau_{11}$ | + | 0 | 0 | + | 0 | 0 |
| $\tau_{11}$ | − | 0 | 0 | − | 0 | 0 |
| $\rho_{00}$ | + | − | 0 | 0 | − | + |
| $\rho_{00}$ | − | + | 0 | 0 | + | − |
| $\rho_{01}$ | + | 0 | 0 | 0 | + | − |
| $\rho_{01}$ | − | 0 | 0 | 0 | − | + |
| $\rho_{11}$ | + | 0 | 0 | 0 | 0 | + |
| $\rho_{11}$ | − | 0 | 0 | 0 | 0 | − |

Thus, each state (T or R) is characterized by an observed equilibrium constant for the binding of the ligand ($K_T$ or $K_R$) and an interaction constant ($\delta_T$ or $\delta_R$) such that cooperativity may also occur within a single quaternary state, localized at the interface between the two subunits forming the cooperon.

It may be noticed that when $m = 1$ the occurrence of $\delta_T = \delta_R = 1$ leads to the MWC formalism [1].

Within this scheme, heterotropic ligands can display their effects on the functional properties of a macromolecule by altering the stabilization factors of the conformational arrangements in the cooperon.

The dependence of $L_0$, $K_T$, $K_R$, $\delta_T$ and $\delta_R$ on the stabilization factors is summarized in table 1 which illustrates, for simplicity, the variations of any one of the stabilization factors, keeping all the others constant. In this respect it should be noted that a pure quaternary heterotropic effect is obtained only when all the stabilization factors in a given state are equally affected by the heterotropic ligand.

## 3. Applications of the model

Although the model is more useful when applied to giant $O_2$ carriers, hemoglobin is briefly considered first because it is certainly the multimeric protein most extensively studied among those which display cooperative ligand binding. Within the nomenclature given above, hemoglobin can be considered as a single tetrameric functional constellation, composed of two dimeric cooperons. Therefore, the binding polynomial for hemoglobin is

$$P(x) = \left\{L_0\left(1 + 2K_T x + \delta_T K_T^2 x^2\right)^2 + \left(1 + 2K_R x + \delta_R K_R^2 x^2\right)^2\right\}/(1 + L_0) \quad (4)$$

Such a formulation makes it also possible to explain equilibrium and kinetic data on valency hybrids and on mutant hemoglobins M [8,9,11,20,21]. For these proteins, the quaternary state of the macromolecule has been shown not to be correlated to the percentage of liganded sites [8], in contrast with the KNF model, whereas the structure of the heme in ferric subunits seems to change on ligand binding to the ferrous subunit [11,20,21], which is in contradiction with the MWC model. However, these two sets of data can be all accounted for by the present model, which allows a ligand-induced variation of the pairwise interactions of the cooperon.

However, except for few intriguing results mentioned above and for the conceptual explanation of the heterotropic interactions reported in the preceding part of this paper, the characteristics of this model are such that in the application to a relatively small oligomeric protein, like hemoglobin, quantitative differences with respect to the original MWC model [1] become apparent only when a detailed analysis is carried out (to be published).

Therefore, in the following we want to show examples of application of this model to other oxygen carriers, such as hemocyanins and erythrocruorins, in order to illustrate its wider applications to oxygen carriers with very different structures. It is important to remark that, for all proteins and all conditions described by the model in this paper, there was no need for $\delta_R \neq 1$ in fitting the data, implying that only within the T state is the ligand binding of an individual cooperon significantly cooperative. Such a condition,

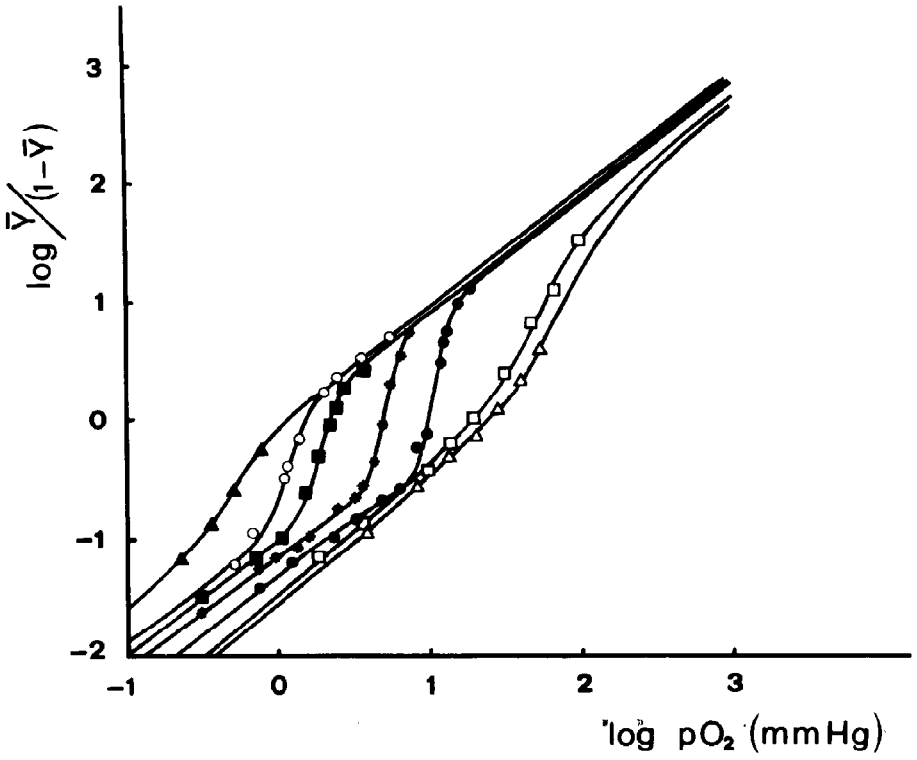


Fig. 1. Hill plots of the oxygen equilbrium curves of *H. pomatia* β-hemocyanin (from ref. 13). The continuous lines have been computed according to the model presented in this paper, using the nonlinear least-squares fitting program described in the text assuming a functional constellation of 16 binding sites (i.e., $z = 8$ dimeric cooperons). (▲) pH 7.00, (○) pH 7.04, (■) pH 7.10, (*) pH 7.18, (●) pH 7.40, (□) pH 8.00, (△) pH 9.00.

which seems to be common to all respiratory proteins analyzed in the present context, implies necessarily that the quaternary transition of the functional constellation brings about a variation of the interactions within individual cooperons.

Fig. 1 depicts the oxygen-binding data of *Helix pomatia* β-hemocyanin at several pH values ranging between 7.0 and 9.0. Previous analysis of these results [13] according to the model of Colosimo et al. [12] allowed definition of the dimensions of the functional constellation (15–16 $O_2$-binding sites) in this macromolecule containing 160 binding sites; nonetheless, in order to have a satisfactory description of the binding isotherms throughout the pH range (especially at pH ⩾ 8.0), the number of sites in a functional constellation had to be reduced to 8. With the present model it has been possible to fit the whole set of data using a constant number of sites (16) for a constellation ($z = 8$) (fig. 1). The pH dependence of the parameters, obtained from the fitting procedure, is reported in fig. 2a and b; although the pH range is too limited to obtain a $pK_a$ value for the proton-induced variation of each parameter, a monotonic change occurs for $K_T$, $\delta_T$ and $L_0$. As a consequence $\tau_{00}$, and possibly also $\tau_{01}$ and $\tau_{11}$, are affected by pH, but an almost absolute independence of pH for $\rho_{00}$ $\rho_{01}$ and $\rho_{11}$ is equally clearcut; thus, only the T state displays a heterotropic effect which is a mixed tertiary and quaternary effect (see table 1). It is worth mentioning that from fig. 2a the value for $\delta_T$ is $> 1$ at pH ⩾ 8.0, but becomes less than unity on lowering of the pH, suggesting a negative cooperativity for T state binding at pH < 8.0.

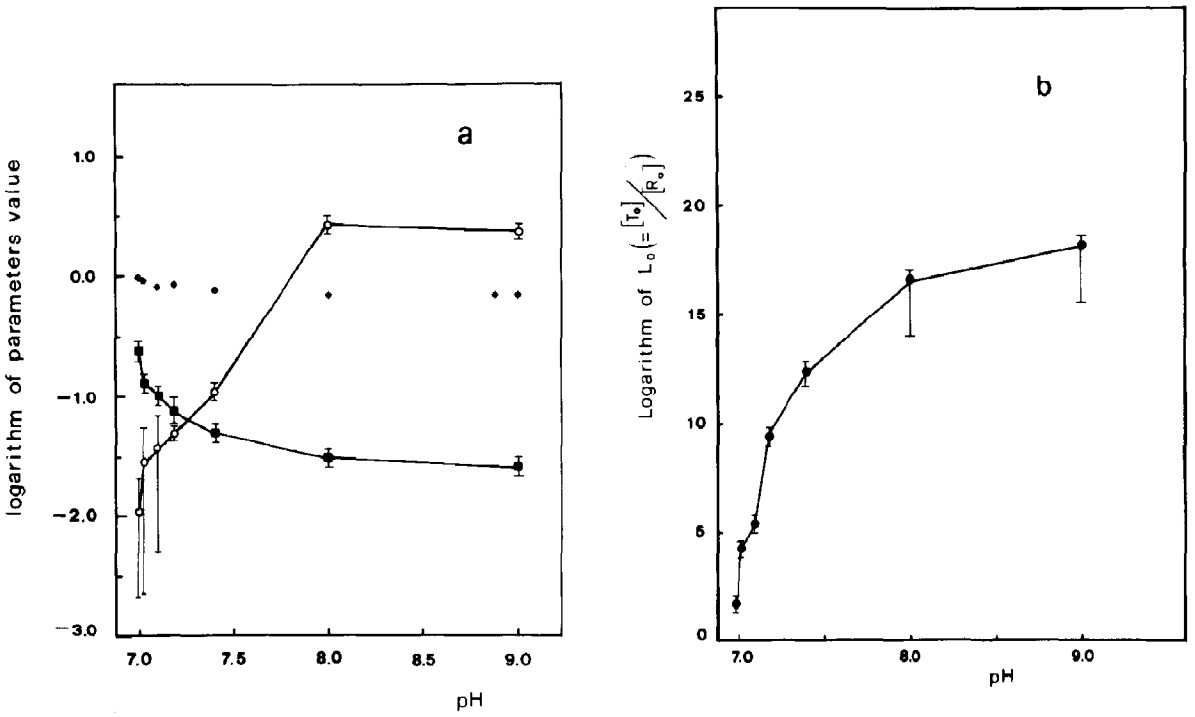


Fig. 2. pH dependence of the parameters, as obtained from the fitting reported in fig. 1. (A) (■) $K_T$ ($mmHg^{-1}$), (○) $\delta_T$, (*) $K_R$ ($mmHg^{-1}$). (B) $L_0$ ( $= [T_0]/[R_0]$). The bars refer to the standard deviations of the parameters from the fitting procedure and are not reported if the deviation is within the size of the corresponding symbol.

Application of the cooperon model to CO and oxygen binding to *Scylla serrata* hexameric hemocyanin [22] provides a rationale for available data and apparent discrepancies. Thus, crab hemocyanin from *S. serrata* displays a small but definite cooperativity ($n_H = 1.2$) in binding CO, at variance with most of hemocyanins investigated up to now, all of which show noncooperative CO-binding behavior [23]. Attempts to explain this result in the framework of the simple MWC model led to a value of $L_0$ very different from that obtained by the analysis of oxygen binding (see ref. 22) and thus a three-state model ($T_0$, $R_0$, $S_0$) was proposed [24]. In fig 3 the CO-binding data have been analyzed according to the cooperon model and the result is shown side by side with the analysis given by Decker et al. [22], a functional constellation of six sites ($z = 3$) being assumed in both cases. Although both fittings are virtually superimposable, our model provides a more rea-

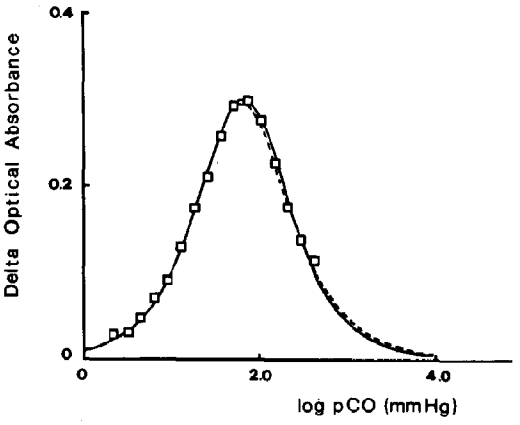


Fig. 3. Carbon monoxide binding to *S. serrata* hemocyanin followed by optical absorption (from ref. 23). (———) Computed best fitting according to the model presented in this paper (parameters reported in table 2). (- - - - -) Best fitting using the MWC model, as reported in ref. 23.

sonable account of the data because it makes use of the same allosteric constant $L_0$ as obtained from the oxygen-binding data [22] and can describe the results without having to imply a quaternary conformational change, in accord with the bridging hypothesis of Brunori et al. [25], but simply imposing a value of $\delta_T > 1$.

A last example we deem worth mentioning is represented by the data on the pH dependence of oxygen binding to *Octolasium complanatum* erythrocruorin, a protein containing 144 binding sites. In this case, similarly to *Helix pomatia* β-hemocyanin, analysis of the oxygen-binding data to this protein with the MWC model required an arbitrary variation of the number of sites forming the functional constellation in order to fit satisfactorily the data at different pH values [14].

In fig 4 the fitting of the data using the model described above and employing a functional constellation of 12 sites ($z = 6$) is reported, the resulting parameters being given in table 2. Within the errors, the value of $K_T$ is essentially pH-independent, which rules out any influence by proton binding in the pH range examined on $\tau_{00}$ and $\tau_{01}$ or implies the same variation on both parameters; therefore, the pH influence on $L_0$ and $\delta_T$ requires a proton-linked variation of $\rho_{00}$ and $\tau_{11}$, respectively, which is further confirmed by the pH dependence of $K_R$; it is worth outlining that a compensating variation of $\rho_{01}$ and $\rho_{11}$ is implied, since $\delta_R \neq 1$ was not required for fitting the data.

From such analyses on ligand-binding data to hemocyanins and erythrocruorins, as well as from the above interpretation of some results on modified hemoglobins, this model appears to be a useful and logical extension of the MWC and KNF models, having the potentialities to describe more satisfactorily larger sets of data under several different experimental conditions and with a limited number of parameters.

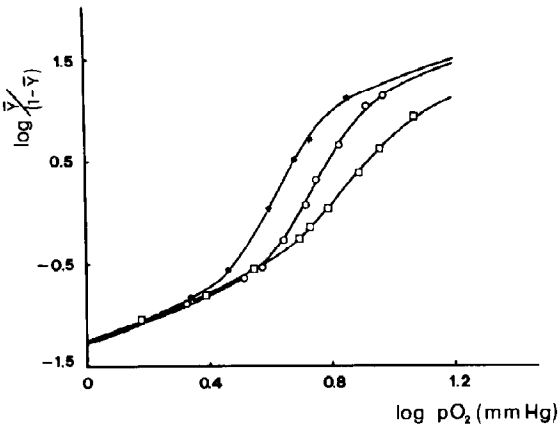


Fig. 4. Hill plots of oxygen binding to *O. complanatum* erythrocruorin. (□) pH 7.0, (○) pH 7.8, (*) pH 8.2. (———) Best fit, using a functional constellation of 12 sites (i.e., $z = 6$ dimeric cooperons), according to the model presented in this paper. The parameters obtained are reported in table 2.

Therefore, from this model it is possible to represent the ligand-binding behavior of largely different oxygen carriers referring to a common functional mechánism, as also recently proposed in qualitative terms [26,27].

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Table 2

Parameters obtained from fitting ligand-binding data on hemocyanins and erythrocruorins to the cooperon model

| | $K_T$ ($mmHg^{-1}$) | $K_R$ ($mmHg^{-1}$) | $L_0$ ( = $[T_0]/[R_0]$) | $\delta_T$ |
|---|---|---|---|---|
| *S. serrata* hemocyanin + CO | | | | |
| pH 8.0 | 0.01 | 0.01 | 5000 | 2.3 |
| *O. complanatum* erythrocruorin + $O_2$ | | | | |
| pH 7.0 | $4.05(\pm 0.1) \times 10^{-2}$ | $1.00 \pm 0.08$ | $8.87(\pm 0.35) \times 10^{8}$ | $8.1 \pm 0.54$ |
| pH 7.8 | $4.29(\pm 0.09) \times 10^{-2}$ | $1.88 \pm 0.29$ | $1.38(\pm 0.38) \times 10^{11}$ | $5.3 \pm 0.8$ |
| pH 8.2 | $4.66(\pm 0.21) \times 10^{-2}$ | $2.05 \pm 0.36$ | $3.22(\pm 2.15) \times 10^{10}$ | $5.3 \pm 0.7$ |

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