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Cooperativity: a unified view

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Abstract

Cooperativity, the departure from hyperbolic behaviour of the fractional saturation of a receptor at equilibrium (Y) for different values of ligand concentration (L), is an essential property of many physiological mechanisms and a first clue to the existence of conformational transitions and allosteric interactions. Here we investigate the properties of a simple and sensitive procedure to test and quantify cooperative behaviour. The measure of cooperativity involved is $\kappa = \mathrm{d}K(L)/\mathrm{d}L$ where $K(L) = (1 - Y)\ L/Y = [\text{free sites}]L/[\text{occupied sites}]$ is called the 'global dissociation quotient' Cooperative behaviour appears when $\kappa \neq 0$, i.e., $K(L)$ is a function of L. We have shown, for several equilibrium models of cooperative behaviour (e.g., Monod–Wyman–Changeux and Koshland–Némethy–Filmer), that $K(L)$ can be expressed as the weighted average of the microscopic dissociation constants (K_i) where the weights are the corresponding fractions of occupied sites (X_i), $K(L) = \sum K_i X_i$. As a consequence, the change in the global dissociation quotient with ligand concentration for a dimer is $\kappa = (K_1 - K_2)\mathrm{d}X_1/\mathrm{d}L$. This result shows that the quantitative importance of a cooperative behaviour in a dimer depends on two factors: (i) the difference of the microscopic dissociation constants of the sites and (ii) the change in the fraction of occupied sites with ligand concentration. We analyze the generality of this unified view concluding that it would be fulfilled by every equilibrium model where there is a one-to-one relationship between free and occupied sites.

Keywords: Cooperativity; Hill number; Koshland–Némethy–Filmer model; Microscopic constant; Monod–Wyman–Changeux model

1. Introduction

A century ago, findings such as the Bohr effect showed that cooperativity was an essential property for respiratory function. In the following decades, methods were developed to analyse this phenomenon. In particular, the Hill number used as a model-free measure of cooperativity was one of the useful tools. Mechanistically, the phenomenon for a long time has been associated with large changes in the structure of the protein known as conformational transitions [1].

More recently, the discovery of the allosteric interactions was one of the most important scientific events in the biochemical sciences [2] (for historical accounts see Judson [3] and Debru [4]). The fact that the activity of enzymes could be affected by metabolites not chemically related to their substrates and products opened new challenges. On the cellular level, it changed our perception of metabolic functioning and regulation. On the molecular level, many questions emerged on the problem of the structure–function relationship which is still only partially understood.

Many authors have contributed to a general theory of cooperative events in biological macromolecules.

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Among these approaches, and in addition to the results reviewed by J. Wyman and S.J. Gill in *Binding and Linkage* [1], we would like to mention the physical description given in the comprehensive book by T.L. Hill [5] and the work of M. Eigen related to the relaxation behaviour of cooperative and allosteric systems [6].

Conformational transitions represent the main physical basis common to cooperative and allosteric phenomena. Therefore, the experimental finding of cooperativity is relevant not only for its own implications but also as a first clue to the existence of conformational changes and allosteric interactions. The quantity most extensively used to quantify cooperative behaviour is the Hill number, alternative measures of cooperativity being seldom used. Here we will recall a simple and sensitive procedure to test and quantify cooperative behaviour [7,8]. We have previously shown that the quantity involved in this procedure, which we call 'global dissociation quotient', is directly related to the Hill number and has an intuitive meaning [9].

Based on the 'global dissociation quotient', in the present work we develop a phenomenological approach that constitutes the basis for a unified microscopic view of cooperativity. This approach is model-free and, as we shall see, applies to very different underlying mechanisms.

2. Cooperativity: an experimental phenomenon

Cooperativity can be unambiguously defined by reference to an experimental procedure. The relevant quantity to measure in a binding experiment is the fractional saturation of the receptor at equilibrium (Y = occupied sites/total sites) for different values of free ligand concentration (L). We say that the binding of a ligand to a protein is not cooperative if, under the particular experimental conditions, the plot Y vs. L is a section of a rectangular hyperbola (Fig. 1). Therefore, the phenomenon of cooperativity is synonymous to deviation from hyperbolic behaviour. To diagnose the existence of cooperativity, several ways to transform the hyperbola into a straight line have been proposed. Three widely used in the scientific literature are: (a) Lineweaver–Burk plot ($1/Y$ vs. $1/L$), (b) Langmuir–Hanes plot (L/Y vs. L) and (c) Eadie–Hofstee plot (Y vs. Y/L). The last plot appears to be the most sensitive to deviations from linearity [10].

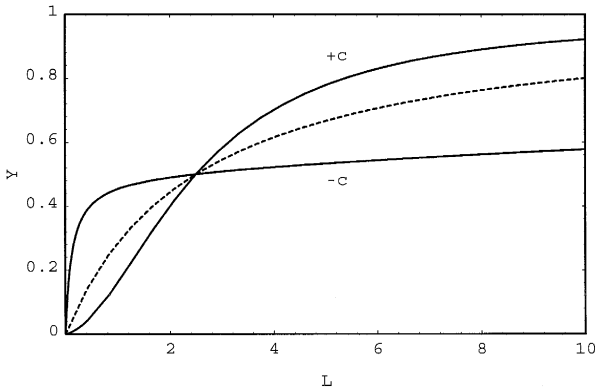

Fig. 1. Plot Y vs. L. Examples of hyperbolic curve (dashed line), positive cooperativity ($+c$) and negative cooperativity ($-c$).

3. A measure of cooperativity: the Hill number

Once the existence of cooperativity has been established, the following step is to determine its quantitative importance. The Hill number (h) is the measure of cooperativity most extensively used. Its prevalence is based on the fact that the Hill equation, i.e.

$$Y = \frac{L^h}{K + L^h} \tag{1}$$

with K and h constants, has been found to fit cooperative data well in the range of 10% to 90% saturation. This means that if we plot $\ln(Y/[1-Y])$ vs. $\ln(L)$ (i.e., the Hill plot), in that range, we obtain a straight line whose slope

$$h = \frac{\mathrm{d}\ln\left(\frac{Y}{1-Y}\right)}{\mathrm{d}\ln(L)} \tag{2}$$

is the Hill number. From the Hill equation we see that in the absence of cooperativity $h = 1$. $h > 1$ is called positive cooperativity and $h < 1$ negative cooperativity. The actual value of h is a quantitative measure of the extent of the cooperative behaviour. It is important to keep in mind that, although h is approximately constant in a wide range of L, strictly speaking, it depends on L (see Fig. 4A).

Historically, the Hill number was introduced in 1910 by A.V. Hill in an attempt to understand the sigmoid binding curve of oxygen to haemoglobin [11]. He suggested that such behaviour could be explained by a model where h molecules of ligand bind to the protein in a single step. A theoretical consequence of this hypothesis was that the fractional saturation and the ligand concentration would be related by the Hill equation where the Hill number would be equal to the number of binding sites. In fact, when this theory was applied to the oxygen binding curve of haemoglobin two interesting results were obtained. The Hill plot was a straight line as predicted by the theory (the data available at that time covered intermediate values of saturation only). In contrast, the value of the Hill number obtained was 2.8, although haemoglobin has four binding sites for oxygen. The first of these results supports the use of the Hill number as a model-free measure of cooperativity. However, the interpretation of the Hill number was obscured by its discrepancy with the number of binding sites.

4. An alternative measure of cooperativity

An alternative way to describe the deviation from hyperbolic behaviour is to use the equation [7,8] ([12], pp. 630–633):

$$Y = \frac{L}{K(L) + L}. \tag{3}$$

In the absence of cooperativity, $K(L)$ is constant (Y vs. L is a rectangular hyperbola). Cooperative behaviour is, therefore, equivalent to change in $K(L)$ with ligand concentration. Rearranging Eq. (3), we obtain:

$$K(L) = \frac{[1 - Y]L}{Y}. \tag{4}$$

By combining this result with the definition of Y, it is easily shown that the experimental value of $K(L)$ can be interpreted as [free sites]L/[occupied sites], and it has the same form as a dissociation constant. In the definition of $K(L)$ free sites and occupied sites are the sum of all the free sites and all the occupied sites of the system respectively. As a consequence, $K(L)$ usually depends on the ligand concentration, being constant only in the absence of cooperativity. Taking into account all these considerations we called $K(L)$: 'global dissociation quotient'. Similarly, the reciprocal of $K(L)$ $(1/K(L))$, the 'global association quotient', represents the overall affinity of the receptor for the ligand. Note that in what follows, we will continue to use the same symbol, $K(L)$, and the same name, global dissociation quotient, for the value calculated from the experimental data (Eq. (4)) and for its interpretation: [free sites]L/[occupied sites].

Eq. (4) can be used for diagnostic purposes. For instance, deviations from a horizontal straight line in the plot $[1 - Y]\ L/Y$ vs. L is a positive test for the existence of cooperativity. The same curve can also be used to evaluate the extent of the cooperative effect. We will use the slope of this curve (i.e., the derivative of $K(L)$ with respect to L, symbolized by κ) as a quantitative measure of cooperativity, i.e.

$$\kappa = \frac{\mathrm{d}K(L)}{\mathrm{d}L}. \tag{5}$$

κ equal, greater or smaller than zero will correspond to absence of cooperativity, negative and positive cooperativity respectively. Under this view, the phenomenon of cooperativity is equivalent to the change in the global dissociation quotient with ligand concentration.

It is important to remember that the Hill number and κ (Eqs. (2) and (5), respectively) can be calculated from a table of experimental values, Y vs. L, and are model-free measures of cooperativity. $K(L)$ is analogous, at least in its form, to a dissociation constant and the meaning of its derivative with respect to L (κ) is transparent. In the next section we will show that there is a close relationship between h and κ which makes it possible to assign to h an intuitive meaning.

5. On the meaning of the Hill number

The two measures of cooperativity described above, h and κ, are closely related. Working with Eqs. (2) and (4) we obtain [9]:

$$h - 1 = -C_L^{K(L)} \tag{6}$$

where

$$C_L^{K(L)} = \frac{L}{K(L)} \frac{\mathrm{d}K(L)}{\mathrm{d}L} = \frac{L}{K(L)} \kappa \qquad (7)$$

$C_L^{K(L)}$ can be interpreted as the relative change in the global dissociation quotient, divided by the relative change in the ligand concentration (when small changes are considered). Similarly, its opposite, $-C_L^{K(L)}$, represents the relative change in the global association quotient $(1/K(L))$ per relative change in the ligand concentration. In essence, $-C_L^{K(L)}$ describes how sensitive to relative changes in ligand concentration is the overall affinity of the system. The Hill number shares this meaning, the only difference being that in the Hill number the non-cooperative behaviour is positioned in one (and not in zero). Therefore, the main difference between the alternative measure of cooperativity, κ, and the traditional measure of cooperativity, h, is that the first one represents the absolute change in global dissociation quotient per absolute change in ligand concentration while the second one is related to the corresponding relative changes.

6. Models for cooperativity

Several models have been proposed to explain the molecular mechanisms responsible for the existence

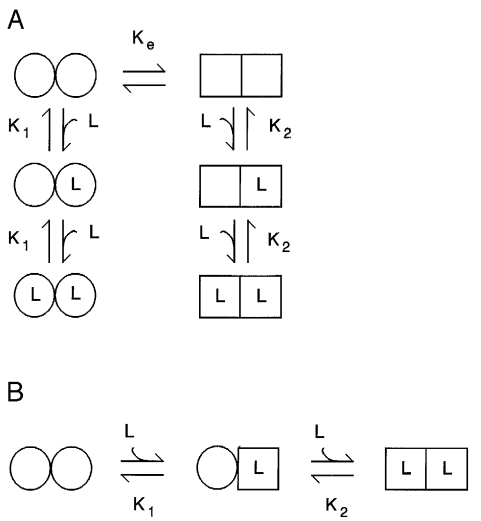

Fig. 2. Reaction schemes. (A) MWC dimer and (B) KNF dimer.

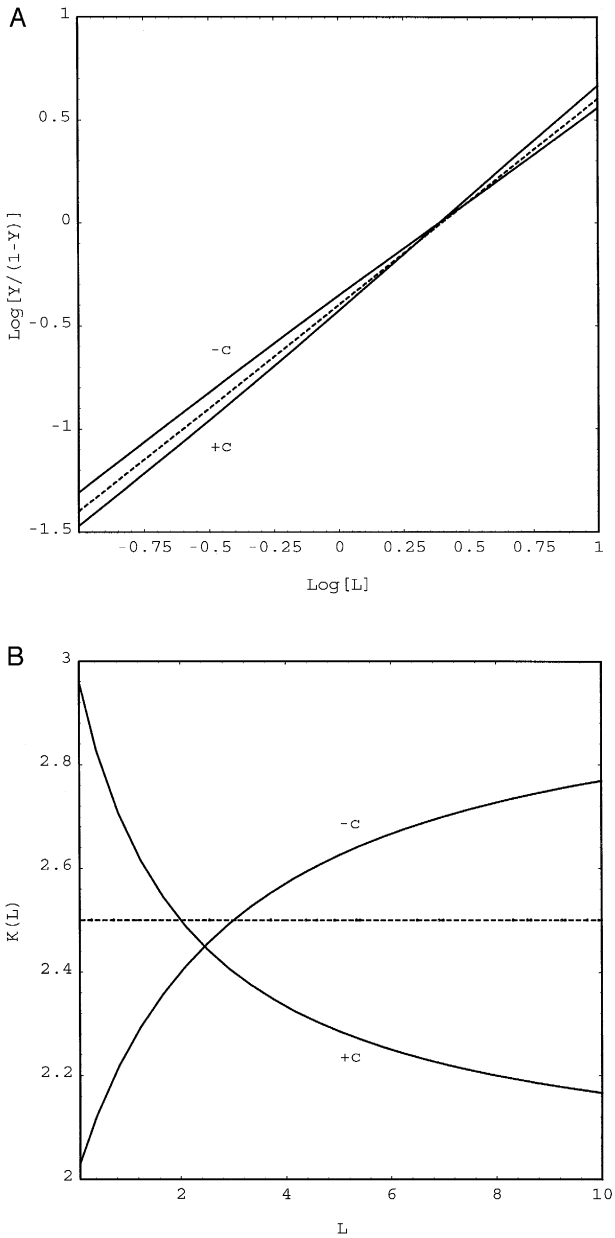

Fig. 3. Plots for diagnose of cooperativity. (A) Hill plots in the case of non-cooperative behaviour (dashed line), positive cooperativity (+c) and negative cooperativity (−c). (B) represents the global dissociation quotient as a function of ligand concentration for the same data as in (A). Note that $K(L)$ vs. L is a more sensitive test than the traditional Hill plot. The curves were generated with a KNF dimer (see Fig. 2B and Table 1) with the microscopic dissociation constants taking the following values: $K_1 = 2.5$ and $K_2 = 2.5$ (dashed line), $K_1 = 25$ and $K_2 = 0.25$ (+c), and $K_1 = 0.25$ and $K_2 = 25$ (−c).

of cooperative behaviours. Here we will consider two famous models: the concerted model of Monod, Wyman and Changeux (MWC) [13] and the sequential model of Koshland, Némethy and Filmer (KNF) [14]. The reaction schemes, in the case of a dimer, are given in Fig. 2 and the corresponding expressions for the fractional saturation (Y), the Hill number (h), the global dissociation quotient ($K(L)$) and its derivative with respect to L (κ) in Table (1). Two types of equilibrium constants are involved in these models: the microscopic dissociation constants of the ligand-binding site complexes (K_1 and K_2) and the constant for the transition between the two states of the protein (K_e). In Fig. 3 we give two plots, suggested above, for diagnostic purposes: $\ln(Y/[1-Y])$ vs. $\ln(L)$ and $K(L)$ vs. L. These plots exemplify a feature of practical importance, namely, $K(L)$ vs. L is a more sensitive test for the existence of cooperative behaviour than the traditional Hill plot. In addition, in Fig. 4, we plot the quantitative measures of cooperativity, h and κ, as a function of L. For high values of L, both measures indicate a decrease in cooperativity when L is increased. However, the analysis of the plots shows an important difference between them. The use of h results in the absence of cooperativity at low ligand concentration. In contrast, κ indicates that, at low values of L, cooperativity is maximum. This seeming contradiction is a consequence of the fact that h measures relative changes and κ absolute changes. For very low L, the relative change in L is very large and therefore the relative change in $K(L)$ per relative change in L ($C_L^{K(L)}$) is very small. In this situation, h is approximately equal to one (see Eqs. (6) and (7)) even if κ could be large. A valid question at this point is: is the system cooperative at very low L? If we are interested in the binding behaviour produced by relative changes in L, h is the relevant measure of cooperativity and the system is not significantly cooperative at low L. On the other hand, if we want to quantify the effect of absolute changes in L, the appropriate measure of cooperativity is κ which is maximum at low L.

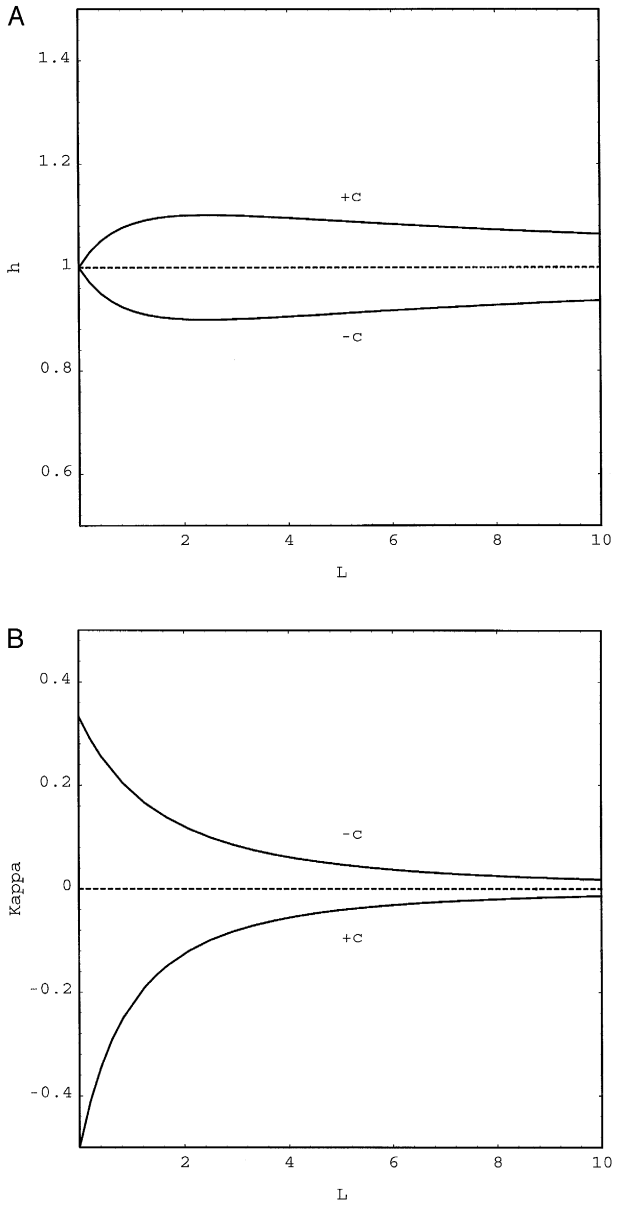

Fig. 4. Quantitative measures of cooperativity. (A) represents h vs. L and (B) represents κ vs. L in the case of non-cooperative behaviour (dashed line), positive cooperativity ($+$c) and negative cooperativity ($-$c). The curves were generated as in Fig. 3.

7. A unified view of cooperativity

The models for cooperativity described above are based on very different molecular mechanisms. The MWC model relies on the existence of two forms of the free oligomeric protein in equilibrium. In contrast, the KNF model postulates that in the absence of ligand there is only one form of free protein and that the binding of the ligand to one site of it affects the binding properties of the adjacent sites. According to these models there are several mechanistic explana-

tions for the phenomenon of cooperativity. Is it possible to summarize in a single idea the diversity of these mechanisms? Is there a unified view of cooperativity? We think that these questions have a positive answer.

In the two models under consideration $K(L)$ can be expressed as follows:

$$K(L) = K_1 X_1 + K_2 X_2 \tag{8}$$

K_1 and K_2 are the microscopic dissociation constants of the two sites. X_1 and X_2 are the fractions of occupied sites with dissociation constants K_1 and K_2 respectively (i.e., X_i = [occupied sites with dissociation constant K_i]/[total occupied sites], $i = 1,2$). These fractions satisfy: $X_1 + X_2 = 1$ and, therefore, $K(L)$ can be interpreted as the 'weighted average' of the microscopic dissociation constants. The validity of Eq. (8) for MWC and KNF models can be tested using the properties given in Table 1.

Now we can calculate a general expression for κ. Differentiating and combining Eq. (8) and $X_1 + X_2 = 1$, we obtain:

$$\kappa = \frac{\mathrm{d}K(L)}{\mathrm{d}L} = [K_1 - K_2]\frac{\mathrm{d}X_1}{\mathrm{d}L} \tag{9}$$

This is a central result. It tells us that the quantitative importance of a cooperative behaviour in a dimer depends on two factors: (1) the difference of the microscopic dissociation constants of the sites and (2) the extent of the change in the fraction of occupied sites with ligand concentration. The existence of two types of sites with different dissociation constants is not a sufficient condition to generate cooperativity. It is also necessary that when L is changed the fraction of occupied sites changes significantly. There is an important difference between the KNF model and the MWC model regarding the factor $\mathrm{d}X_1/\mathrm{d}L$ in Eq. (9). In the first model, the cooperativity (described by κ) can only be abolished by increasing ligand concentration while, in the second one, it is also suppressed at very high or very low values of K_e, the constant for the transition between the two states of the protein. For these extreme values of K_e, $\mathrm{d}X_1/\mathrm{d}L$ will approach zero and the protein will show hyperbolic behaviour for all values of L, even if it has two sites with very different microscopic dissociation constants (see Table 1). The definition of cooperativity proposed by Forsén and Linse [15], namely the free energy coupling, is related to the first factor in Eq. (9). Since their measure of cooperativity only includes one of the two aspects involved in a cooperative behaviour (in a dimer) it does not allows a full description of the phenomenon as traditionally defined.

Table 1
Properties of the Monod–Wyman–Changeux (MWC) and Koshland–Némethy–Filmer (KNF) dimers

Property	Model	
	MWC	KNF
Y	$\dfrac{\left[K_2^2(K_1+L)+K_eK_1^2(K_2+L)\right]L}{K_2^2(K_1+L)^2+K_eK_1^2(K_2+L)^2}$	$\dfrac{(K_2+L)L}{L^2+2K_2L+K_1K_2}$
h	$1+\dfrac{K_eK_1K_2(K_1-K_2)^2L}{\left[K_2(K_1+L)+K_eK_1(K_2+L)\right]\left[K_2^2(K_1+L)+K_eK_1^2(K_2+L)\right]}$	$1+\dfrac{(K_1-K_2)L}{(K_1+L)(K_2+L)}$
$K(L)$	$\dfrac{K_1K_2^2(K_1+L)+K_2K_eK_1^2(K_2+L)}{K_2^2(K_1+L)+K_eK_1^2(K_2+L)}$	$\dfrac{K_1K_2+K_2L}{K_2+L}$
κ	$-\dfrac{K_eK_1^2K_2^2(K_1-K_2)^2}{\left[K_2^2(K_1+L)+K_eK_1^2(K_2+L)\right]^2}$	$-\dfrac{K_2(K_1-K_2)}{(K_2+L)^2}$
X_1	$\dfrac{K_2^2(K_1+L)}{K_2^2(K_1+L)+K_eK_1^2(K_2+L)}$	$\dfrac{K_2}{K_2+L}$
$\dfrac{\mathrm{d}X_1}{\mathrm{d}L}$	$-\dfrac{K_eK_1^2K_2^2(K_1-K_2)}{\left[K_2^2(K_1+L)+K_eK_1^2(K_2+L)\right]^2}$	$-\dfrac{K_2}{(K_2+L)^2}$

The interpretation of the global dissociation quotient as the weighted average of the microscopic or elementary dissociation constants is valid for macromolecules with N different sites:

$$K(L) = K_1 X_1 + \ldots + K_N X_N = \sum_i K_i X_i \qquad (10)$$

We have demonstrated (see Appendix Section A.1) that in all the models whose fractional saturation can be put in the form of an Adair equation [16] — e.g., the MWC and KNF models for N subunits — $K(L)$ can be decomposed according to Eq. (10). The decomposition was also successful for models where the structure of the oligomeric receptor exhibits association–dissociation equilibrium (see Appendix Section A.2). The only requirements for the fulfillment of Eq. (10) appear to be that the system is at equilibrium and that the occupied sites are in a one-to-one relationship with the free sites from which they are formed by ligand binding. A more detailed discussion on the generality of the unified view of cooperativity is given in Appendix Section A.3.

The quantitative measure of cooperativity, κ, used above was based on the global dissociation quotient. Alternatively, we could have done a similar treatment in terms of the derivative of the global association quotient ($1/K(L)$) with respect to L (see also [25]). The advantage of using the global dissociation quotient, however, is that κ is a dimensionless quantity and therefore its value is independent of the units used.

8. Cooperativity in enzymes

The analysis given above was concerned with equilibrium situations. Enzyme catalysis is intrinsically a non-equilibrium process and requires a special treatment. The diagnose of cooperativity is done using the same plots that linearize the hyperbola (i.e., Lineweaver–Burk, Langmuir–Hanes and Eadie–Hofstee plots). For this purpose the fractional saturation at equilibrium (Y) is replaced by the steady-state initial velocity (v) and the ligand concentration (L) by the substrate concentration (S). To quantify the cooperative behaviour, the procedure usually applied requires, in addition, the measurement of the maximum velocity (V_m). h and κ, defined in Eqs. (2) and (5) respectively, can be calculated by substituting in those equations Y by v/V_m and L by S. The values obtained in this way are empirical measures of the departure from hyperbolic behaviour but, as we shall see, their interpretation is more difficult than in a binding experiment.

A general way to express the dependence of the velocity with the concentration of a substrate in the absence of products is:

$$v = \frac{V(S)S}{Q(S) + S} \qquad (11)$$

where $Q(S) = [\text{free sites}]S/[\text{occupied sites}]$ and $V(S) = (\sum k_i\ X_i)E_t$. k_i are the catalytic constants, X_i the fraction of occupied sites with catalytic constant k_i (i.e., $X_i =$ occupied sites with catalytic constant k_i/total occupied sites) and E_t the total concentration of sites (free plus occupied). Eq. (11) shows how to represent a general rate equation using two meaningful quantities: $Q(S)$ and $V(S)$. $Q(S)$ is equal to [free sites]S/[occupied sites] (i.e., the global dissociation quotient) but is not necessarily equal to the experimental value determined by $[1 - (v/V_m)]S/(v/V_m)$. This is the reason why, for the description of cooperativity in enzymes, we use two different notations: $K(S)$ for the empirical value and $Q(S)$ for the global dissociation quotient. On the other hand, the quantity between parentheses in the expression for $V(S)$ represents the weighted average of the catalytic constants and, therefore, $V(S)$ can be interpreted as the average maximum velocity. In the simplest case where $Q(S)$ and $V(S)$ are constants the system is not cooperative and the equation is of the Michaelis–Menten type [17,18]. Cooperativity — i.e., the deviation from hyperbolic behaviour — can be originated by changes in $Q(S)$, $V(S)$ or both. The first case is what Monod et al. [13] called K-system. It could be found when k_i has the same value (k) for all the occupied sites. As a result $V(S)$ is independent of S and equal to the maximum velocity V_m ($V(S) = k\ E_t = V_m$). In this case, the empirical value $K(S)$ is equal to $Q(S)$ and can be interpreted as a global dissociation quotient. Therefore, to be able to identify the value of $K(S)$ with $Q(S)$ we must demonstrate that $V(S) = V_m$. A piece of evidence in this direction could be to study the dependence of the maximum velocity with the concentration of an allosteric effector of the enzyme

(M). If when using saturating values of S the velocity does not depend on M then we can assume that we are working with a pure K-system. In the case of V-systems (for a well studied experimental system see [19]), $K(S)$ cannot be identified with $Q(S)$ and it is not possible to assign a general meaning to the values of κ and h.

In the theoretical ground, we have shown (Acerenza and Mizraji, unpublished results) that there are some steady-state models where $Q(S)$ cannot be expressed as the weighted average of the microscopic or elementary dissociation constants of the sites (see Eq. (10)). A possible scenario to explain this difficulty could be that the equilibrium constants are not the appropriate description for the steady-state dissociation properties. The development of a unified view of cooperativity that includes all non-equilibrium situations remains an open problem.

9. Final remarks

The study of cooperative and allosteric isolated proteins has given us a clearer picture of the conformational transitions and domain interactions taking place at the molecular level. Our present image of these proteins is closer to a complex molecular machine than to a small active site maintained in a large inert structure. At the cellular level cooperative and allosteric proteins have been found in virtually all functional domains. For instance, in the context of neural function, large conformational transitions have even been related to the consolidation of long-term memories ([1], p. 268). This ubiquity suggests that the peculiar binding and kinetic properties of cooperative and allosteric proteins would be essential to the phenomenon of life or, at least, would give the organism that possess them a notorious advantage. The type of advantage would vary with the biological function. For instance, the introduction of new allosteric interactions in a metabolic system confers to it the potential ability to respond in a qualitatively richer way to changes in the environment [20–22].

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Appendix A. On the generality of the unified view

The aim of this appendix is to analyse the generality of Eq. (10). This will be done in three steps. First, we will prove that Eq. (10) holds for the general Adair model. Secondly, we will show an association–dissociation model, not belonging to the general Adair form, that fulfills Eq. (10). Finally, we will uncover the general conditions required for the fulfillment of the unified view of cooperativity.

A.1. Adair cooperativity model

The Adair cooperativity model [16] assumes the existence of all the partly liganded intermediates. In a receptor with N sites for the ligand the total concentration of free sites (R_f) and the total concentration of occupied sites (R_o) are given by:

$$R_f = \sum_{i=1}^{N} (N-(i-1))[ML_{i-1}] \tag{12}$$

$$R_o = \sum_{i=1}^{N} i[ML_i]. \tag{13}$$

where L is the ligand concentration and $[ML_i]$ is the concentration of receptor bound to i molecules of ligand. The fractional saturation is:

$$Y = \frac{R_o}{R_f + R_o} = \frac{\sum_{i=0}^{N} i[ML_i]}{N\sum_{i=0}^{N}[ML_i]} \tag{14}$$

At equilibrium, the species $[ML_{i-1}]$ and $[ML_i]$ are related by the expression:

$$K'_i = \frac{L[ML_{i-1}]}{[ML_i]} \quad i = 1,\ldots,N. \tag{15}$$

where K'_i are the macroscopic dissociation constants. Applying Eq. (15) i times we obtain:

$$[ML_i] = c_i[ML_0]L^i \quad i = 1,\ldots,N. \tag{16}$$

where

$$c_i = \frac{1}{\prod_{j=0}^{i} K'_j} \quad i = 1, \ldots, N. \tag{17}$$

and $K'_0 = 1$. Substituting Eq. (16) into Eq. (14), we obtain the fractional saturation in terms of the macroscopic dissociation constants:

$$Y = \frac{\sum_{i=0}^{N} i c_i L^i}{n \sum_{i=0}^{N} c_i L^i} \tag{18}$$

The global dissociation quotient, $K(L)$, for the Adair model is given by:

$$K(L) = \frac{LR_f}{R_o} = \frac{L \sum_{i=1}^{N} (N-(i-1))[ML_{i-1}]}{\sum_{i=1}^{N} i[ML_i]} \tag{19}$$

Combining this equation with Eq. (15), we obtain:

$$K(L) = \frac{\sum_{i=1}^{N} (N-(i-1)) K'_i [ML_i]}{\sum_{i=1}^{N} i[ML_i]}. \tag{20}$$

The microscopic or intrinsic dissociation constants (K_i) are defined by:

$$K_i = \frac{N-(i-1)}{i} K'_i \tag{21}$$

where $(N-(i-1))/i$ is called the statistical factor. The expression for $K(L)$ in terms of the microscopic dissociation constants is:

$$K(L) = \frac{\sum_{i=1}^{N} K_i i[ML_i]}{\sum_{i=1}^{N} i[ML_i]}. \tag{22}$$

Finally, the last equation can be rearranged to the form:

$$K(L) = \sum_{i=1}^{N} K_i X_i \tag{23}$$

where

$$X_i = \frac{i[ML_i]}{\sum_{i=1}^{N} i[ML_i]}. \tag{24}$$

is the fraction of occupied sites with microscopic dissociation constant K_i. We conclude that in every model whose fractional saturation can be put in the form of an Adair equation (Eq. (18)) $K(L)$ can be decomposed according to Eq. (23) (i.e., Eq. (10)).

We would like to mention that the saturation function (Eq. (14)) can be expressed in terms of the binding polynomial (also called partition function) [23,1]. As a consequence, the relationship between $K(L)$ and the binding polynomial is readily obtainable. In addition, the work of Bardsley and colleagues shows that the factorizability of these polynomials is related to the type of cooperativity and also provides valuable information about site–site interactions [24,25].

Finally, we will make some comments on the meaning of the microscopic dissociation constants. Let us consider a receptor molecule where i of the N sites are bound to ligand. If these i occupied sites are identical their individual dissociation constants would also be identical and their values can be calculated using Eq. (21). On the other hand, if the occupied sites are different the dissociation constant of each of the i occupied sites in the molecule could in principle have a different value. In this last situation the microscopic dissociation constant defined by Eq. (21) could be expressed in terms of the i different 'elementary' dissociation constants corresponding to the i occupied sites. To exemplify this case we consider a KNF dimer with different subunits. The reaction scheme for this model is:

$$\begin{aligned} R_1R_2 + L &\rightleftharpoons LR_1R_2 & K_{11} \\ R_1R_2 + L &\rightleftharpoons R_1R_2L & K_{12} \\ R_1R_2L + L &\rightleftharpoons LR_1R_2L & K_{21} \\ LR_1R_2 + L &\rightleftharpoons LR_1R_2L & K_{22} \end{aligned}$$

where K_{11}, K_{12}, K_{21} and K_{22} are the elementary dissociation constants. It can be shown that, for the model under consideration, the expressions for the

microscopic dissociation constants K_1 and K_2 in terms of the elementary dissociation constants are:

$$K_1 = \frac{2K_{11}K_{12}}{K_{11}+K_{12}} \tag{25}$$

$$K_2 = \frac{K_{21}+K_{22}}{2} \tag{26}$$

The microscopic dissociation constants could therefore be interpreted as 'mean' values of the *i* elementary dissociation constants. In fact, in this particular example, K_1 is the harmonic mean between K_{11} and K_{12}, and K_2 is the arithmetic mean between K_{21} and K_{22}. It is important to note that while there is one microscopic dissociation constant associated to each macroscopic dissociation constant, there is in principle no upper limit to the number of different elementary constants.

A.2. Association–dissociation models

Frieden [26] and Nichol et al. [27] showed that the existence of association–dissociation equilibrium between the subunits of a receptor could generate a cooperative binding curve. Here we will show in a particular example that, although the fractional saturation of this type of models can not be put under the form of an Adair equation, the global dissociation quotient, $K(L)$, can be expressed as the weighted average of the microscopic dissociation constants. Let us consider the following reaction scheme:

$$\begin{aligned} 2M &\rightleftharpoons M_2 && K_e \\ M+L &\rightleftharpoons ML && K_1 \\ M_2+L &\rightleftharpoons M_2L && K_2 \\ M_2L+L &\rightleftharpoons M_2L_2 && K_2 \end{aligned}$$

where M represents the monomer, M_2 the dimer and L the ligand. K_e is the association constant of two subunits of monomer to give the dimer and K_1 and K_2 are the microscopic dissociation constants of the ligand from the monomer and the dimer respectively. After cumbersome calculations we obtained the expression for the fractional saturation:

$$Y = \frac{(1+\alpha)L}{K_1+L+(K_2+L)\alpha} \tag{27}$$

where

$$\alpha = \frac{K_1\left[\sqrt{\beta^2+2K_e m_t}-\beta\right]}{K_2} \tag{28}$$

$$\beta = \frac{K_2(K_1+L)}{2K_1(K_2+L)} \tag{29}$$

and m_t is the total concentration of sites. This fractional saturation is not a quotient of polynomials and, therefore, does not belong to the Adair type (Eq. (18)).

The global dissociation quotient defined in Eq. (4) is:

$$K(L) = \frac{K_1+K_2\alpha}{1+\alpha} \tag{30}$$

The fractions of occupied sites with microscopic dissociation constants K_1 and K_2 are:

$$X_1 = \frac{1}{1+\alpha} \tag{31}$$

$$X_2 = \frac{\alpha}{1+\alpha} \tag{32}$$

Combining Eqs. (30)–(32) it is immediate to show that $K(L)$, X_1 and X_2 satisfy Eq. (10).

A.3. General requirements for the fulfillment of Eq. (10)

In the first section of this appendix we have proven that Eq. (10) applies to all those equilibrium systems whose fractional saturation can be put under the form of an Adair equation. In the second section we showed that the validity of this relationship could be extended to association–dissociation models. Our aim in this last section is to show what are the general requirements for the fulfillment of Eq. (10).

The definition for the global dissociation quotient is:

$$K(L) = \frac{LR_f}{R_o} \tag{33}$$

where R_f and R_o are the concentrations of total free sites and total occupied sites respectively. We will assume that each type of occupied site decomposes to give a single type of free site and that each type of

free site combines with the ligand to give a single type of occupied site. This one to one relationship allows to assign a unique elementary dissociation constant to each type of occupied site. We will call $R_{o,j}$ the concentration of occupied sites with elementary dissociation constant K_j and $R_{f,j}$ the concentration of the corresponding free sites. At equilibrium we have:

$$K_j = \frac{R_{f,j} L}{R_{o,j}} \tag{34}$$

With this condition the concentration of total free sites can be expressed as follows:

$$R_f = \sum_j R_{f,j} = \sum_j \frac{K_j R_{o,j}}{L} \tag{35}$$

Substituting the last expression into Eq. (33) we obtain:

$$K(L) = \sum_j \frac{K_j R_{o,j}}{R_o} = \sum_j K_j X_j \tag{36}$$

We conclude that the fulfillment of Eq. (10) requires that the system is at equilibrium and that there is a one to one relationship between free sites and occupied sites.

The importance of the one to one relationship between free and occupied sites for the fulfillment of Eq. (10) can be illustrated with a simple example. Let us consider the reaction scheme:

$$R + L \rightleftharpoons C_1 \qquad K_1$$
$$R + L \rightleftharpoons C_2 \qquad K_2$$

where R represents the free receptor and C_1 and C_2 two forms of the occupied receptor. K_1 and K_2 are the dissociation constants of C_1 and C_2 respectively. This simple model does not fulfil Eq. (10), since:

$$K(L) = \frac{K_1 K_2}{K_1 + K_2} \tag{37}$$

$$X_1 = \frac{K_2}{K_1 + K_2} \tag{38}$$

$$X_2 = \frac{K_1}{K_1 + K_2} \tag{39}$$

and therefore

$$\sum_{j=1}^{2} K_j X_j = 2K(L) \tag{40}$$

Finally, it is important to remember that the number of discrete states that one includes in a reaction scheme is somehow arbitrary. In this sense, the scheme described above could be considered a simplified version of the more detailed scheme:

$$R_1 \rightleftharpoons R_2 \qquad K_e$$
$$R_1 + L \rightleftharpoons C_1 \qquad K_1$$
$$R_2 + L \rightleftharpoons C_2 \qquad K_2$$

The simplification from this scheme to the previous one could be based, for example, in the assumption that the interconversion of R_1 and R_2 is faster than all the other reaction steps [28]. Under this assumption, the free forms of the receptor, R_1 and R_2, can be fusioned in one form (R) obtaining a scheme with the same kinetic behaviour. In this situation one is tempted to apply a one-sided view of the 'principle of parsimony' in the sense of choosing the smallest of the two diagrams previously shown compatible with the steady-state kinetic measurements. However, the expanded scheme has the advantage that makes explicit the existence of the two different forms of free sites and is the simplest structural description. Importantly for our approach, the expanded diagram fulfills Eq. (10). If this type of expansion is applied to allow a one to one relationship between free sites and occupied sites (every time this one-to-one relationship is not satisfied) it appears that Eq. (10) will be fulfilled by all equilibrium receptors, cooperative and non-cooperative, without exceptions.

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