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On the Relation of Different Definitions of Cooperative Binding for Systems with Two Binding Sites

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Abstract

The concept of cooperativity is popular in the context of ligand binding. However, it has been shown that in the framework of the grand canonical ensemble, different established definitions of cooperativity do not coincide if systems with more than two binding sites are considered. This can cause problems if for instance the Hill coefficient is used to infer interaction energies, but also makes a conceptual environment-independent classification into "positive" and "negative" cooperativity difficult. Here, we derive formal proofs for the fact that for systems with only two binding sites, several of the considered definitions of cooperativity are indeed equivalent, and that all except for one criterion coincide, if the two sites are energetically identical. This work shall complement the currently re-emerging discussion on cooperative phenomena and highlight that insights gained by considering the frequently stressed two-binding-sites example may be restricted to this particular case.

1 Introduction

Cooperative phenomena in ligand binding have been recurrently discussed since the investigation of the binding behavior of oxygen to hemoglobin [4, 12, 13] and different equations and frameworks have been used to describe the binding of a ligand to its target molecule (for a review see for instance [28]). A standard framework is the setup of the

grand canonical ensemble in which equilibrium (or steady state) binding is considered and it is assumed that an uptake of a ligand by a target molecule does not reduce the thermodynamic activity of the ligand in the environment (no ligand depletion, see for instance [14] or [26]). This situation is approximately given if the number of ligand molecules is much larger than the number of target molecules in solution. A quantity which is often considered in the context of cooperative binding is the Hill coefficient which is defined by the slope of the Hill plot

$$H_\Psi(\log(\lambda)) := \log\left(\frac{\Psi(\lambda)}{n-\Psi(\lambda)}\right) \tag{1}$$

at half-saturation (Ψ the "overall titration curve" or "average occupation" or "isotherm"; λ the thermodynamic activity of the ligand; n the number of binding sites; H as a function of the natural logarithm of λ). It is widely known that in the setup of the grand canonical ensemble, the Hill coefficient is related to the variance of the number of occupied sites in the system under consideration (for instance [14]). Abeliovich [1] highlighted that for systems with independent identical subunits, the variance of the number of occupied sites is the variance of the binomial distribution, and that the slope of the Hill plot gives the ratio between the variance in the number of occupied sites of the system under consideration, and the binomial reference variance (see in this context also the work by Briggs [5, 6, 7] and Wyman and Gill [29] and their references). Consequently, if the slope of the Hill plot deviates at any ligand activity from the reference value 1, a considered system of identical binding sites has non-negligible interaction. This observation is conceptually important, since it emphasizes that not only the slope of the Hill plot at a fixed ligand activity such as at half-saturation or at the maximal variance is important, but that the whole slope function characterizes cooperativity.

This saturation-dependent boundary is unidirectionally also valid for systems with different, non-identical independent binding sites. More specifically, the variance in the number of occupied sites of any independent system is bounded by the variance of the binomial distribution with expected value Ψ. This means that with non-identical binding sites, a value of the slope of the Hill plot smaller than 1 does not necessarily imply interaction in the system, since this can also be a result of the sites being different (but independent). However, if the slope exceeds the threshold 1, indeed this means that the system cannot consist of independent sites [17]. This result has also been confirmed in a

different context, by the observation that the Hill coefficient of a system of independent sites is maximal when the sites are equal [10].

Analogously to the illustrated connections between the slope of the Hill plot, the variance in the number of occupied sites and stochastic dependence, we recently discussed the relation of several characterizations of cooperativity generally [17]. A main result was that not any pair of the considered definitions of cooperativity is fully equivalent for non-identical multiple-sites-systems. Thus, it may be difficult for instance to connect interaction energies of subunits to the Hill coefficient of the overall titration curve ([17]; Abeliovich recently discussed this connection for the case of identical subunits [2]).

Tangentially to this conceptual discussion on the relation of different definitions of cooperativity, and the fundamental question of whether a good unifying definition exists which also makes a quantification of cooperativity or at least its ligand-activity-independent classification in the two classes "negative" and "positive" possible, several papers of the last years have addressed other aspects of interacting binding sites. Interaction energies were addressed in the context of kinetics of ligand binding without ligand depletion [18, 19] or with ligand depletion [8]. Moreover, Ha and Ferrell [11] investigated response curves given by the number of fully occupied receptors (as a variant to considering the average occupation) for two-sites-systems and under conditions in which ligand depletion is possible. Other work addressed the equilibrium situation without ligand depletion but with two types of ligands [20, 21], the stochastic interpretation of results derived in the polynomial framework of the grand partition function [22] or limit behavior for very large systems [9].

In the context of recent discussions on cooperativity [27], we will complement results on the non-equivalence of different definitions of cooperativity in bigger systems, by formal proofs for several equivalences when systems with only two sites are considered (in the framework of the grand canonical ensemble, which means without ligand depletion). As a side effect, this work shall recall that insights gained from the repeatedly treated two-sites case, which is often also used to define cooperativity illustratively [15], are very limited and thus not suitable to understand different symptoms arising from binding site interaction in larger systems.

After having recapitulated the setup of the grand canonical ensemble, we formally prove equivalences of different definitions of cooperativity for the case of two binding

sites.

2 The grand canonical ensemble

To recapitulate the grand canonical ensemble, we follow the structure of the same section of our earlier work [17].

The grand canonical ensemble defines a parameterized set of probability measures on the microstates of a molecule which can exchange energy and particles (ligands) with its environment. Thus, the parameters are two quantities: temperature T and thermodynamic activity of the ligand λ (also called "ligand activity" in this text). For a molecule with n ligand binding sites, the set of microstates can be described by $\{0,1\}^n$, where for state $k = (k_1, ..., k_n) \in \{0,1\}^n$, the i-th entry k_i indicates whether site i is occupied (1) or not (0). The probability assigned to microstate k is then given by

$$P(\{k\}) := \frac{g(k)\lambda^{|k|}}{\sum_k g(k)\lambda^{|k|}}. \tag{2}$$

Here,

$$g(k) := \exp(-G(k)/RT) \tag{3}$$

is called the Boltzmann factor. Moreover, $G(k)$ denotes the free energy of the molecule in state k compared to a fixed, arbitrarily chosen reference state, R is the Boltzmann constant and $|k|$ the number of occupied sites in microstate k (the macrostate of microstate k). The overall titration curve is given by the average number of bound ligands as a function of ligand activity at constant temperature:

$$\Psi(\lambda) = \frac{n \cdot a_n\lambda^n + (n-1) \cdot a_{n-1}\lambda^{n-1} + ... + a_1\lambda}{a_n\lambda^n + a_{n-1}\lambda^{n-1} + ... + a_1\lambda + 1} \tag{4}$$

The denominator $\Phi(\lambda) = a_n\lambda^n + ... + 1$ is called the "binding polynomial" or the "grand partition function". Its coefficients a_i are the sum of all Boltzmann factors $g(k)$ with i occupied binding sites. The parameterization of the overall titration curve as in Eq. (4) is also called the "Adair equation" [3].

Before we restrict ourselves to the two-sites case, let us recapitulate some interesting general statements which we will use afterwards. For this, for any function $f(\lambda)$, let $f' := \frac{df}{d\log\lambda}$ denote its derivative with respect to the natural logarithm of λ. Then we have the following lemma.

Lemma 1 *a)* $\Psi = \frac{\Phi'}{\Phi}$

b) The variance of the number of occupied sites as a function of λ *is* $\mathbf{V}(\lambda) = \Psi'$.

c) The slope of the Hill plot is

$$\left(\log\left(\frac{\Psi(\lambda)}{n-\Psi(\lambda)}\right)\right)' = \frac{\mathbf{V}(\lambda)}{n\frac{\Psi}{n}\left(1-\frac{\Psi}{n}\right)}$$

which means it is the variance of the number of occupied binding sites in the system under consideration, divided by the variance of the binomial distribution with the same mean occupation.

Proof (Lemma 1)

a) Φ is a polynomial in λ. Its derivative is the sum of the derivatives of each monomial. For each monomial, a derivative with respect to $\log\lambda$ means we differentiate $\exp(i\log\lambda)$, which means the power i creates an additional factor i. Only the constant term 1 vanishes. Thus, Φ' is the numerator of Ψ.

b) A monomial of Φ divided by Φ gives the probability of the respective macrostate at the respective ligand activity. For instance $\frac{a_2\lambda^2}{\Phi}$ is the probability of being occupied with two ligands. This means that Φ'/Φ is the expectation and Φ''/Φ is the second moment of the distribution of the macrostates. With a), we have $\Psi' = \frac{\Phi''\Phi-\Phi'\Phi'}{\Phi^2} = \Phi''/\Phi-(\Phi'/\Phi)^2$, which is the second moment minus the squared first moment, which is the variance.

c) With b) we have $\left(\log\left(\frac{\Psi(\lambda)}{n-\Psi(\lambda)}\right)\right)' = \frac{n-\Psi}{\Psi}\frac{\mathbf{V}(\lambda)(n-\Psi)+\Psi\mathbf{V}(\lambda)}{(n-\Psi)^2} = \frac{\mathbf{V}(\lambda)}{n\frac{\Psi}{n}\left(1-\frac{\Psi}{n}\right)}$. ■

3 Characterizations of cooperative binding

We present some characterizations of cooperative binding behavior which can be found in literature and discuss their interpretations and relations for the case of n non-identical binding sites. The criteria are based on a macroscopic description, in particular on properties of the overall titration curve. For the case of two binding sites, we will add characterizations of cooperativity based on microscopic properties as well.

C1 The slope of the Hill plot at half-saturation is larger than 1.

C2 The maximal variance in the number of occupied sites is larger than $0.25n$.

C3 The slope of the Hill plot has a value larger than 1.

C4 The overall titration curve is sigmoidal in a linear plot.

C5 The binding polynomial of the system has a non-real root.

C1 is the most frequently used indicator of cooperativity, the slope of the Hill plot at half-saturation (or half-maximal response). T. L. Hill proposed to compare the maximal variance to $0.25n$ (C2) [14], which is the highest variance a binomial distribution can have for fixed n. C3 represents the criterion considered by Abeliovich [1] for systems with identical binding sites (as deviation from 1, which means larger or smaller than 1), and which also defines an upper bound for the variance (in the number of bound ligands) for general systems with non-identical independent sites [17]. Criterion C4 describes the sigmoidal shape which was observed in the context of oxygen binding to hemoglobin [4]. We illustrated that the appearance of non-real roots of the binding polynomial (C5), which has for instance been used by Briggs [5, 6, 7] or Onufriev and Ullmann [25], is a unifying definition [17].

Proposition 1 (Implications for general systems)

a) If a molecule satisfies C1, it also satisfies C2.

b) If a molecule satisfies C2, it also satisfies C3.

c) Satisfying C3 does not imply satisfying C4, nor vice versa.

d) If a molecule satisfies C3 or C4, it also satisfies C5.

Proof (Proposition 1)

a) With Lemma 1 c), C1 means $\mathbf{V}(\lambda) > 0.25n$ at half-saturation. Consequently C2 is fulfilled.

b) With Lemma 1 c), C2 states that at the point of maximal variance, the slope of the Hill plot is larger than 1, since the Binomial variance $n\Psi/n(1-\Psi/n)$ is smaller than or equal to $0.25n$.

c) See the counterexamples provided by Martini *et al.* [17].

d) The decoupled sites representation [23, 24, 25] illustrates that the overall titration curve of a binding polynomial can be interpreted as being generated by a system of independent binding sites with binding constants defined by the negative inverse of the roots of the polynomial. Thus, in the case of only real roots, a hypothetical system exists which consists of independent sites and which gives the same overall titration curve. C3 and C4 have been shown to indicate that the system under consideration cannot consist of independent sites [17]. In more detail, C3 was shown to imply for any system possessing the considered titration curve, that the sum of the covariances of its sites is positive (which means that there is no independent system with this titration curve). Moreover, regarding C4, it was shown that any system consisting of independent sites does not generate an overall titration curve of sigmoidal shape in a linear plot, which again implies that a sigmoidal shape in a linear plot indicates non-negligible dependence. ■

4 The two–sites case

Considering a molecule with only two binding sites, we are dealing with four different states

$$(0,0) \quad (0,1) \quad (1,0) \quad (1,1) \tag{5}$$

Choosing the energy level of state $(0,0)$ as reference, and with $g(k)$ of Def. (3), we can describe the system fully by $g_1 := g((1,0))$, $g_2 := g((0,1))$ and interaction $w := \frac{g((1,1))}{g_1 g_2}$. We will assume that all occupational states are possible, that is that g_1, g_2 and w are positive numbers (not infinity). Its overall titration curve is then given by

$$\Psi = \frac{2g_1g_2w\lambda^2 + (g_1+g_2)\lambda}{g_1g_2w\lambda^2 + (g_1+g_2)\lambda + 1} \tag{6}$$

and its binding polynomial is the denominator $\Phi = \underbrace{g_1g_2w}_{=:a_2}\lambda^2 + \underbrace{(g_1+g_2)}_{=:a_1}\lambda + 1$. Moreover, we use the notation $\mathbf{Cov}(k_1,k_2)$ for the covariance between the states of the two sites as a function of λ. The following lemma for systems with two identical sites will be helpful for proving some statements afterwards:

Lemma 2 (Covariance for identical sites)

a) For a molecule with two identical binding sites and negative interaction energy, which means $w > 1$, the covariance of the state of the sites is larger than 0, for any

positive ligand activity:

$$\mathbf{Cov}(k_1, k_2) > 0, \qquad \forall \lambda \ > 0.$$

Moreover, the covariance is maximal at half-saturation.

b) Analogously, for a molecule with two identical sites and $w < 1$,

$$\mathbf{Cov}(k_1, k_2) < 0, \qquad \forall \lambda \ > 0$$

and the covariance is minimal at half-saturation.

Proof (Lemma 2) a) With $g := g_1 = g_2$, some calculation gives

$$\mathbf{Cov}(k_1, k_2) = \mathbf{E}(k_1 k_2) - \mathbf{E}(k_1)\mathbf{E}(k_2) = \frac{g^2 w \lambda^2}{\Phi} - \frac{(g^2 w \lambda^2 + g\lambda)^2}{\Phi^2} = (w-1)\frac{g^2 \lambda^2}{\Phi^2}.$$

The second factor of the right-hand side is always larger than zero, which gives the first statement. To show that the covariance has a maximum at half-saturation, we calculate the derivative with respect to λ. Having factored out some terms which are positive for $\lambda > 0$, we receive

$$\Phi - 2g^2 w \lambda^2 - 2g\lambda = 0 \Longleftrightarrow 1 = g^2 w \lambda^2.$$

The last expression means that the microstates $(0,0)$ and $(1,1)$ are equally probable, which implies half-saturation at this ligand activity.

b) Analogous. ■

With the introduced notation, we define the following additional characterizations of ("positive") cooperativity for molecules with two binding sites.

C6 The coefficients of the binding polynomial fulfill $4a_2/a_1^2 > 1$.

C7 The interaction w satisfies $w > \frac{(g_1+g_2)^2}{4g_1 g_2} \geq 1$.

C8 The occupation of the two sites is positively correlated for all $\lambda > 0$.

C9 The probability of both sites being occupied is larger than the product of the marginal probabilities of the sites being occupied for all $\lambda > 0$:

$$\frac{P(1,1)}{P(1,\bullet)P(\bullet,1)} > 1,$$

($P(1,\bullet) = P(1,0) + P(1,1)$ denotes the marginal occupation probability of site 1.)

C6 is an additional indicator for cooperativity mentioned by Hunter and Anderson [15], which is based on the macroscopic observation only (since only the coefficients a_i are considered and not the binding and interaction energies). We will see the very simple statement that this indicator is identical to having non-real roots, for the case of two binding sites. How to generalize this criterion to systems with more binding sites is not obvious, but approaches might be built on more general connections between coefficients of polynomials and their roots (for instance [16]). C7-C9 are criteria for cooperativity on the microscopic level and motivated by the descriptive sentence "the binding of the ligand to one site enhances the binding of the ligand to another binding site", which is frequently stated in different variants to describe cooperativity. These criteria based on microscopic behavior can also be applied to the case of n binding sites by pairwise considerations. However, it is possible that for instance the correlation will change its sign at a certain non-trivial ligand concentration [17].

C7 describes the threshold of negative interaction energy between the two sites, which is required to cause phenomena observed on the macroscopic level, which cannot be caused by a system consisting of two independent sites. The interaction constant w being larger than 1 is not sufficient to cause the appearance of non-real roots of the binding polynomial, if the sites are energetically not identical. The interaction w has to compensate the asymmetry of the system first and thus will only cause macroscopic behavior usually associated to cooperativity above the threshold $\frac{(g_1+g_2)^2}{4g_1g_2}$ (to confirm that $\frac{(g_1+g_2)^2}{4g_1g_2} \geq 1$, note that $(g_1+g_2)^2 - 4g_1g_2 = (g_1-g_2)^2 \geq 0$).

C8 and C9 are two different formulations, but as we will see, equivalent to w being larger than 1.

We will show that for the two-sites case, we have three groups of equivalent statements. A satisfaction of the sigmoidal shape criteria C4 implies that the equivalent statements {C1–C3, C5–C7} are fulfilled. Moreover, if one of the latter class holds, {C8,C9} are true as well. The converse statements are not true: C8 does not imply C5 which again does not imply that C4 holds. In more detail, Proposition 1 described the general implications

$$\text{C1} \Rightarrow \text{C2} \Rightarrow \text{C3} \Rightarrow \text{C5 and C4} \Rightarrow \text{C5}.$$

Proposition 2 will additionally demonstrate the implications

$$\text{C5} \Leftrightarrow \text{C6} \Leftrightarrow \text{C7} \Leftrightarrow \text{C1} \Rightarrow \text{C8} \Leftrightarrow \text{C9} \Leftrightarrow w > 1,$$

for two-sites systems. The equivalence of C1 and C7 is the crucial point which closes a circle and reduces all criteria to three equivalence classes for two binding sites.

In the case of two-sites-systems with identical binding sites all criteria except for C4 are one equivalence class.

Proposition 2 (Additional implications for two-sites systems)

a) A molecule with two binding sites satisfies C5 if and only if it satisfies C6.

b) A molecule with two binding sites satisfies C6 if and only if it satisfies C7.

c) A molecule with two binding sites satisfies C8 if and only if $w > 1$.

d) A molecule with two binding sites satisfies C9 if and only if $w > 1$.

e) A molecule with two binding sites satisfies C8 if and only if it satisfies C9.

f) If a molecule with two binding sites satisfies C7 it also satisfies C8 and C9.

g) A molecule with two binding sites satisfies C7 if and only if it satisfies C1.

h) Satisfying C5 does not imply that C4 is fulfilled.

i) For a molecule with two identical binding sites, satisfying C8 or C9 also implies that C1-C3 and C5-C7 are fulfilled.

Proof (Proposition 2)

a) C5 means that the discriminant of the polynomial is negative, which implies $a_1^2 - 4a_2 < 0$, which gives $4a_2/a_1^2 > 1$. Analogously for the converse.

b) $4a_2/a_1^2 > 1$ means $4wg_1g_2/(g_1+g_2)^2 > 1 \Leftrightarrow w > \frac{(g_1+g_2)^2}{4g_1g_2}$. If $g_1 = g_2$, this boundary is equal to 1.

c) An analogous calculation to the proof of Lemma 2, but with different g_1 and g_2 gives $\mathbf{Cov}(k_1, k_2) = \frac{(w-1)g_1g_2\lambda^2}{\Phi^2}$, which shows that the correlation is positive for any value $\lambda > 0$, if $(w-1) > 0$ (and conversely).

d) The probability of both sites being occupied is $g_1g_2w\lambda^2/\Phi$, which satisfies

$$\frac{g_1g_2w\lambda^2}{\Phi} > \frac{g_1g_2w\lambda^2 + g_1\lambda}{\Phi}\frac{g_1g_2w\lambda^2 + g_2\lambda}{\Phi} \Leftrightarrow w > 1.$$

e) Obviously a consequence of C8 $\Leftrightarrow w > 1 \Leftrightarrow$ C9.

f) A result of $\frac{(g_1+g_2)^2}{4g_1g_2} \geq 1$.

g) From the previous statements it is clear that C1 implies C7. What remains to be shown is that C7 implies C1. C7 means that $w > \frac{(g_1+g_2)^2}{4g_1g_2}$. We use an identical sites representation (ISR) of the overall binding behavior by defining $\tilde{g} := g_1/2 + g_2/2 = a_1/2$ and $\tilde{w} := a_2/\tilde{g}^2$. Then

$$\tilde{w} = \frac{g_1 g_2 w}{(g_1/2 + g_2/2)^2} = w\frac{4g_1g_2}{(g_1+g_2)^2} > 1,$$

because $w > \frac{(g_1+g_2)^2}{4g_1g_2}$. Thus, the overall titration curve can result from a system with energetically identical binding sites with binding constant $\tilde{g}$ and $\tilde{w} > 1$ and we can represent the variance in the number of occupied sites as a sum of the variance of the two identical sites and twice the covariance:

$$\mathbf{V} = 2\mathbf{V}(k_1) + 2\mathbf{Cov}(k_1, k_2).$$

We consider $\mathbf{V}(k_1)$ which is

$$\mathbf{V}(k_1) = \frac{a_2\lambda^2 + \tilde{g}\lambda}{\Phi} - \left(\frac{a_2\lambda^2 + \tilde{g}\lambda}{\Phi}\right)^2 = \frac{a_2\lambda^2 + \tilde{g}\lambda}{\Phi}\ \frac{1 + \tilde{g}\lambda}{\Phi}.$$

The last expression equals the probability of site 1 being occupied multiplied by the probability of site 2 being unoccupied. At half-saturation and in a symmetric system, both probabilities are 0.5, which means $\mathbf{V}(k_1) = 0.25$. Consequently $\mathbf{V} = 2 \cdot 0.25 + 2\mathbf{Cov}(k_1, k_2) > 0.5$, due to Lemma 2 and $\tilde{w} > 1$. Thus, with Lemma 1 c), C1 is fulfilled.

h) Consider a molecule with $g_1 = g_2 = 10^6$ and $w = 1.5$. Being sigmoidal means that $\frac{\mathbf{V}}{\lambda}$ has a local maximum [17]. Calculating the derivative with respect to λ of $\frac{\mathbf{V}}{\lambda}$ of this example, gives a ratio with a polynomial of degree three as numerator. Its roots are all real but negative, which means $\frac{\mathbf{V}}{\lambda}$ does not have a local maximum for any positive ligand activity.

i) In the case of two identical binding sites, $w > 1$ implies that $w > \frac{(g_1+g_2)^2}{4g_1g_2}$, which means that C8 and C9 are equivalent to all other criteria except for C4.

5 Conclusion

We have seen that for two-sites-systems, several of the considered definitions of cooperativity coincide. In contrast to systems with more binding sites, criteria C1–C3, C5–C7 are fully equivalent for the two sites case. The most important statement to receive this result is Proposition 2 g), which allows to close a circle of implications. For this step, we used an identical sites representation (ISR) to reduce the general problem to an easier case to which Lemma 2 applies. This might also be a useful strategy for other theoretical problems of ligand binding. Moreover, we have seen, that positive correlation (C8) or a relatively high probability to find both sites occupied (C9), are consequences of C1–C7, but weaker criteria and not sufficient to cause cooperative behavior on the macroscopic level. However, they imply C1 (and thus C2–C3,C5–C7), in the case of identical sites. Finally, recall that the sigmoidal shape criteria C4 implies that all others are fulfilled but that any converse implication is impossible. Also in the case of only two binding sites, C4 is a stronger criterion than the others. The contrast between the illustrated equivalences of definitions of cooperativity for two-sites system, and their non-equivalence for larger systems highlights that inconsistencies might arise when results are transferred from the two-sites case to larger systems. Insights gained from considering the two-sites example may be restricted to this situation.

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