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Cooperative equilibrium curves generated by ordered ligand binding to multi-site molecules

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Abstract

The sigmoid shape of equilibrium curves in normal axes and Hill coefficients higher than unity, are indexes of cooperativity or homotropic allostery where the affinity for the ligand increases as saturation progresses. The mathematical transformation of the Adair scheme of equilibria in the Hill plot, reveals that sigmoid binding curves can also be generated by ordered ligand binding to a receptor with multiple binding sites of identical microscopic association constants. This mechanism only based on the law of mass action, could participate to some extent to certain cooperative effects observed in non-biological systems and perhaps in the physiological binding of oxygen to heme proteins.

Keywords: Adair equation; Allostery; Cooperative binding; Hill coefficient; Hill plot; Intrinsic binding constants

1. Introduction

The concept of cooperativity has arisen from the original observation that saturation of tetrameric hemoglobin (Hb) by oxygen does not fit a hyperbola, contrary to monomeric myoglobin [1]. The sigmoid oxygenation equilibrium curve of hemoglobin is physiologically important since it allows to reconcile an efficient loading of oxygen in the lungs and, even more importantly, an efficient release of oxygen in the tissues where the oxygen concentration is low. The more pronounced the sigmoid shape, the greater the fraction of oxygen that can be unloaded. This shape was explained by the fact that the filling of the first oxygen binding site increases the affinity for oxygen of the remaining sites [2]. Structural studies allowed to identify the conformational changes underlying this communication between the binding sites [3]. The data obtained at equilibrium with Hb have then been applied to the study of the kinetic cooperativity of many enzymes under steady-state conditions. Elegant mathematical models have been proposed to fit the observed saturation curves of Hb, assuming that each binding site exists in two possible states, with low and high affinity for oxygen. The Monod–Wyman–Changeux (MWC) model [4], postulates that Hb subunits change shape in a concerted manner upon fixation of the first oxygen, so that the deoxy-Hb is switched and locked into a higher affinity form, while retaining the overall symmetry of the whole molecule. The Koshland–Nemethy–Filmer model (KNF) [5], postulates that the subunits can change their conformation one at a time in response to oxygen binding. This non-symmetrical model also called sequential, can also fit the cases of negative cooperativity.

Cooperativity cannot be exclusively attributed to complex evolutionary-selected protein behaviors since sigmoid equilibrium curves can also be obtained using non-physiological systems. The mere examination of the law of mass action shows that filling multi-site molecules in an ordered manner, is in fact sufficient to generate a sigmoid saturation curve, when eliminating the statistical distinction between microscopic and macroscopic binding constants. This result which can be theorized through several ways, is deduced here from an elaboration of the most popular tools for analyzing cooperativity: the Hill plot, the Hill coefficient and the Adair general formula.

2. The Hill coefficient

The Hill coefficient has originally been introduced as the slope of the linear plot $\mathrm{Log}\,\frac{Y}{1-Y} = n\ \mathrm{Log}\,x + \mathrm{Log}\,K$, derived from the Hill's equation $Y = \frac{Kx^n}{1+Kx^n}$ [6], where x is the

Abbrevations: Hb, hemoglobin; *Y*, fractional saturation; $\bar{n}$, Hill coefficient.

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concentration of a ligand L and Y is the fractional saturation of a macromolecule M capable of binding n molecules of L. This equation describes the improbable case where the n binding sites of M are filled at the same time according to the following equilibrium $M+nL \leftrightarrow MLn$, whose binding constant is $K=\frac{[ML_n]}{[M][L]^n}$. Though this assumption does not fit the actual fixation of oxygen on hemoglobin, where the Hill coefficient is always lower than the number of hemes n, the maximal slope $\bar{n}$ of the Hill plot is used as an index of the degree of cooperativity. While the Hill coefficient derived from the Hill's equation where the intermediate species are neglected, is clearly empirical, it could become calculable when starting from the Adair scheme [7] taking into account all the successive bimolecular association reactions.

3. Formulation of the Hill plot derived from the Adair scheme

One can show for a molecule with n binding sites for the same substrate in an ideal solution, that the fractional saturation Y, ranging from 0 to 1, is

$$Y=\frac{\sum_{i=1}^{n} i\left(\prod_{j=1}^{i} Kj\right)x^i}{n\left(1+\sum_{i=1}^{n}\left(\prod_{j=1}^{i} Kj\right)x^i\right)}, \tag{1}$$

where Kj is the macroscopic, or apparent, binding constant for the jth binding step. Each constant Kj can be defined by statistical balancing of the microscopic, or intrinsic, constant Kj^*: $Kj=\left(\frac{n-j+1}{j}\right)Kj^*$.

Eq. (1) is thus equivalent to

$$Y=\frac{\sum_{i=1}^{n} i\,C_n^i\left(\prod_{j=1}^{i} Kj^*\right)x^i}{n\left(1+\sum_{i=1}^{n} C_n^i\left(\prod_{j=1}^{i} Kj^*\right)x^i\right)}, \tag{2}$$

which is the so-called Adair general formula [7], in fact written in its general form by Ferry and Green in 1929 [8]. This phenomenological equation is independent of any mechanistic model and does not require foreseeing the values of the n stepwise formation constants. An other curve, the Hill plot, has been selected as a very useful tool for plotting results of cooperative binding experiments. In this representation, the logarithm of the ratio $Y/(1-Y)$, is plotted against the logarithm of the ligand concentration. The maximal slope in the central part of this curve, known as the Hill coefficient ($\bar{n}$), has been selected as a useful empirical factor to compare the data, for example obtained with hemoglobins from various origins. By analogy with the Hill plot obtained with myoglobin, which gives straight line with unity slope, it is admitted that values of $\bar{n}$ lower than, equal to, or higher than unity, reflects negative, zero or positive cooperativity respectively. For a theoretical examination of the Hill plot, Eq. (1) may be rearranged to yield:

$$\frac{Y}{1-Y}=\frac{\sum_{i=1}^{n} i\left(\prod_{j=1}^{i} Kj\right)x^i}{n+\sum_{i=1}^{n}(n-i)\left(\prod_{j=1}^{i} Kj\right)x^i}. \tag{3}$$

To study the Hill curve with normal axes, introduce novel coordinates:

$$X=\mathrm{Log}\,x \text{ and } H=\mathrm{Log}\,\frac{Y}{1-Y}$$

Eq. (3) becomes:

$$H=\mathrm{Log}\left(\sum_{i=1}^{n} i\left(\prod_{j=1}^{i} Kj\right)e^{iX}\right) - \mathrm{Log}\left(n+\sum_{i=1}^{n}(n-i)\left(\prod_{j=1}^{i} Kj\right)e^{iX}\right). \tag{4}$$

Considering that each binding constant Ki can be expressed as $Ki=e^{Ai}$, with $Ai=-\frac{\Delta Gi}{RT}$ where ΔGi is the free energy of association for the ith binding step, R is the gas constant and T the temperature (K), Eq. (4) can be rewritten:

$$H=\mathrm{Log}\left(\sum_{i=1}^{n} i e^{iX+\sum_{j=1}^{i} Aj}\right)-\mathrm{Log}\left(n+\sum_{i=1}^{n}(n-i)e^{iX+\sum_{j=1}^{i} Aj}\right). \tag{5}$$

This function $H=f(X)$ displays the actual appearance of the experimental Hill plots. For each point on this curve, the value of the slope is given by the derivative:

$$\frac{dH}{dX}=\frac{\sum_{i=1}^{n} i^2 e^{iX+\sum_{j=1}^{i} Aj}}{\sum_{i=1}^{n} i e^{iX+\sum_{j=1}^{i} Aj}}-\frac{\sum_{i=1}^{n} i(n-i)e^{iX+\sum_{j=1}^{i} Aj}}{n+\sum_{i=1}^{n}(n-i)e^{iX+\sum_{j=1}^{i} Aj}}, \tag{6}$$

which could be written in the form $\frac{dH}{dX}=P(X)-Q(X)$.

We have

$$\lim_{X\to+\infty} P(X)=n \quad \text{and} \quad \lim_{X\to-\infty} P(X)=1$$

$$\lim_{X\to+\infty} Q(X)=n-1 \qquad \lim_{X\to-\infty} Q(X)=0$$

and then,

$$\lim_{X\to+\infty}\frac{dH}{dX}=1 \quad \text{and} \quad \lim_{X\to-\infty}\frac{dH}{dX}=1$$

Hence, for very low and very high ligand concentrations, the curve $H=f(X)$ approaches asymptotes with unit slopes, whatever the number of binding sites n and the values of the constants Ki may be. This feature is inherent in the multireceptor system and rigorously independent of the nature of molecules studied and the existence of cooperativity. On the contrary, the extreme value of this slope $\bar{n}$ provided for in Eq. (6), is maximal or minimal depending on the cases. It can vary between two theoretical limits, from 0, when for every i, $A1 \gg Ai$, to n, when for every i, $An \gg Ai$.

The normal distance between the two asymptotes,

$$D = \frac{\sqrt{2}}{2}\left|\mathrm{Log}\frac{Kn}{K1} + 2\mathrm{Log}\,n\right|, \tag{7}$$

is set with only three parameters: the binding constants for the first and the last binding steps and the number of sites. If the n binding sites are equivalent ($K1^*=Ki^*=Kn^*=K^*$), Eq. (2) reduces to

$$Y = \frac{K^*x}{1+K^*x} \tag{8}$$

and Eq. (4) to $H=X+\mathrm{Log}K^*$, with a unity slope. The distance D defined in Eq. (7) is zero since $Kn=K^*/n$ and $K1=nK^*$.

4. The particular case of an ordered ligand binding

If the filling of the equivalent sites is ordered, then macroscopic and microscopic binding constants are identical, so that for every i, $A1=Ai=An=A$. In this case, Eqs. (1) and (5) reduce to

$$Y = \frac{\sum_{i=1}^{n}(Kx)^i}{n\left(1+\sum_{i=1}^{n}(Kx)^i\right)} \tag{9}$$

and

$$H = \mathrm{Log}\left(\sum_{i=1}^{n} i\mathrm{e}^{i(A+X)}\right) - \mathrm{Log}\left(n+\sum_{i=1}^{n}(n-i)\mathrm{e}^{i(A+X)}\right) \tag{10}$$

respectively.

One can observe that Eq. (9) gives an S-shaped curve and that Eq. (10) a curve with the typical shape of a Hill plot, with unity slopes at each extreme. To visualize these features, Fig. 1B shows this curve computed for $n=4$. In order to predict the Hill coefficient, the maximal slope of the curve should be determined. The second derivative of the plot is

$$\frac{\mathrm{d}^2H}{\mathrm{d}X^2} = \frac{\left(\sum_{i=1}^{n} i^3\mathrm{e}^{[i(A+X)]}\right)\left(\sum_{i=1}^{n} i\mathrm{e}^{[i(A+X)]}\right) - \left(\sum_{i=1}^{n} i^2\mathrm{e}^{[i(A+X)]}\right)^2}{\left(\sum_{i=1}^{n} i\mathrm{e}^{[i(A+X)]}\right)^2} - \frac{\left(\sum_{i=1}^{n} i^2(n-i)\mathrm{e}^{[i(A+X)]}\right)\left(\sum_{i=1}^{n}(n-i)\mathrm{e}^{[i(A+X)]}\right) - \left(\sum_{i=1}^{n} i(n-i)\mathrm{e}^{[i(A+X)]}\right)^2}{\left(n+\sum_{i=1}^{n}(n-i)\mathrm{e}^{[i(A+X)]}\right)^2} \tag{11}$$

The half saturation is obtained when $Y=1/2$ and $H=0$, that is to say when $X=1/K$ and $X=-A$. At this point, Eq. (11) simplifies to:

$$\frac{\mathrm{d}^2H}{\mathrm{d}X^2} = \frac{2\sum_{i=1}^{n} i^3 - 3n\sum_{i=1}^{n} i^2 + n^2\sum_{i=1}^{n} i}{\sum_{i=1}^{n} i} \tag{12}$$

substituting the sums by the following values:

$$\sum_{i=1}^{n} i = \frac{n(n+1)}{2}, \sum_{i=1}^{n} i^2 = \frac{n(n+1)(2n+1)}{6} \text{ and } \sum_{i=1}^{n} i^3 = \frac{n^2(n+1)^2}{4},$$

Eq. (12) becomes:

$$\frac{\mathrm{d}^2H}{\mathrm{d}X^2}0 = 0, \tag{13}$$

regardless of the n value, indicating that the point of inflection of this plot is located at the intersection with the abscissa. At this point, the Hill coefficient ($\overline{n}_0=\overline{n}_{max}=\overline{n}_{50}$), is given by Eq. (6) when $X=-A$, and simplifies to

$$\overline{n}_0 = \frac{2\sum_{i=1}^{n} i^2 - n\sum_{i=1}^{n} i}{\sum_{i=1}^{n} i} = \frac{n+2}{3}. \tag{14}$$

5. Comparison with experimental data

Fitting theoretical predictions with experimental results (but not the contrary) is generally used as a validation of the most accurate models. Many cases of cooperative binding data could be examined to evaluate the possible participation of this mechanism. On can cite the binding of calcium to the fours sites of the protein p26olf since it is assumed to be sequential and ordered [9]. This system displays cooperativity with a Hill coefficient of 2.0 that is to say, perhaps fortuitously, $(n+2)/3$ for a tetramer [9]. But the paradigm of cooperative binding for which the Hill and Adair schemes have initially been conceived is the oxygenation of Hb. It is thus tempting to determine if an ordered filling of multimeric heme proteins by oxygen could somehow participate to this cooperative binding, but it is clear that the numerous experimental data available for Hb suggest that no single mathematical model could entirely explain all aspects of structure-function relationship in Hb. Theoritical curves can match the data obtained with Hb only in certain ranges of experimental conditions. Comparison of experimental data is bothered by a wide variety of physicochemical conditions affecting Hb oxygenation including, among others, temperature, pH, CO_2, salts and diphosphoglycerate, which can in some cases yield a $\overline{n}$ lower than unity. The situation is further complicated in the case of human Hb because of the presence of two types of subunits, α and β. In fact, no simple rule can be drawn from the relationship between n and $\overline{n}$, which fluctuates between reports, experiments and heme proteins. The $\overline{n}$ value around 2.8, most often reported in the literature to highlight cooperativity, is likely to reflect ideal experimental conditions, including optimal intrinsic and heterotropic parameters. One can notice that earlier assessments of $\overline{n}$ for human Hb, in blood as well as in distilled water [7] and in Ringer's solution [6], yielded values around 2.0. To determine more accurately if an ordered binding oxygen to Hb could be responsible for the sigmoid shape of

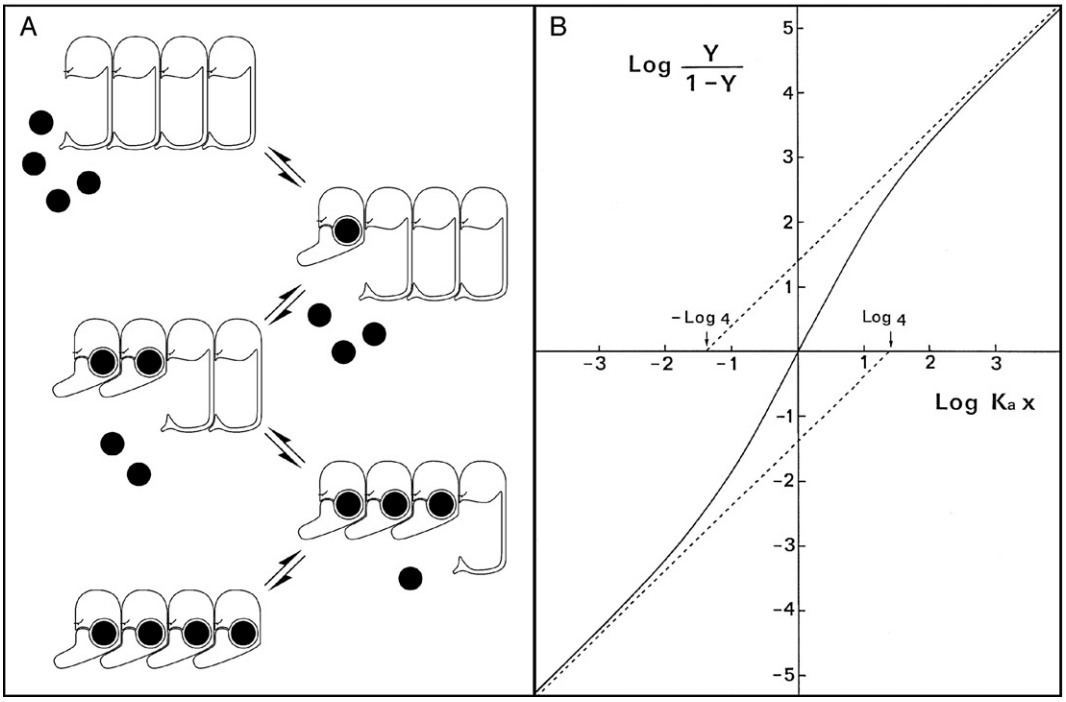

Fig. 1. Theoretical ordered ligand binding to a tetrameric receptor. (A) Representative scheme of an idealized ordered fixation of a ligand (black disks) to four sites successively available for binding. At low ligand concentration, the fractional saturation expected for subunits arranged in multimers is lower than would be for independent subunits. (B) Sigmoid Hill plot expected from the binding scheme shown in panel A and calculated from Eq. (10), for an ordered binding of a ligand at concentration x to the four binding sites of a multireceptor with an identical association constant Ka. The normal distance between the asymptotes is $\sqrt{2}$ Log 4. The predicted Hill coefficient $\bar{n}$ max can be measured at the intersection with abscissa and is equal to 2.0.

saturation curves, it would be useful to determine the ratios of Hill coefficients values yielded by associations of various numbers of identical subunits in the same experimental conditions. Such modular hemoglobins are unfortunately rare, but an interesting case provided in the literature is the Hb from the shellfish *Scapharca inaequivalvis*, where the same subunit can be arranged in tetramers or dimers. Authors found a Hill coefficient equal to 2.0 for a tetramer and to 1.5 for a dimer [10], in rough agreement with the expected ratio of $(n+2)/3$ values.

Certain giant respiratory proteins from invertebrates devoid of blood cells, such as molluscan hemocyanins and worm hemoglobins, reconcile large values of Hill coefficients, with relatively small values of the normal distance between the Hill plot asymptotes [11]. These features are consistent with the present hypothesis. On the one hand, because of the large number n, the Hill coefficient remains high, even in the presence of negative site–site interaction. On the other hand, the distance D between asymptotes is limited as predicted by Eq. (7), since the ratio $Kn/K1$ is smaller than unity when there is negative interaction between the binding sites. A large number of heme groups present in the same molecule is likely to cause reciprocal inhibitory effects because of the decrease of the surface/volume ratio as the number of subunits increases. According to this view, the cooperative-like behavior generated by subunit multimerization would not result, as classically admitted, from the stepwise increase in affinity for oxygen as oxygen binding proceeds, but conversely from a reciprocal inhibition between the associated binding sites observable at low ligand concentration. These data thus make it plausible that the emergence of sigmoid dissociation curves could mainly reflect the multimeric organization of Hb, as anticipated by Archibald Vivian Hill in the title of his pioneering article [6]. This organization could itself result from some evolutionary pressures unrelated to cooperativity since the sigmoid saturation of Hb in invertebrates is likely to not have the same physiological advantages than described in mammals. Notably, in certain invertebrates devoid of blood cells, the high multimerization level of pigment proteins ensures another important function: confining them in the circulatory system.

6. Discussion

Sigmoid Hill plots are classically explained by postulating an increase of intrinsic affinity constants and methods to calculate these constants from the Hill plot have been proposed. The theoretical analysis provided here shows that the sigmoid shape of the Hill plot can also result from an ordered filling of a macromolecule with different binding sites of identical intrinsic affinity for a ligand. Such a situation could be obtained in several circumstances, due for example to: i) the decreased accessibility of certain binding sites at low ligand concentration, which could for example result from a steric inhibition between subunits when aggregated, as compared to free subunits. ii) the relative organization of the binding sites, making them available only one by one for ligand binding (Fig. 1A). These cases do not correspond to an absence of neighboring information transfer but, at the opposite, to an extreme case of interaction between the successive binding sites, shifting their affinity from 0 to a

given microscopic constant identical for all steps. Such a process is somehow related to the KNF model, also termed sequential or "induced fit", which postulates that ligand molecules can bind one by one to the macromolecule [5]. But while this transfer of energy can affect several adjacent subunits in the KNF model, the theoretical ordered binding mechanism presented here corresponds to an idealized system where each subunit induces changes in a single adjacent one.

As discussed for giant invertebrate respiratory pigments, the non-availability of certain binding sites in a multimeric molecule could result from a reciprocal masking of the associated subunits. Such a possibility agrees with the observation that for Hb from several species including human, an isolated subunit has greater affinity for oxygen than multimeric deoxy-Hb. This notion tends to minimize the view of allostery as a sophisticated property of proteins, contrary to the classical models of homotropic allostery, but it is supported by the lot of cooperative binding data reported in the literature for non-biological molecular systems which cannot result from an evolutionary selection. To remain in the field of oxygen binding, one can cite the cooperative oxygen binding obtained with a protein-free system composed of two connected metalloporphyrins, which has an estimated hill coefficient of 1.5 [12].

The theoretical development proposed in this article applies to an idealized molecular system (Fig. 1A). It is unlikely that any real system is exclusively governed by this mechanism, but conversely its possible participation in the generation of sigmoid equilibrium curves should be at least taken into consideration beside more elaborated interpretations. Even if it is virtually impossible to quantify its precise contribution in cooperativity, the eventuality of an ordered filling of multi-site macromolecules could be first tested for example in favorable cases through chemical shift NMR ligand titration. Finally, whether or not it fully applies to certain biological systems, this theoretical mechanism could be useful for the conceptual design of artificial fluid transporters such as blood substitutes.

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