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Contributions of Individual Molecular Species to the Hill Coefficient for Ligand Binding by an Oligomeric Protein

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New insights into the Hill coefficient (n) as a measure of cooperativity are obtained by resolving \bar{Y} , the fractional ligand binding to an oligomeric protein, into a series of integral n^{th} -order reactions. For identical sites within a single conformational state, the weighted sum of each reaction multiplied by its net order gives a Hill coefficient at $\bar{Y} = 0.5$ of $n_{50} = 1.0$, indicative of non-cooperative binding. However, the disappearance of unliganded oligomers (S_0) reflects the higher-order reactions, with their weighted sum (for a tetramer) leading to a Hill coefficient at $S_0 = 0.5$ of $n_{50}^* = -1.27$. For an oligomer with two conformational states (such as represented by the T and R states in the Monod-Wyman-Changeux model) capable of generating highly cooperative binding, the same n^{th} -order reactions apply, but with different weights. For oxygen binding to hemoglobin, n_{50} is resolved into three components with net reaction orders of $n = -2, 2,$ and 4 (with weights of $0.067, 0.15,$ and 0.754 corresponding, respectively, to the contributions of singly, triply and quadruply liganded molecules) to give $n_{50} = 3.18$. However, the cooperativity of the "state" function, \bar{R}' (the normalized fraction of molecules in the R state), as characterized by n'_{50} (the Hill coefficient at $\bar{R}' = 0.5$) is distinct from n_{50} . If the T - R equilibrium lies very far in favor of either state, then even when the two states differ widely in their intrinsic affinity for ligand, the lower limit of cooperativity for \bar{Y} is $n_{50} = 1.0$, but the Hill coefficient for \bar{R}' cannot fall below $n'_{50} = 1.27$ (for a tetramer). Hence, the lower limit of n'_{50} is equal to the absolute value of n_{50}^* describing the disappearance of S_0 for an oligomer with a single conformational state.

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Early in the century, Hill (1910) proposed that the sigmoidal curve for oxygen binding by hemoglobin (Hb) could be explained on the basis of higher-order reactions: $\text{Hb} + nX \rightleftharpoons \text{HbX}_n$, with integral values of $n > 1$. When the tetrameric structure of hemoglobin was established, the oxygenation reaction was described by Adair in terms of four successive binding steps (Adair, 1925). As a result, the non-integral values of n in the range 2.5 to 3 obtained by fitting the oxygenation data to the Hill equation have been assumed to provide only an empirical index of cooperativity. However, by re-

solving ligand binding into a series of integral n^{th} -order reactions, new insights into the Hill coefficient are obtained, and a simple method for calculating n_{50} (the Hill coefficient at 50% saturation) is generated.

In this analysis, we consider an oligomeric protein with N distinct, but equivalent binding sites. We define the fractional population of each molecular species, S_i (for $i = 0, 1, 2, \dots, N$), where S_i represents the concentration of protein molecules with i ligands divided by the total protein concentration, such that $0 \leq S_i \leq 1$ and $S_0 + S_1 + S_2 + \dots + S_N = 1$. The individual S_i can be used to define Y_i , the "species fractions" (Wyman & Gill, 1990), since $Y_i = iS_i/N$. The Y_i are the

Abbreviation used: hb, hemoglobin.

contributions of the separate species to the overall fractional saturation, \bar{Y} . Hence, $Y_0=0$ and $\bar{Y}=Y_1+Y_2+\dots+Y_N$. These parameters can be used to characterize the cooperativity of ligand binding from the slope of the Hill plot, n , which relates \bar{Y} to the ligand activity, X , by the equation:

$$n = \frac{d \log [\bar{Y}/(1 - \bar{Y})]}{d \log X} \quad (1)$$

Since n varies with X , it is convenient to define n_{50} , the value of the slope of the Hill plot at half saturation ($\bar{Y}=0.5$), which occurs at the ligand concentration defined as X_{50} .

The conventional view of ligand binding to an oligomeric protein depicts the process by a series of sequential reactions involving molecular species (S_i) at progressively higher degrees of ligation, as shown in Figure 1(a). For identical sites and a single conformational state, a sole intrinsic equilibrium constant (K_D) applies and defines the value of each of the four individual constants: $K_1=K_2=K_3=K_4=K_D$. With two conformational states, T and R in the MWC model (Monod *et al.*, 1965), each S_i represents the sum of the species T_i and R_i , as presented in Figure 1(b). Irrespective of the origin or the magnitudes of the K_i values, the overall fractional saturation (\bar{Y}) is given by:

$$\bar{Y} = \frac{\frac{X}{K_1} + \frac{3X^2}{K_1K_2} + \frac{3X^3}{K_1K_2K_3} + \frac{X^4}{K_1K_2K_3K_4}}{1 + \frac{4X}{K_1} + \frac{6X^2}{K_1K_2} + \frac{4X^3}{K_1K_2K_3} + \frac{X^4}{K_1K_2K_3K_4}} \quad (2)$$

In the case of the MWC model, the values of K_i are fixed by L , c , and K_R , according to the equations presented in Figure 1(b). However, for both the single-state and two-state cases, the relationships between the individual S_i can also be formulated as a series of progressively higher-ordered reactions, from S_0 to S_i , corresponding to integral order coefficients of $n=i$, as described in Figure 1(c). Moreover, the intermediate species S_1 to S_3 will disappear in favor of S_4 with negative integral order coefficients of $n=i-N$ (where N is the number of ligand-binding sites), as also indicated in Figure 1(c).

Ligand binding can be related to the appearance or disappearance of the various S_i components when \bar{Y} is separated into the individual Y_i species fractions. It may be noted that each Y_i is given by the i^{th} term of the numerator divided by the denominator of equation (2). A striking feature of the analysis of binding in terms of the Y_i components that has not been reported concerns the observation that cooperativity can be represented in a simple form. A formal mathematical derivation of

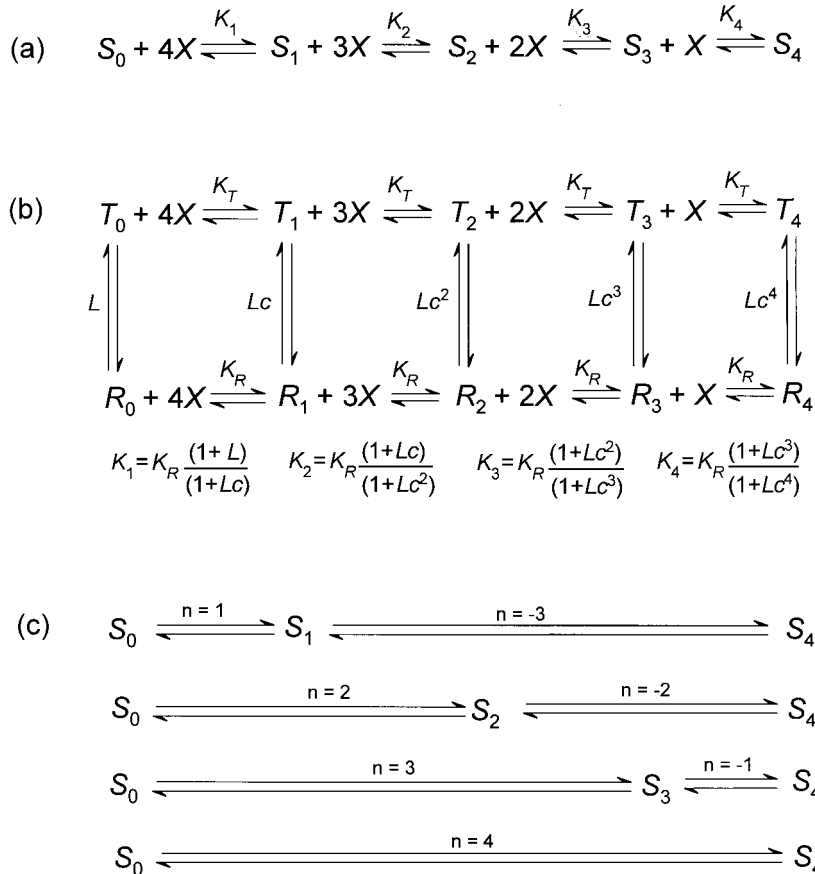


Figure 1. Ligand binding steps for a four-site protein. (a) Conventional description as sequential steps with four intrinsic dissociation constants (K_1 to K_4) governing the binding of ligand to form the successive S_i species. For identical sites, all four K_i values are equal to the intrinsic dissociation constant (K_D) characterizing the sites. (b) The case of two conformational states, corresponding to the MWC model (Monod *et al.*, 1965), with each molecular species S_i resolved into T_i and R_i components, such that each K_i may be defined as shown, i.e. $K_i = K_R(1 + Lc^{i-1})/(1 + Lc^i)$, where K_R is the intrinsic dissociation constant for the R state, c is the ratio of dissociation constants of the R and T states ($c = K_R/K_T$), K_T is the intrinsic dissociation constant for the T state, and L defines the equilibrium between T and R in the absence of ligand ($L = [T_0]/[R_0]$). (c) Representation of binding as a series of n^{th} -order reactions. For the intermediate degrees of ligation (S_1 to S_3), each species S_i appears by a reaction of the order indicated to its left and disappears by a reaction of the negative order indicated to its right.

the simplified relationship is presented in the Appendix, but initially it can be presented schematically by noting that for a tetramer the contributions of each Y_i to n_{50} may be expressed as its relative magnitude multiplied by the net order for that component:

$$n_{50} = \frac{[1-3]Y_1 + [2-2]Y_2 + [3-1]Y_3 + [4-0]Y_4}{0.5} \quad (3)$$

where each Y_i refers to its value at $\bar{Y} = 0.5$ and the reaction orders for the formation and disappearance of each species depicted in Figure 1(c) are in bold (since S_4 only accumulates, its disappearance order is set at zero). When the net order for each component is calculated, the term for Y_2 disappears and the equation for n_{50} reduces to:

$$n_{50} = \frac{[-2]Y_1 + [2]Y_3 + [4]Y_4}{0.5} \quad (4)$$

The significance of formulating multi-step binding as a series of progressively higher-order reactions is first illustrated with binding curves for a tetramer with identical sites and a single conformational state (Figure 2). The binding function, \bar{Y} is separated into the individual components, Y_i , as presented in Figure 2(a), along with the disappearance of the unliganded species, S_0 . On this linear scale the individual Y_i curves display similar properties, as noted previously (Wyman & Gill, 1990), but when the same components are examined using the Hill plot in Figure 2(b), a distinctive pattern appears. Although the overall process is non-cooperative ($n=1$, as indicated by the slope of the Hill plot), the individual Y_i components possess cooperative phases, with the Hill plot slopes varying from $n=1$ to $n=-3$ for Y_1 , from $n=2$ to $n=-2$ for Y_2 , and from $n=3$ to $n=-1$ for Y_3 , recalling the reaction orders for the appearance and disappearance of the species as described in Figure 1(c) and equation (3). For Y_4 , formation occurs with slopes varying from $n=4$ to $n=1$. At $\bar{Y} = 0.5$ the distribution of the Y_i components for a one-state tetramer with identical sites corresponds to the simple binomial relationships (see legend to Figure 2), yielding the value for n_{50} :

$$n_{50} = \frac{[-2]0.0625 + [2]0.1875 + [4]0.0625}{0.5} = 1.0000 \quad (5)$$

Thus, the weighting of the components eliminates overall cooperativity and $n_{50} = 1$.

It may also be noted that the progressive disappearance of the unliganded species S_0 in Figure 2(b) occurs with values from $n = -1$ to $n = -4$, corresponding to the appearance, respectively, of the S_1 to S_4 components presented in Figure 1(c). The cooperativity for the disappearance of S_0 at the

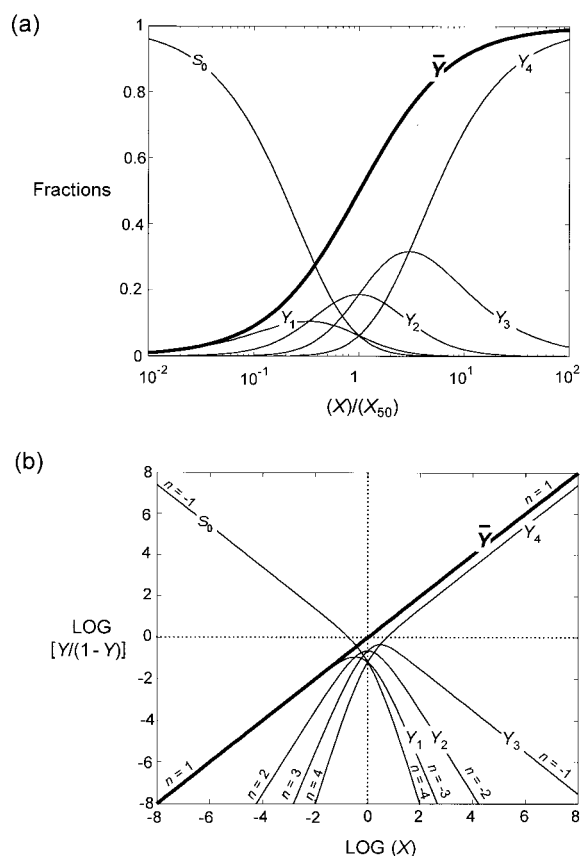


Figure 2. Ligand binding to a tetramer with a single conformational state. (a) The overall binding, \bar{Y} (thick line), and the individual binding components, Y_i (thin lines), as a function of (X) , the ligand concentration. (b) The data in the form of a Hill plot, with broken horizontal and vertical lines that intersect the curve for $\log [\bar{Y}/(1-\bar{Y})]$ at $\bar{Y} = 0.5$. Data for the disappearance of the unliganded species S_0 are also presented. Each individual binding fraction is calculated on the basis of equation (2), but using only the i^{th} term of the numerator for the corresponding Y_i . For a single conformational state with identical binding sites, all K_i values are identical: $K_1 = K_2 = K_3 = K_4 = K_D$. The value of K_D is set at 1.0 in this example, but any value of K_D may be used, since (X_{50}) the concentration of ligand at 50% binding will scale accordingly and at $\bar{Y} = 0.5$ the ratio of X/K_D in equation (2) will be always be unity. The values of the Y_i components at $\bar{Y} = 0.5$ are $Y_1 = 0.0625$; $Y_2 = 0.1875$; $Y_3 = 0.1875$; $Y_4 = 0.0625$, i.e. a purely binomial (1:3:3:1) distribution. With equivalent binding sites, equation (2) reduces to $\bar{Y} = (X)/[(X) + K_D]$, which corresponds to the overall behavior of the system, but ignores the contributions of the individual Y_i components.

ligand concentration corresponding to $S_0 = 0.5$ may be characterized by n_{50}^* , where:

$$n_{50}^* = \frac{d \log [S_0/(1-S_0)]}{d \log X} \quad (6)$$

The weights of the various fractional S_i components lead to a simple equation for n_{50}^* :

Table 1. The n_{50}^* values and the weights of the fractional S_i components for the disappearance of S_0

N	(X)	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7-S_{10}	n_{50}^*
1	1.0000	0.5000								-1.0000
2	0.4142	0.5000	0.4142	0.0858						-1.1716
3	0.2599	0.5000	0.3899	0.1013	0.0088					-1.2378
4	0.1892	0.5000	0.3784	0.1074	0.0135	0.0006				-1.2728
5	0.1487	0.5000	0.3717	0.1106	0.0164	0.0012	$<10^{-4}$			-1.2945
6	0.1225	0.5000	0.3674	0.1125	0.0184	0.0017	0.0001	$<10^{-4}$		-1.3092
⋮										
10	0.0718	0.5000	0.3589	0.1159	0.0222	0.0028	0.0002	$<10^{-4}$	$<10^{-4}$	-1.3393

For oligomers with N sites, the concentration of ligand corresponding to $S_0 = 0.5$ is given by $(X) = K_D(\sqrt[N]{2} - 1)$, with K_D set to 1.0. The value of n_{50}^* is obtained from equation (7) with N terms, and with each S_i value obtained from equation (2) with the numerator replaced by the i^{th} term of the denominator.

$$n_{50}^* = \frac{[-1]S_1 + [-2]S_2 + [-3]S_3 + [-4]S_4 + \dots}{0.5} = 2 \sum_{i=1}^N iS_i \quad (7)$$

A proof of equation (7) is presented in the Appendix. The fractional populations of successive S_i decrease sharply, with the values for a tetramer (Table 1) yielding $n_{50}^* = -1.27$. The values for other oligomeric species show a shallow dependence on N , the number of sites: $n_{50}^* = -1$ for $N = 1$, and n_{50}^* varies from -1.17 to -1.34 in the range of $N = 2$ to $N = 10$ (Table 1). Hence, for an oligomer with two or more sites, the disappearance of unliganded molecules, even for a protein with non-cooperative overall ligand binding, occurs with a small but finite degree of cooperativity.

The analysis of binding in terms of the individual Y_i components may also be applied to highly cooperative binding. For tetrameric human hemoglobin with parameters based on fitting to the MWC two-state model (Edelstein, 1971, 1996; Mills *et al.*, 1976; Ackers & Johnson, 1981; Edelstein & Edsall, 1986; Ackers *et al.*, 1992), the data for \bar{Y} and for the individual Y_i components, presented as binding curves in Figure 3(a) or in the form of the Hill plot in Figure 3(b), reveal a different pattern than observed in Figure 2 for a single conformation. As expected for the cooperative system, the intermediate species are less populated. Nevertheless, for the Y_i Hill plots, the series of curves display the same initial and final Hill slopes as observed for the one-state example in Figure 2(b). Hence, the same elementary n^{th} order reactions are present in both cases, but with different weights. With two-states, the Y_i values (see the legend to Figure 3) represent the fractional binding to the T_i and R_i molecules. In this case, equation (4) for n_{50} becomes:

$$n_{50} = \frac{[-2]0.03365 + [2]0.07493 + [4]0.3772}{0.5} = 3.1827 \quad (8)$$

Correcting for the denominator of 0.5, the three integral n components (-2 , 2 , and 4) contributing to n_{50} in equation (8) have weights of 0.0673, 0.14986, and 0.7544, respectively.

When the value of n_{50} obtained with equation (8) is compared with the Hill slope calculated using an equation based on the Hessian of the binding polynomial (Bardsley & Waight, 1978), exactly the same value is obtained (see Appendix). Therefore, the linear combination of Hill components provides a simple description of a highly cooperative system. The analysis is not model-dependent, since simulations with K_i values derived from formulations other than the MWC model, such as the KNF model (Koshland *et al.*, 1966), as well as random values, give perfect agreement between equation (8) and the Hessian equation.

With respect to the disappearance of S_0 , for the highly cooperative case (Figure 3), equation (7) also applies and yields a value of $n_{50}^* = -2.81$. Thus, the disappearance of S_0 displays a high Hill coefficient for an oligomer with strongly cooperative binding, while retaining low but finite cooperativity for a system with non-cooperative overall binding, i.e. $n_{50}^* = -1.27$ for a one-state tetramer (Table 1). An equation of the same form as equation (7), but with positive coefficients, can also be used for an oligomer with two states to evaluate the lower limits of n'_{50} (the Hill coefficient for the state function, \bar{R}' , at 50%), where \bar{R}' is defined as the fraction of molecules in the R state, normalized to a scale of 0 to 1 (Rubin & Changeux, 1966; Changeux & Rubin, 1968). At the high and low extremes of L , the lower limit of n'_{50} for \bar{R}' (for $c \ll 1$) is the same as the absolute value of the lower limit of n_{50}^* for S_0 (Table 1). Hence, for a tetramer with two states that differ widely in their affinity for ligand, the Hill coefficient for \bar{R}' cannot fall below the value of $n'_{50} = 1.27$, in contrast to n_{50} for \bar{Y} , which drops to 1.0 at high and low L values (Rubin & Changeux, 1966). However, if the ligand-binding affinities of the two states are similar, the lower limit of n'_{50} approaches 1.0 as c approaches 1.0, with the minimum value for a tetramer decreasing, for example, to $n'_{50} = 1.25$ for $c = 0.1$ and to $n'_{50} = 1.1$ for $c = 0.5$. In this context, the cooperativity of the dose-response curves of the homopentameric $\alpha 7$ nicotinic acetylcholine receptor predicted by a two-state model, $n'_{50} = 1.27$, is effectively at the lower limit for $c = 0.1$ (Galzi *et al.*, 1996; Edelstein & Changeux, 1996).

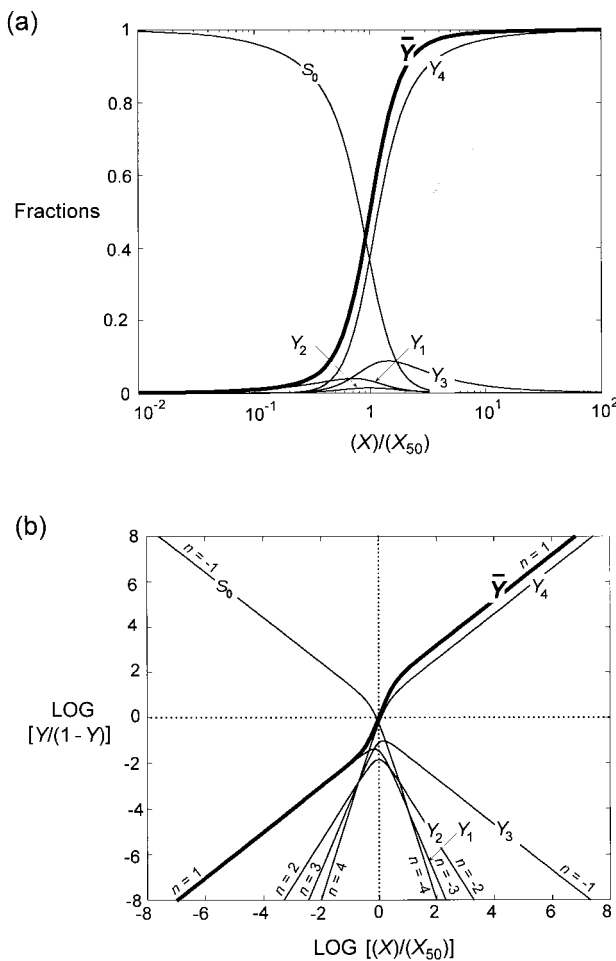


Figure 3. Cooperative ligand binding to a two-state tetramer representing human hemoglobin. (a) Data for the overall binding, \bar{Y} (thick line), and the individual binding components, Y_i (thin lines), as a function of $(X)/(X_{50})$, the ligand concentration normalized to the value at $\bar{Y}=0.5$. (b) The data in the form of a Hill plot. Other details are as for Figure 2, with the exception that $K_1 \neq K_2 \neq K_3 \neq K_4$. For the example selected, the K_i values are calculated to correspond to data for hemoglobin represented by the two-state allosteric model (Monod *et al.*, 1965) with $L = 52,000$, $c = 0.0061$, and $K_R = 1.5 \times 10^{-6}$ M, as previously described (Edelstein, 1971; Mills *et al.*, 1976; Ackers & Johnson, 1981; Edelstein & Edsall, 1986; Ackers *et al.*, 1992; Edelstein, 1996). With these parameters the four K_i values, as defined in Figure 1(b), are fixed at $K_1 = 2.45 \times 10^{-4}$ M, $K_2 = 1.63 \times 10^{-4}$ M, $K_3 = 4.35 \times 10^{-6}$ M, and $K_4 = 1.52 \times 10^{-6}$ M. The curves for \bar{Y} and the individual Y_i components are calculated with the above K_i values using equation (2). The Y_i values at $\bar{Y}=0.5$ are: $Y_1 = 0.03365$; $Y_2 = 0.014225$; $Y_3 = 0.07493$; $Y_4 = 0.3772$. The ligand concentration scale (abscissa) is normalized to $X_{50} = 2.29 \times 10^{-5}$ M.

The resolution of the Hill coefficient into individual components as described in equation (4) is not limited to tetramers, but can be applied to any protein with N sites. In each case, the Y_i components will be multiplied by the net integral order for the

binding of the i^{th} ligand, $[i - (N - i)]$, equivalent to $[2i - N]$, leading to the general equation:

$$n_{50} = \frac{[2-N]Y_1 + [4-N]Y_2 + \cdots + [N]Y_N}{0.5} = 2 \sum_{i=1}^N [2i - N]Y_i \quad (9)$$

A formal mathematical derivation of this equation is presented in the Appendix. As observed in the tetrameric case, for any oligomeric protein with an even number of sites, the net Hill value multiplying the $Y_{N/2}$ term will be zero and the term disappears.

The analysis of the Hill coefficient developed here is applicable at 50% binding or response. The Hill coefficient at other points may be of interest, such as the maximal value which does not necessarily occur at 50%, but the n_{50} values are generally the most useful for characterizing cooperativity with experimental data, since the precision of the data is rarely sufficient to determine the position of maximal n . For the hemoglobin data set analyzed, the value of $n_{50} = 3.1827$ is close to the value of $n_{\text{max}} = 3.1851$ at $\bar{Y} = 0.537$. In the case of the MWC model, progressively larger differences between n_{50} and n_{max} occur as L diverges from $c^{-N/2}$ (Rubin & Changeux, 1966). An average value of $n = 3.05$ is obtained when the hemoglobin data set analyzed in Figure 3 is fit by least squares to the Hill equation over the range of $\bar{Y} = 0.05$ to 0.95 (Edelstein, 1996).

Hill plots for experimental data have not been examined at values of \bar{Y} as low as presented in Figures 2(b) and 3(b); this range was selected to illustrate the n values at the extremes of the slopes. However, by using low temperature electrophoresis to separate individual Y_i components (Perrella *et al.*, 1990; Perrella & Denisov, 1995) and radioactive ligands, it should be possible to approach such low values. Hence, the description in terms of Y_i components could aid in the quantitative estimation of the intermediate species, of prime importance in the evaluation of mechanistic models of cooperativity (Ackers *et al.*, 1992; Holt & Ackers, 1995; Edelstein, 1996). Similar methods could test the predictions for the cooperativity of the disappearance of S_0 by examining, for example a hemoglobin variant locked in the R state (Edelstein, 1975). The results reported for the inactivation of the nicotinic acetylcholine receptor $\alpha 7$ by methylcaconitine are consistent with non-cooperative binding combined with cooperative disappearance of S_0 (Palma *et al.*, 1996).

From an historical perspective, the analysis presented here partially restores the original principal of Hill (1910) by placing it in a different context. Rather than explaining cooperativity by a weighted sum of n -mers, as Hill proposed, a more physically meaningful explanation is provided by the weighted sum of n^{th} order reactions for a monodisperse oligomer. While this analysis contributes

to clarifying the role of the various S_i forms in generating cooperativity, for any protein-ligand system resolving the mechanism responsible for the distribution of these forms is a separate problem. A considerable effort over the last 30 years has been directed towards distinguishing specific theories of cooperative ligand binding, including the concerted (Monod *et al.*, 1965) and sequential (Koshland *et al.*, 1966) models, or combinations of both (Eigen, 1967; Ackers *et al.*, 1992; Edelstein, 1996). Experimental approaches that discriminate between the different mechanistic models in certain cases, particularly where \bar{Y} and \bar{R} can be measured separately, have recently been reviewed (Edelstein & Changeux, 1996).

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Appendix: Mathematical relations concerning the Hill coefficient

Evaluation of the value of the Hill coefficient calculated with equation (8) and a formal derivation of equations (7) and (9) can be achieved by considering the binding polynomial, P :

$$P = \phi_0 + \phi_1 X + \phi_2 X^2 + \dots + \phi_N X^N \quad (10)$$

with $\phi_0 = 1$, which is equivalent to the denominator of equation (2) for $N = 4$. Alternatively, P may be expressed with respect to the individual terms as $P = \sum_{i=1}^N P_i$, where N is the number of ligand-binding sites and $P_i = \phi_i X^i$. It follows from the definition of P that the fractional saturation, \bar{Y} , can be represented as:

$$\bar{Y} = \frac{XP'}{NP} \quad (11)$$

where the prime indicates differentiation with respect to X ; hence, for $N = 4$ equation (11) is identical to equation (2). Furthermore, the Hill slope, n , is given by:

$$\frac{d \log [\bar{Y}/(1 - \bar{Y})]}{d \log X} = \frac{X\bar{Y}'}{\bar{Y}(1 - \bar{Y})} \quad (12)$$

leading to an expression that can be readily evalu-

ated quantitatively:

$$n = 1 + \frac{XH}{P'(NP - XP')} \quad (13)$$

where H refers to the Hessian of the binding polynomial: $H = N P P' - (N - 1)P'^2$ (Bardsley & Waight, 1978). This formulation in terms of the Hessian has provided insights into cooperative ligand binding in relation to limits on the Hill slope, its asymptotes, and the factorability of binding polynomials (Bardsley & Waight, 1978; Bardsley *et al.*, 1980; Bardsley & Wood, 1985). A form of equation (13) based on the parameters of the MWC model may also be derived (Levitzki, 1978; W.G. Bardsley, cited by Edelstein & Changeux, 1996).

With respect to the findings presented in this report, equation (13) can be used to verify the value of n_{50} predicted for a cooperative system using the simpler equation (8). In this case, for the four values of K_1 to K_4 presented in the legend to Figure 3 and the ligand concentration at $\bar{Y}=0.5$ given by $X = 2.29 \times 10^{-5}$ M, with $N=4$ the values for the parameters of equation (13) and its Hessian are: $P = 2.7791$, where P is the denominator of equation (2); $P' = 2.425 \times 10^5$, where $P' = 4/K_1 + 12X/(K_1K_2) + 12X^2/(K_1K_2K_3) + 4X^3/(K_1K_2K_3K_4)$; and $P'' = 2.742 \times 10^{10}$, where $P'' = 12/(K_1K_2) + 24X/(K_1K_2K_3) + 12X^2/(K_1K_2K_3K_4)$. Incorporating these values into the Hessian yields $H = 1.286 \times 10^{11}$ and a Hill slope of $n = 3.1827$, exactly the same value as obtained using equation (8).

The binding polynomial can also be used to construct a formal derivation for the dependence of n_{50} on Y_i presented in equation (9). It follows from the relations defined above that $P'_i = iP_i/X$ and $\bar{Y}_i = iP_i/NP$, with the latter leading to the relation $\bar{Y} = \sum_{i=1}^N \bar{Y}_i$. In order to express the Hill slope in terms of Y_i , the following derivatives are required:

$$Y'_i = \frac{i}{N} \left\{ \frac{(iP_i/X)P - P_iP'}{P^2} \right\} \quad (14)$$

$$XY'_i = i \left\{ \frac{iP_i}{NP} - \frac{XP_iP'}{NP^2} \right\} \quad (15)$$

When the appropriate terms are replaced by \bar{Y} or \bar{Y}_i , equation (15) becomes:

$$XY'_i = i \left\{ \bar{Y}_i - \frac{N\bar{Y}\bar{Y}_i}{i} \right\} = [i - N\bar{Y}]\bar{Y}_i \quad (16)$$

Consequently, the Hill slope defined in equation (12) may be expressed as:

$$\frac{X\bar{Y}}{\bar{Y}(1-\bar{Y})} = \frac{1}{\bar{Y}(1-\bar{Y})} \sum_{i=1}^N [i - N\bar{Y}]\bar{Y}_i \quad (17)$$

which, for the special case of $\bar{Y}=0.5$, yields:

$$n_{50} = 2 \sum_{i=1}^N [2i - N]\bar{Y}_i \quad (18)$$

corresponding to the general expression for n_{50} presented as equation (9).

Concerning the relationship that relates n_{50}^* to S_i , as expressed in equation (7), we observe that each $S_i = P'_i/P$, so that their individual Hill slopes are given by:

$$\begin{aligned} \frac{d \log[S_i/(1-S_i)]}{d \log X} &= X \left\{ \frac{P'_i}{P_i} - \frac{(P' - P'_i)}{(P - P_i)} \right\} \\ &= X \left\{ \frac{PP'_i - P'P_i}{P_i(P - P_i)} \right\} \end{aligned} \quad (19)$$

The expression on the right of equation (19) may also be transformed as follows:

$$\begin{aligned} X \left\{ \frac{PP'_i - P'P_i}{P_i(P - P_i)} \right\} &= \frac{iP - \sum_{j=1}^N jP_j}{P - P_i} \\ &= \frac{i - \sum_{j=1}^N jS_j}{1 - S_i} \end{aligned} \quad (20)$$

which yields equation (7) when $i = 0$ and $S_0 = 0.5$.

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