# Ligand-Dependent Aggregation and Cooperativity: A Critique

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ABSTRACT: Ligand-dependent site–site (or subunit–subunit) interactions provide the basis for explaining cooperativity in chemical reactions. Even in the simplest possible nonaggregating system, interpretation of the interactions in terms of structural details requires an explicit assumption (or model) for the binding of the ligand to the sites when there are no interactions. This paper develops in detail the processes by which aggregation will yield ligand-dependent cooperativity. Two conceptually distinct free energy differences may contribute to cooperativity in an aggregation reaction. One is the free energy difference in ligand binding between the monomer and the aggregate. The other is derived from ligand-dependent interactions between the sites of the aggregate. In this analysis an explicit distinction is made between the experimentally accessible constants and those derived from assumed models. Experimental measurements of an aggregation cycle in which all of the species in equilibrium are defined do not allow for an evaluation of the energies of interaction without some model (or assumption). In the analysis presented, an explicit assumption is employed relating the constant for binding of the ligand to the isolated monomer and the constant for the binding of the ligand to aggregate under conditions where thare are no ligand-dependent interactions.

One of the earliest proposals for explaining the deviations of binding isotherms from those predicted by the equation P + X ⇌ PX was aggregation (Hill, 1910). Later studies have expanded on this idea particularly with reference to proteins such as hemoglobin and some enzymes (Benesch et al., 1965; Guidotti, 1967; Briehl, 1968; Sawula & Suzuki, 1970). Equations have been derived describing ligand-dependent aggregation related to cooperativity (Wyman, 1964; Nichol et al., 1967; Klapper & Klotz, 1968; Dessen, 1973; Levitski & Schlessinger, 1974; Ackers & Halvorson, 1974; Colosimo et al., 1976; Kacser et al., 1990). However, these equations usually have been applied in a manner to emphasize only some specific aspect of the reactions.

The recent general treatment of Kacser et al. (1990) on reaction rates has emphasized the role of aggregation in invalidating the usual assumption of proportionality of rate and enzyme concentration. Nichol et al. (1967) and later Colosimo et al. (1976) assumed that the free energy of binding of ligand to a monomer and its aggregate differed and then developed equations implying that within the aggregate there were no site–site (or subunit–subunit) interactions. With these assumptions the aggregation reaction and concerted allosteric equilibria (Monod et al., 1965) showed (as expected) similar cooperativity characteristics.

For a monomer with a single site in equilibrium with a dimer with the sites preserved on aggregation, there are five possible species requiring four constants to describe the system. Levitski and Schlessinger (1974) chose these four equilibrium constants to explore the characteristics of the system in relation to ligand-dependent cooperativity. These four constants tend to blur the distinction between experimentally accessible and assumed values required for describing the reactions. Because of the difficulty in exploring all aspects of the aggregation in terms of these constants, some correct (but incomplete) conclusions have been drawn along with a misleading conclusion.[1]

[1] For example, it has been stated (Levitski & Schlessinger, 1974): "Some proteins dissociate upon ligand binding, and others associate... In such cases the ligand binding is always positively cooperative, as was already pointed out (Klotz et al., 1970)." This is correct only for the special case of eq 5. We show that ligand binding in an aggregating system can yield both positive and negative cooperativity.

Since the number of parameters characterizing an associating system in equilibrium with its monomer rises rapidly with the degree of aggregation, some of the detailed studies of the derived equations have been limited to a monomer–dimer reaction. A monomer–dimer system does, in fact, illustrate many of the properties of ligand-dependent cooperativity, and this aggregating reaction will be used below.

This paper explores the general consequences of aggregation in relation to the property of ligand-dependent cooperativity. The present analysis differs from prior analyses in that an explicit distinction is made between the experimentally accessible constants and those derived from assumed models. In particular, it is emphasized that two conceptually distinct free energy differences contribute to the process of generating cooperativity in an aggregation reaction. One is the free energy change derived from the difference in the ligand-binding affinity between the monomer and the aggregate. The other is derived from the ligand-dependent interactions between sites (or subunits) of the aggregate. In this paper I treat each of these effects separately and then in combination.

## Equations for Two Sites

An equilibrium mixture of a monomer P and a dimer $P_2$, both binding the ligand X, may be described as follows:

$$\begin{array}{ccc} (c_P)\ P & \rightleftharpoons & P_2\ (k_G c_P^2) \\ \downarrow\uparrow & & \downarrow\uparrow \\ (c_P k_X c_X)\ PX & & P_2X\ (k_G c_P^2 2 k_X c_X \beta_1) \\ & & \downarrow\uparrow \\ & & P_2X_2\ (k_G c_P^2 k_X^2 c_X^2 \beta_2) \end{array} \qquad (1)$$

The expression inside the parentheses beside each of the molecular species represents the equilibrium concentration of that species. In these expressions the symbols $c_P$ and $c_X$ represent the concentrations (acitivity coefficients assumed to be unity) of the free monomer and free ligand, respectively; $k_G$ is the equilibrium constant for the formation of the unliganded dimer from the unliganded monomer; $k_X$ is the constant for the binding of the ligand X to the monomer; $k_X\beta_1$ is the constant

for the binding of the first ligand to the dimer. A formulation of the reaction $PX + P \rightleftharpoons P_2X$ is redundant since $[P_2X]/([PX][P]) = 2k_Gc_P\beta_1$. Thus the factor $\beta_1$ represents the combined perturbations of both $k_G$ and $k_X$, which are ligand dependent for the formation of the species $P_2X$ in the equilibrium mixture of monomer and dimer. Formulation of the reaction $PX \rightleftharpoons P_2X_2$ is also redundant since $[P_2X_2]/[PX]^2 = k_G\beta_2$. $\beta_2$ is the combined interaction factor summarizing the ligand-dependent perturbations for the formation of the species $P_2X_2$ in the equilibrium mixture.

In an aggregating system of this kind, the value of $k_X$ may be experimentally determined by carring out the liganding reaction in sufficiently dilute solutions. Also the value of $k_G$ may be measured in the absence of the ligand X. Similarly, the quantity $k_G\beta_2$ (this product may be labeled as another aggregation constant) may be evaluated experimentally at sufficiently high concentrations of the free ligand X. The feasibility of evaluating these quantities independently, of course, depends upon the relative values of the constants involved.

At each value of $c_X$ the total concentration of the binding molecule, P, in units of the monomer, is

$$c_t = c_P(1 + k_Xc_X) + 2k_Gc_P^2\,(1 + 2k_Xc_X\beta_1 + k_X^2c_X^2\beta_2) \quad (2)$$

The concentration of monomeric P is then given by

$$c_p = [-(1 + k_Xc_X) + \sqrt{(1 + k_Xc_X)^2 + 8k_Gc_t(1 + 2k_Xc_X\beta_1 + k_X^2c_X^2\beta_2)}]/[4k_G(1 + 2k_Xc_X\beta_1 + k_X^2c_X^2\beta_2)] \quad (3)$$

The number of ligands X binding to a total of the molecules P and $P_2$ in units of the monomer is

$$\bar{\nu}_X = \frac{k_Xc_X + 2Qk_Xc_X\beta_1 + 2Qk_X^2c_X^2\beta_2}{1 + k_Xc_X + 2Q(1 + k_Xc_X\beta_1 + k_X^2c_X^2\beta_2)} \quad (4)$$

where $Q = c_Pk_G$.

Equations 2, 3, and 4 facilitate the exploration of the effects of the free energy changes represented by the factors $\beta_i$ on the characteristics of the ligand binding isotherm. When $\beta_1 = \beta_2 = 1$ (a) the concentrations of the monomeric and dimeric species are independent of $c_X$ and depend only on the values of $k_G$ and $c_t$ and (b) binding of ligand is not affected by the aggregaton reactions; that is, the normalized binding isotherm is indistinguishable from that of the monomer.

When $\beta_1 \neq \beta_2$, (a) concentrations of the monomeric and dimeric species are a function of $c_X$ as well as $k_G$ and $c_t$ and (b) the binding of ligand is affected, as evidenced by a change in shape and/or position of the normalized binding isotherm relative to that of the monomer.

The free energy changes represented by the expressions $-RT \ln \beta_i$ may be divided into two essentially different contributions. One is the free energy representing the change in ligand binding to a site in the dimer relative to that of binding to a site in the monomer. The second is the ligand-dependent free energy of interaction between the two binding sites (subunits or domains) of the dimer. To explore the effects of these two energetic quantities separately, I first treat the limiting case in which there are no ligand-dependent interactions between the two binding sites of the dimer (independent sites).

## No Ligand-Dependent Interactions between Sites on the Dimer

In eq 4 above binding of the ligand to the monomer is characterized with the constant $k_X$, which is assumed to be experimentally accessible. Binding of the ligand to the dimer

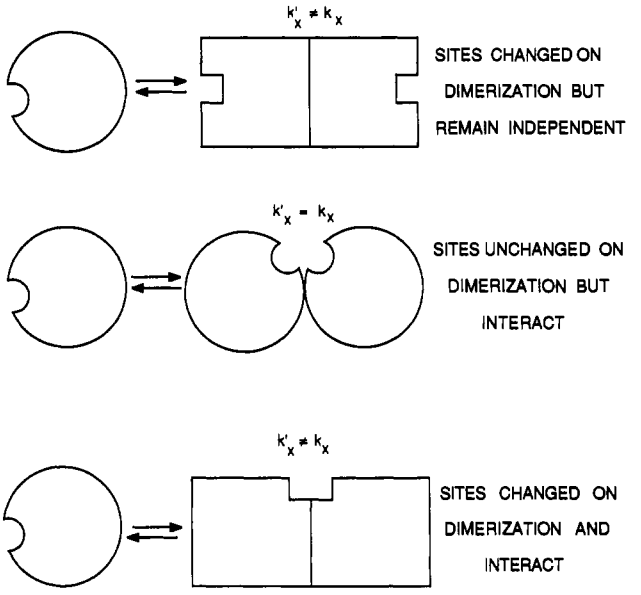


FIGURE 1: Schematic representation of the three idealized treatments in the text. (Top panel) Aggregation with a change in the binding of ligand but with the sites noninteracting or independent. (Middle panel) Aggregation with interacting sites maintaining the same binding affinity as that in the monomer. (Bottom panel) Aggregation accompanied by both a change in affinity and ligand-dependent interactions between the sites.

may be characterized with some different constant, $k'_X$, defined as the unperturbed constant. The unperturbed constant obtains when there are no ligand-dependent interactions. No ligand-dependent interactions between the two sites or subunits of the dimer specifies that, for the dimer, the (unoccupied, site a) ↔ (unoccupied, site b) interactions, the (occupied, site a) ↔ (unoccupied, site b) interactions, the (occupied, site b) ↔ (unoccupied, site a), and the (occupied, site a) ↔ (occupied, site b) interactions are all identical. This set of assumptions specifies that, while the aggregation changes the binding affinity at each site (subunit), the relationship between the sites is such that the ligand binds at each site within the dimer independently. A schematic representation of this idealized assumption is illustrated in the top panel of Figure 1. With these assumptions, the factors $\beta_1$ and $\beta_2$ of equations 2, 3, and 4 become

$$\beta_1 = k'_X/k_X$$

and

$$\beta_2 = \beta_1^2 \quad (5)$$

The simple assumptions of eq 5 are sufficient to generate ligand-dependent cooperativity in the binding isotherms. These isotherms may be generated with values of the ratio $k'_X/k_X$ (or $\beta_1$) both greater than and less than unity. Figures 2 and 3 illustrate binding isotherms, as well as values for the fraction of monomer present in the equilibrium mixtures, and values of the Hill $n$ as saturation proceeds. The $n$ values of Hill ($n_H$) are defined (Wyman, 1964) as

$$n_H = \frac{\partial \ln\left(\frac{Y}{1 - Y}\right)}{\partial \ln c_X} \quad (6)$$

where $\partial$ represents partial differentiation and $Y$ is the fractional saturation. Values of $n_H$ measure the deviation of binding isotherms from those of unperturbed isotherms representing a single site or a set of identical and independent or noninteracting sites. A single or noninteracting set of identical sites

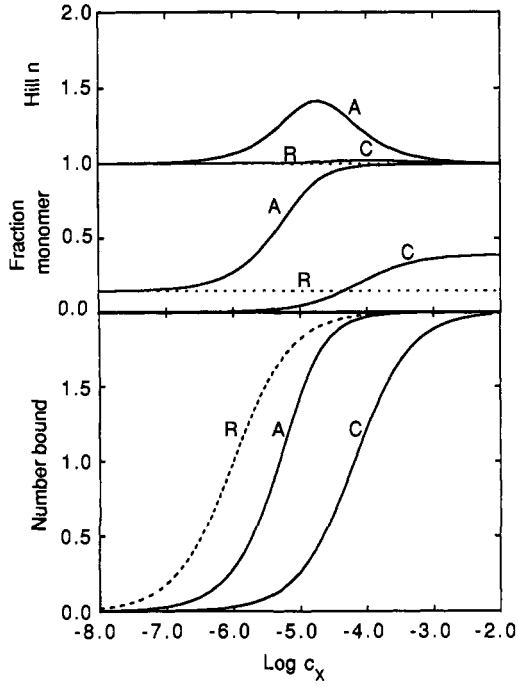


FIGURE 2: Ligand binding isotherms with their associated curves for values of fraction of monomer and $n$ value of Hill for parameters listed in Table I with $\beta_1 < 1$. Values for $\bar{\nu}_X$ are given for total concentration in units of the dimer. R designates the reference or unperturbed curves. A and C refer to the different concentrations specified in Table I.

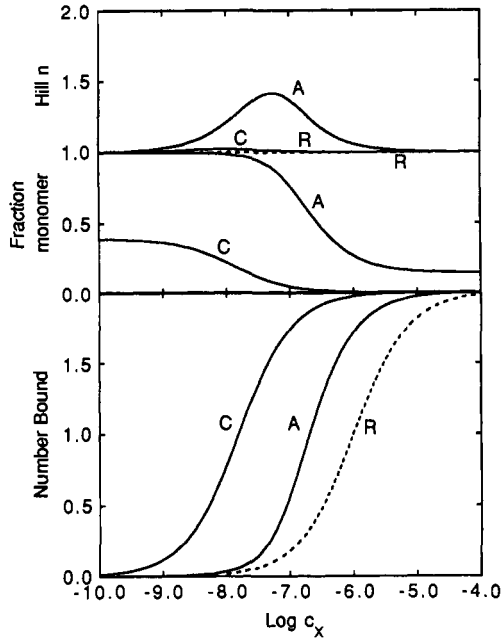


FIGURE 3: Ligand binding isotherms with their associated curves for values of fraction of monomer and $n$ value of Hill for parameters listed in Table I with $\beta_1 > 1$. Values for $\bar{\nu}_X$ are given for total concentration in units of the dimer. R designates the reference or unperturbed curves. A and C refer to the different concentrations specified in Table I.

yields values of $n_H$ which are unity at all levels of saturation. Perturbed or interacting sites may generate values of $n_H$ greater than or less than unity at some level(s) of saturation. Values of $n_H$ greater than unity are commonly labeled as positive cooperativity and those less than unity, negative cooperativity.

Table I lists the parameters explored in Figures 2 and 3 with the resulting concentrations at half saturation, the median ligand concentrations, and total energy of interaction.

Concentrations at half saturation, $p_{50}$, are the concentrations of free ligand at which $\bar{\nu}_X$ is one-half of saturation. The value of $p_{50}$ may be different from the median ligand concentration, $p_{med}$.

Table I: Values of Parameters To Generate Isotherms of Figures 2 and 3 for a Monomer–Dimer Aggregating System, Equation 4[a]

| curve | unperturbed R | $\beta_1 < 1$ A | $\beta_1 < 1$ C | $\beta_1 > 1$ A | $\beta_1 > 1$ C |
|---|---|---|---|---|---|
| log $k_G$ | 4.0 | 4.0 | 4.0 | 0.0 | 0.0 |
| $c_t$ (M) | 0.002 | 0.002 | 2.0 | 0.002 | 2.0 |
| $\beta_1$ | 1.0 | 0.01 | 0.01 | 100 | 100 |
| log $p_{50}$ | −6.00 | −5.31 | −4.19 | −6.69 | −7.81 |
| log $p_{med}$ | −6.00 | −5.35 | −4.19 | −6.65 | −7.81 |
| $(n_H)_{max}$ | 1.00 | 1.42[b] | 1.02 | 1.42 | 1.02 |
| $\Delta G_I$ ($RT$ units) | | +2.99 | +8.34 | −2.99 | −8.34 |

[a] For all isotherms, $c_t$ is listed in units of the monomer, $\beta_2 = \beta_1^2$, and $k_X = 10^6$ M$^{-1}$. [b] The largest value of $(n_H)_{max}$ for log $k_G = 4$ and $\beta_1 = 0.01$ is 1.426 at $c_t = 0.00364$.

The median ligand concentration is the concentration of free ligand, $\bar{X}$, at which the two integrals in the following equation are equal (Wyman, 1964).

$$\int_0^{\bar{X}} Y \, d \ln c_X = \int_{\bar{X}}^{\infty} (1 - Y) \, d \ln c_x \qquad (7)$$

The sum of these two integrals (areas) for an unperturbed binding isotherm is log 4 or 0.60206. When the binding isotherms are steeper than the unperturbed curve the sum of these integrals is less than log 4.

The total energy of interaction, $\Delta G_I$, may be evaluated from the median ligand concentration (Minton & Saroff, 1974) as follows for the monomer–dimer binding isotherm.

$$\Delta G_I = -2RT(\ln p_{med} - \ln k_X) \qquad (8)$$

Positive values of $\Delta G_I$ shift the binding isotherm to the right. Negative values of $\Delta G_I$ shift the binding isotherm to the left (see Figures 2 and 3). When the value of $\beta_1$ is less than unity, values of $k_G$ and $c_t$ may be chosen (see Table I), to yield a positive energy of interaction. Under these conditions the fraction of monomer increases as the binding of ligand approaches saturation. When the value of $\beta_1$ is greater than unity, conditions may be chosen to illustrate the reverse behavior on saturation.

The values of $k_G$ and $\beta_1$ are properties of a given molecule, while the total concentration, $c_t$, may be manipulated experimentally. For a given pair of values for $k_G$ and $\beta_1$, there exists at most a finite range (window) of values of $c_t$ where cooperativity on binding of ligand can be observed. This is illustrated in Figures 2 and 3. When $k_G = 10^4$ M$^{-1}$ and $\beta_1$ is unity, the binding isotherm is unperturbed at all values of $c_X$ but with the fraction of monomer remaining constant at 0.15 when $c_t = 2 \times 10^{-3}$ M. However, when the value of $\beta_1$ is lowered to 0.01, as illustrated in Figure 2, a steep curve is generated with a maximum $n_H$ of 1.42 and a free energy of interaction of $+2.99RT$ units. As saturation proceeds, the fraction of monomer changes from 0.15 to approximately 1.0. When $k_G$ and $\beta_1$ are held to their previous values of $10^4$ M$^{-1}$ and 0.01, respectively, and $c_t$ is increased to 2 M, the binding isotherm moves to the right and loses most of its cooperativity while the fraction of monomer varies from approximately 0 to 0.15 as saturation proceeds.

A similar window is illustrated in Figure 3 for values of $\beta_1 > 1$.

## Ligand-Dependent Interactions between Sites with $K'_X = K_X$

A schematic diagram to illustrate aggregation assuming the same value of $k_X$ for both monomer and dimer, and with ligand-dependent interactions between the sites, is found in

Table II: Values for Binding of a Ligand to an Aggregating Monomer–Dimer System: Figure 4, 5, and 6 with Equation 4[a]

| | case I steep | | | case II normal | | case III shallow | | |
|---|---|---|---|---|---|---|---|---|
| | A | B | C | A | C | A | B | C |
| $\beta_1$ | 0.7 | 0.0155 | 0.0 | 4.531 | 0.257 | 20.1 | 5.116 | 1.964 |
| $\beta_2$ | 30.0 | 1.0 | 0.04 | 20.1 | 0.049 | 20.1 | 1.0 | 0.05 |
| log $p_{50}$ | −6.665 | −6.0 | −5.596 | −6.564 | −5.643 | −6.525 | −6.0 | −5.727 |
| $(n_H)_{max}$ | 1.655 | 1.655 | 1.470 | 1.0 | 1.0 | 0.427 | 0.427 | 0.427 |
| log $p_{med}$ | −6.647 | −6.0 | −5.630 | −6.564 | −5.643 | −6.564 | −6.0 | −5.644 |
| $\Delta G_1$ ($RT$ units) | −2.98 | 0 | +1.70 | −2.60 | +1.64 | −2.60 | 0 | +1.64 |

[a] For all isotherms, $c_t = 2 \times 10^{-3}$ M in units of the monomers, $k_X = 10^6$ M$^{-1}$, and $k_G = 10^{3.5}$.

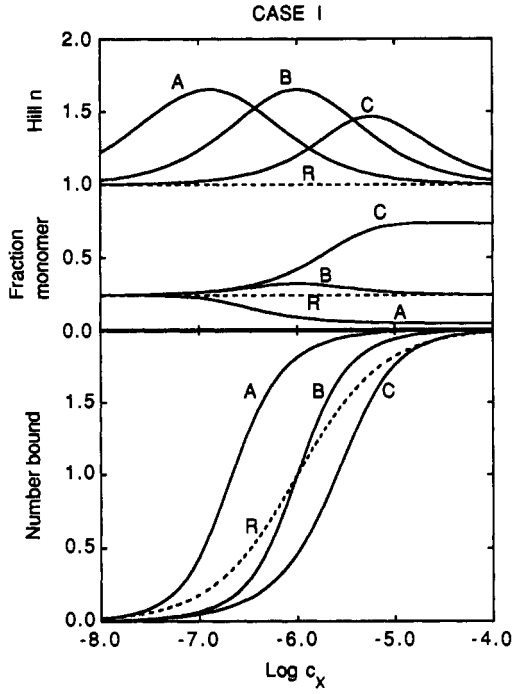


FIGURE 4: (Case I) Steep ligand binding isotherms with their associated curves for values of fraction of monomer and $n$ value of Hill for parameters listed in Table II. Values of $\bar{\nu}_X$ are given for total concentration in units of the dimer. Curves R in all panels represent the reference of unperturbed characteristics. Curves A, B, and C illustrate curves with negative, zero, and positive total energies of interaction, respectively.

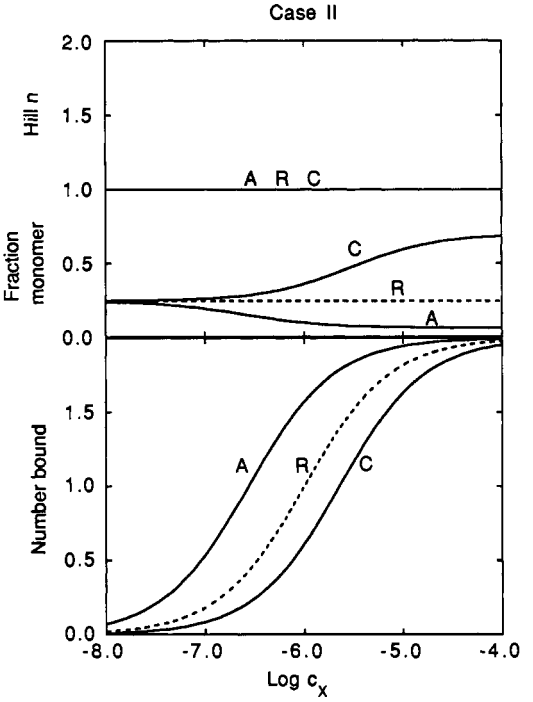


FIGURE 5: (Case II) Normal (in shape) ligand binding isotherms with their associated curves for values of fraction of monomer and $n$ value of Hill for parameters listed in Table II. Values of $\bar{\nu}_X$ are given for total concentration in units of the dimer. Curves R in all panels represent the unperturbed characteristics. Curve A and C illustrate negative and positive total energies of interaction, respectively.

the middle panel of Figure 1. Equation 4 generates three limiting types of binding isotherm derived from the values of $\beta_i$. Depending on the values of $\beta_i$, the binding isotherms will be steeper (case I), have the same contour (case II), or be shallower (case III) than those of the identical independent (noninterating or unperturbed) sites. Table II lists a set of values generating binding isotherms for these three cases. These binding isotherms are illustrated in Figures 4, 5, and 6. The general characteristics of these binding isotherms are similar to those of a nonaggregating system with multiple interacting sites (Saroff & Yap, 1972).

*Case I.* Steeper binding isotherms may occur with negative, zero, and positive total energies of interaction. Values of $\beta_1$ near unity and $\beta_2$ greater than unity yield steep curves with a negative interaction energy. The value of $\beta_1$ will be close to unity when the (unoccupied) ↔ (unoccupied) interactions approximate the (unoccupied) ↔ (occupied) interactions. For simplicity the (unoccupied, site a) ↔ (occupied, site b) interactions are assumed to be equal to the (unoccupied, site b) ↔ (occupied, site a) interactions; that is, the sites are assumed to be identical and isotropic. The value of $\beta_2$ will be greater than unity when the (occupied) ↔ (occupied) energy level is lower than that of the (unoccupied) ↔ (unoccupied) state, resulting in a negative free energy change ($-RT \ln \beta_2$). These conditions yield the curve A of Figure 4, where, as saturation precedes, the fraction of monomer decreases.

An extreme case representing the reverse of that of curve A of case I is illustrated in isotherm C of case I where $\beta_1 = 0$ and $\beta_2 <$ unity. Under these conditions a steep curve results with a positive free energy of interaction and with the fraction of monomer increasing on saturation with ligand.

When the (occupied) ↔ (occupied) interactions are the same as the (unoccupied) ↔ (unoccupied) interactions, $\beta_2$ is unity with a zero total free energy of interaction. This is illustrated in the steep curve B of case I with $\beta_1 < 1$. When the total interaction energy is zero, the fraction of monomer first increases and then decreases to give the same value at the two extremes of saturation with ligand.

*Case II.* Interactions may be such that the shape of the binding isotherm remains unchanged but with a shift in the binding isotherm representing positive and negative free energies of interaction. A positive free energy of interaction results in an increase in fractions of monomer on saturation with ligand. When the interaction energy is negative, the fraction of monomer decreases on ligand binding (see Figure 5 and Table II).

*Case III.* Interactions may occur to generate shallow curves (with values of $n_H$ less than unity). These are illustrated in Figure 6 with representative values of the parameters listed in Table II. The same general rule holds with reference to

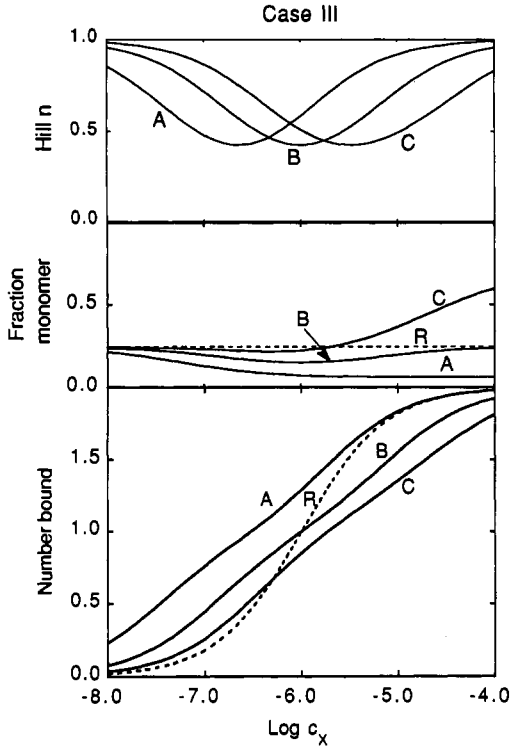


FIGURE 6: (Case III) Shallow ligand binding isotherms with their associated curves for values of fraction of monomer and $n$ value of Hill for parameters listed in Table II. Values of $\bar{\nu}_X$ are given for total concentration in units of the dimer. Curves R in all panels represent the unperturbed characteristics. Curves A, B, and C illustrate curves with negative, zero, and positive total energies of interaction, respectively.

the change in fraction of monomer and the interaction energy. Increases in the fraction of monomer present on ligand binding are associated with positive free energies of interaction with the reverse being the case when the fraction of monomer decreases.

Finally, note that the aggregation reactions (1) are those suggested by Hill (1910). He did not write the conservation of mass (eq 2 for $c_t$) and abandoned the concept for the nonintegral values of $n$ in his equation

$$Y = \frac{K_c^n}{1 + K_c^n} \tag{9}$$

The basic process suggested by A. V. Hill is contained in the reactions $P \rightleftharpoons PX \rightleftharpoons P_2X_2$. The idea of A. V. Hill may be generated by taking $\beta_1 = 0$ and $\beta_2 > 0$ in eq 4 without the necessity of introducing a nonintegral value of $n$.

## Aggregation and Evaluation of Interactions

In formulating eq 4, the interaction factors $\beta_i$ were used with only a general specification of the nature of the interactions. The free energy terms $-RT \ln \beta_1$ and $-RT \ln \beta_2$ contain the free energy changes $-RT \ln (k'_X/k_X)$ and $-2RT \ln (k'_X/k_X)$, respectively where the unperturbed constant, $k'_X$, describes the binding of the ligand X to the dimer and $k_X$ is the constant for binding the ligand X to the monomer. In addition, each of the free energy terms $-RT \ln \beta_1$ and $-RT \ln \beta_2$ may contain an increment of energy derived from ligand-dependent interactions between sites or subunits. These two simplified formulations were treated separately above.

When $k'_X$ and $k_X$ are not equal, it is possible to evaluate $k'_X$ only when there are no ligand-dependent site–site interactions (or when the ligand-dependent site–site interactions are linear with the binding of ligand). The absence of site–site interactions may be explored experimentally by increasing the total concentration to investigate whether the binding isotherm reverts to the contour of noninteracting sites. A binding isotherm with a contour of interacting sites (cooperative isotherm) persisting at sufficiently high total concentrations indicates ligand-dependent site–site interactions in the aggregate. Illustrated in the bottom panel of Figure 1 is a schematic representation of the presence of both effects ($k'_X$ not equal to $k_X$ and ligand-dependent interactions between the sites or subunits).

When ligand-dependent interactions between sites occur, the evaluation of these quantities requires an assumption for the value of $k'_X$. The value $k'_X$ is not experimentally accessible in the presence of ligand-dependent site–site interactions. The assumption that $k'_X = k_X$ is a reasonable one, but it remains an assumption and interpretations of interaction energies hinge on this very important qualification.

Distinction between the values of $k'_X$ and $k_X$ becomes important when evaluations are made of interaction energies at a given step of liganding. Consider the following definitions for use in eqs 2, 3, and 4:

$$k_X = k'_X/\gamma \qquad \beta_1 = \gamma\omega_1 \qquad \beta_2 = \gamma^2\omega_2 \tag{10}$$

These definitions explicitly define the factors $\beta_i$ where $k'_X$ is the unperturbed constant for the binding of ligand X to the assembled dimer; $-RT \ln \omega_1$ and $-RT \ln \omega_2$ are the free energy changes for the (occupied) ↔ (unoccupied) and (occupied) ↔ (occupied) interactions, respectively, compared to the (unoccupied) ↔ (unoccupied) interactions in the assembled dimer.

The free energy change for the reaction $P_2X + X \rightleftharpoons P_2X_2$ is

$$-RT \ln \frac{k'_X\omega_2}{2\omega_1} \quad \text{or} \quad -RT \ln \frac{k_X\gamma\omega_2}{2\omega_2}$$

The ratio $\gamma\omega_2/2\omega_1$ may be explored by writing the reactions in eq 1 as follows to emphasize the aggregation phenomena:

$$\begin{array}{lll} & & P_2 \ (k_G c_P^2) \\ (c_P) \ P & \rightleftharpoons & \\ \Big\| & & \Big\} \rightleftharpoons P_2X \ (2^1K_2 c_P^2 k_X c_X) \\ (c_P k_X c_X) \ PX & & \\ & & P_2X_2 \ (^2K_2 c_P^2 k_X^2 c_X^2) \end{array} \tag{11}$$

where $^1K_2$ is the aggregation constant for the reaction $P + PX \rightleftharpoons P_2X$ and $^2K_2$ is the constant for the reaction $2PX \rightleftharpoons P_2X_2$. Note that with the formulation of reactions in eq 11 the constant $^1K_2$ contains the (occupied) ↔ (unoccupied) interaction factor, while the constant $^2K_2$ contains the (occupied) ↔ (occupied) interaction factor. The equilibrium concentrations of the species $P_2X$ and $P_2X_2$ in the formulations in eqs 1 and 11 may be equated as follows:

$$[P_2X] = 2^1K_2 c_P^2 k_X c_X = k_G c_P^2 2k_X\gamma c_X\omega_1$$

$$[P_2X_2] = {}^2K_2 c_P^2 k_X^2 c_X^2 = k_G c_P^2 k_X^2\gamma^2 c_X^2\omega_2 \tag{12}$$

yielding the ratio

$$^2K_2/{}^1K_2 = \gamma\omega_2/\omega_1 \tag{13}$$

The ratio $^2K_2/{}^1K_2$ contains terms which reflect both the ligand-dependent interactions and the change in binding constant on the formation of the aggregate. *When $\gamma$ is unity*, $-RT \ln (^2K_2/{}^1K_2)$ evaluates difference in the site or subunit interaction energies between the species $P_2X_2$ and $P_2X$. This method (assuming $\gamma = 1$) for evaluating the subunit interactions en-

ergies of oxygenated tetramers of hemoglobin was employed by Smith and Ackers (1985).

## A Hypothetical Experiment

So far in this presentation the binding isotherm for a dissociating dimer have been constructed from assumed parameters. When confronted with an experimental isotherm and its associated aggregation characteristics, one cannot decide on a unique model. Consider a hypothetical experiment with the following results: measured value for the binding constant of ligand to the monomer, $k_X = 10^6\ M^{-1}$; measured value for the association constant for dimerization of the unliganded monomer, $k_G = 10^{3.5}\ M^{-1}$. The ligand binding isotherm and ligand-dependent aggregation data were collected at a total concentration of 0.002 M in units of the monomer. A total of 24 points were collected for the ligand binding isotherm varying from 0.004 to 0.984 in the values of fractional binding; 22 points were collected for the ligand-dependent fraction of monomer present at equilibrium with the values ranging from 0.24 to 0.86. The 46 points were fit without error (assumed, of course) with the following parameters: $\omega_1 = 0.025$, $\omega_2 = 0.015$, and $\log k'_X = 5.98$. All of these points were also fit to a precision of at least 1 part in 10000 with the following parameters: $\omega_1 = 1.0$, $\omega_2 = 24.0$, and $\log k'_X = 4.378$. This is just one of the additional fits. The value of one of the parameters, $\omega_1$, $\omega_2$, or $k'_X$, must be assumed before a unique solution is possible.

## Concluding Remarks

The unperturbed binding constant $k'_X$ (unperturbed by the presence of neighboring sites) in a multisite binding system is an experimentally inaccessible quantity. Evaluation of interaction energies which can then be interpreted in terms of important structural and functional details must depend upon an assumption or model defining this unperturbed constant. One of the earliest studies of this kind was that by Bjerrum (1923) relating the structure (charge separation) of malonic acid to the cooperativity (negative) on the binding of protons. Even in such a well-defined symmetrical compound as malonic acid the problem exists in defining the unperturbed constant for the binding of protons [see Dygert et al. (1970)].

Experimental binding data for a single site in an interacting system are available with such spectral measurements as ultraviolet absorption (Kallen, 1971). Measurements of this kind yield the ratios of the unperturbed constants, not their individual values (Saroff, 1987).

Similar difficulties apply to studies of aggregation where data are analyzed for evaluating interaction energies. Such studies of aggregation in a multicomponent system add data which may make the necessary assumptions more reasonable, but they do not remove the necessity for explicitly specifying that the value of the unperturbed binding constant in the aggregate is an ad hoc assumption.

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