Supplementary Material

A comparison of different multisite binding models

Several studies that used both the Hill [1-7] equation and Adair [2-4, 6] equations to describe Ca^{2+}-dependent CaM activation. In this section, we describe in detail the similarities and differences between these well known models for multisite ligand binding and the model proposed in this paper. A recent study by Weiss [8] suggested that ligand molecules bind to multisite proteins differently according to their biochemical mechanisms. Figure 1 summarizes and compares several possible ligand binding mechanisms for a protein with *n* binding sites. Figure 1A shows the mechanism of independent ligand binding where each binding site is not influenced by any other site and has a unique dissociation constant. Figure 1B illustrates a hypothetical case when all ligand molecules simultaneously bind to a multisite protein. Figure 1C shows the case of sequential "bottle" binding mostly observed in channels. In this case, all ligand molecules that bind to the "sequential" sites on the protein also have different individual dissociation constants. The independent (Figure 1A) and sequential (Figure 1C) ligand access mechanisms may or may not have a degree of cooperativity. By cooperativity we mean here the alteration in the affinity of a binding site, when other sites are occupied by a ligand, rather than just different sets of affinities for various binding sites. Simultaneous ligand binding can be considered as a case of extreme overall cooperativity when any ligand bound to the protein dramatically increases the affinity of all binding sites so that all ligand molecules bind the protein almost simultaneously. We now derive the equations corresponding to the different binding schemes outlined in Figure 1.

The equation for independent ligand binding has already been described in the Materials and Methods of this paper. It shows the multiplication of probabilities representing individual binding events for each site. The corresponding equation for a protein to be fully bound is given by:

$$p_n(u) = \prod_{i=1}^{n} \frac{u}{K_i + u} \tag{1}$$

The probability of a multisite protein with independent access to be free from ligand is given by:

$$p_n(u) = \prod_{j=1}^{n} \frac{K_j}{K_j + u} \tag{2}$$

The intermediate states of partially bound species with j sites occupied by ligand are described by:

$$p_n(u) = \prod_{i=1}^{j} \frac{u}{K_i + u} \cdot \prod_{i=n-j}^{n} \frac{K_i}{K_i + u} \tag{3}$$

If all dissociation constants are equal, then the equation describing fully bound species with independent ligand access becomes:

$$p_n(u) = \frac{u^n}{(K + u)^n} \tag{4}$$

The simultaneous binding of ligand molecules (Figure 1B) can be described by the following equations:

$$\frac{dL_n}{dt} = (k^+)^n \cdot u^n \cdot L - (k^-)^n \cdot L_n \tag{5}$$

where L is a multisite protein, L_n is the multisite protein with all binding sites occupied by a ligand, u is the concentration of ligand molecules, and k^+ and k^- are

association and dissociation constants, respectively. The equation (5) describes a process when *n* ligand molecules simultaneously occupy all *n* sites of a multisite protein. The sum of fully bound and ligand free multisite protein species in this case is equal to the total protein concentration $L0$:

$$L + L_n = L0 \tag{6}$$

The steady-state solution of the equation (5) for simultaneous ligand binding results in the well known Hill equation [9]:

$$p_{Hill}(u) = \frac{L_n}{L0} = \frac{u^n}{u^n + K^n} \tag{7}$$

where $K = \frac{k^-}{k^+}$ is the equilibrium dissociation constant.

The system of differential equations for sequential binding where only the most recently bound ligand can be released (Figure 1C) can be described by the following differential equations:

$$\begin{aligned}
&\frac{dL}{dt} = -k_1^+ \cdot u \cdot L + k_1^- \cdot L_1, \\
&\frac{dL_1}{dt} = k_1^+ \cdot u \cdot L - k_1^- \cdot L_1 + k_2^- \cdot L_2 - k_2^+ \cdot u \cdot L_1, \\
&\ldots \\
&\frac{dL_n}{dt} = k_n^+ \cdot u \cdot L_{n-1} - k_n^- \cdot L_n.
\end{aligned} \tag{8}$$

where L, L_1, L_{n-1}, L_n are the multisite protein species with no ligand, one molecule, *n*-1 and *n* ligand molecules, respectively. This system of differential equations (unlike the equation (5)) describes binding of ligand molecules "in turn". The sum of all protein-ligand complexes gives the total number of protein molecules:

$$L + L_1 + \ldots + L_{n-1} + L_n = L0 \tag{9}$$

The steady-state solution of equations (8) is given by:

$$p_i(u) = \frac{L_i}{L0} = \frac{\frac{u^i}{\prod_{j=0}^{i} K_j}}{\sum_{i=0}^{n} \frac{u^i}{\prod_{j=0}^{i} K_j}}, \; i = 0,1,...,n \qquad (10)$$

where, $K_j = \frac{k_j^-}{k_j^+}$ are the equilibrium dissociation constants for individual sites.

For a fully occupied multisite protein species the equation (10) takes the following form:

$$p_n(u) = \frac{L_n}{L0} = \frac{\frac{u^n}{\prod_{j=0}^{n} K_j}}{\sum_{i=0}^{n} \frac{u^n}{\prod_{j=0}^{n} K_j}} \qquad (11)$$

The equation (11) is the well known Adair equation [10] for multisite binding and it has been derived based on the sequential binding scheme (Figure 1C).

The generalized representation of the Adair equation also includes binomial coefficients or "statistical factors" which are introduced to indicate that there are multiple ways of a ligand molecule binding to a multisite protein [11]:

$$p_{Adair}(u) = \frac{L_n}{L0} = \frac{\frac{u^n}{\prod_{j=0}^{n} K_j}}{\sum_{i=0}^{n} \frac{\frac{n!}{i! \cdot (n-i)!} u^n}{\prod_{j=0}^{n} K_j}} \qquad (12)$$

A recent study by Weiss highlighted the differences between the mechanisms of simultaneous, sequential and independent binding and proposed the following equation to describe the independent ligand binding mechanism:

$$p_{Weiss}(u) = \frac{\dfrac{u^n}{\prod_{j=1}^{n} K_j}}{1 + \sum_{i=1}^{n} \dfrac{u^i \cdot \dfrac{n!}{(n-i)!i!}}{\prod_{j=1}^{i} K_j}} \qquad (13)$$

The equation proposed by Weiss is different from the equations (3) and (4) for independent binding that have been obtained in this study by multiplication of probabilities for individual binding sites, as will be shown below.

In order to highlight the similarities and differences between the above mentioned models, while minimizing the complexity of the equations, in the following we will restrict ourselves to considering one, two and three site models.

For a protein with one binding site all the models converge to the Michaelis-Menten equation:

$$p_{Hill}(u) = \frac{u}{K+u}$$

$$p_{Adair}(u) = \frac{u}{K+u} \qquad (14)$$

$$p_{Weiss}(u) = \frac{u}{K+u}$$

$$p_{ind}(u) = \frac{u}{K+u}$$

The equations for a protein with two sites are far more illustrative. The probabilities for a fully occupied protein are given by:

$$p_{Adair}(u) = \frac{\frac{u^2}{K_1 \cdot K_2}}{1 + 2 \cdot \frac{u}{K_1} + \frac{u^2}{K_1 \cdot K_2}} = \frac{u^2}{K_1 \cdot K_2 + 2 \cdot K_2 \cdot u + u^2}$$

$$p_{Weiss}(u) = \frac{\frac{u^2}{K_1 \cdot K_2}}{1 + 2 \cdot \frac{u}{K_1} + \frac{u^2}{K_1 \cdot K_2}} = \frac{u^2}{K_1 \cdot K_2 + 2 \cdot K_2 \cdot u + u^2} \quad (15)$$

$$p_{independent}(u) = \frac{u^2}{(K_1 + u) \cdot (K_2 + u)} = \frac{u^2}{K_1 \cdot K_2 + (K_1 + K_2)u + u^2}$$

It is discussed in [11] that there are two types of dissociation constants; molecular and group dissociation constants. The same type molecular equilibrium dissociation constants have been used for derivation of equations (3), (7) and (12).

If $K_1 = K_2 = K$ then all the above equations would merge into the same equation:

$$p(u) = \frac{u^2}{K^2 + 2 \cdot K \cdot u + u^2} \quad (16)$$

The Hill equation does not allow dissociation constants at different binding sites to be distinguished. The Hill model for two sites is given by:

$$p_{Hill}(u) = \frac{u^2}{K^2 + u^2} \quad (17)$$

The equations for a fully bound protein species with three sites are given by:

$$p_{Adair}(u) = \frac{u^3}{K_1 \cdot K_2 \cdot K_3 + 3 \cdot K_2 \cdot K_3 \cdot u + 3 \cdot K_3 \cdot u^2 + u^3}$$

$$p_{Weiss}(u) = \frac{u^3}{K_1 \cdot K_2 \cdot K_3 + 3 \cdot K_2 \cdot K_3 \cdot u + 3 \cdot K_3 \cdot u^2 + u^3}$$

$$p_{independent}(u) = \frac{u^3}{(K_1 + u) \cdot (K_2 + u) \cdot (K_3 + u)} =$$

$$\frac{u^3}{K_1 \cdot K_2 \cdot K_3 + (K_1 \cdot K_2 + K_1 \cdot K_3 + K_2 \cdot K_3) \cdot u + (K_1 + K_2 + K_3) \cdot u^2 + u^3}$$

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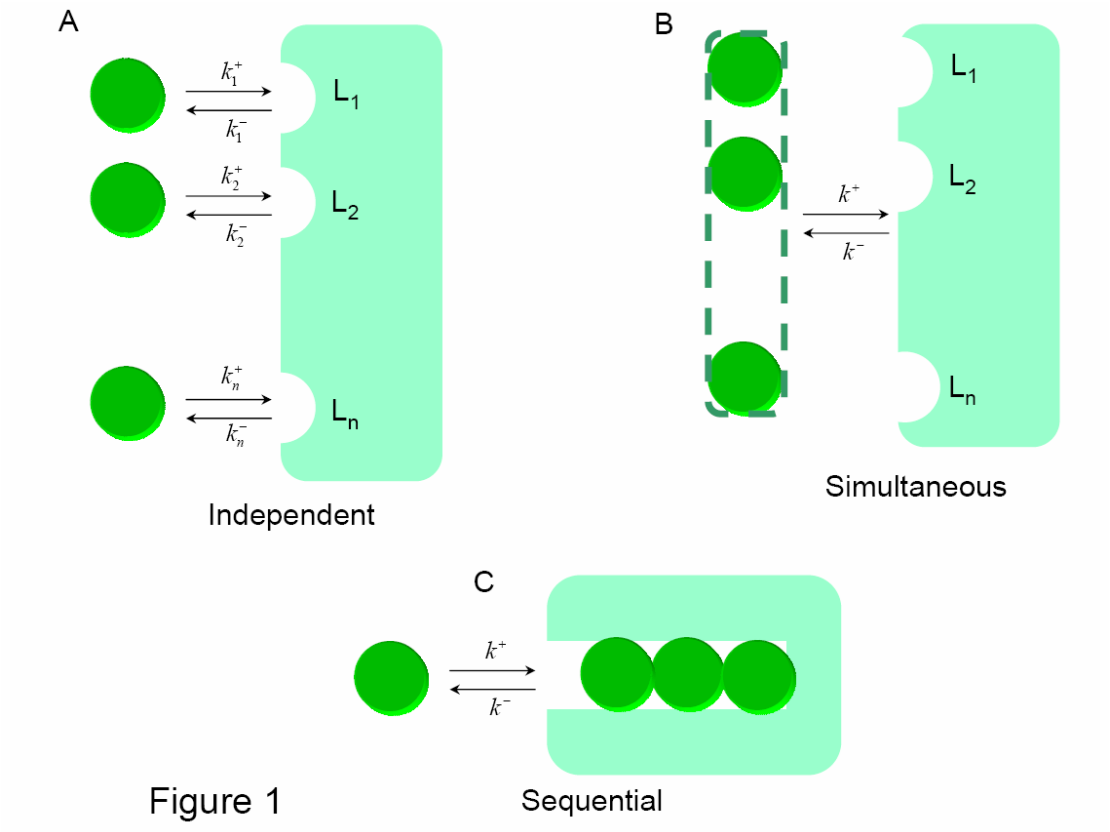
A
k_1^+
k_1^-
L_1
k_2^+
k_2^-
L_2
k_n^+
k_n^-
L_n
Independent
B
L_1
L_2
k^+
k^-
L_n
Simultaneous
C
k^+
k^-
Sequential

Figure 1