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ORIGINAL ARTICLE

Analysis of Biochemical Equilibria Relevant to the Immune Response: Finding the Dissociation Constants

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Abstract This paper analyzes the biochemical equilibria between bivalent receptors, homo-bifunctional ligands, monovalent inhibitors, and their complexes. Such reaction schemes arise in the immune response, where immunoglobulins (bivalent receptors) bind to pathogens or allergens. The equilibria may be described by an infinite system of algebraic equations, which accounts for complexes of arbitrary size n (n being the number of receptors present in the complex). The system can be reduced to just 3 algebraic equations for the concentrations of free (unbound) receptor, free ligand and free inhibitor. Concentrations of all other complexes can be written explicitly in terms of these variables. We analyze how concentrations of key (experimentally-measurable) quantities vary with system parameters. Such measured quantities can furnish important information about dissociation constants in the system, which are difficult to obtain by other means. We provide analytical expressions and suggest specific experiments that could be used to determine the dissociation constants.

Keywords Antibody · Aggregation · Bivalent ligand · Immune system

1 Introduction

In the immune response, multivalent antibodies and antigens (or pathogens) bind together and form noncovalent complexes, which may be large. The efficacy of the

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response of the immune system toward a pathogen is often related to the number of antibodies that bind to a pathogen (Murphy et al. 2008). This immune response can be particularly profound in an allergic reaction (Goldstein 1988), where the antigen is an allergen such as pollen; and the allergic response is a useful and much-studied model system for the immune response.

In this paper, we follow a model proposed by Mack et al. (2011), which itself generalizes one presented in a classic paper by Dembo and Goldstein (1978). We consider a system containing identical symmetric bivalent receptors (antibodies, or immunoglobulins), bivalent ligands (antigens; pathogens or allergens), and monovalent ligands. Bivalent ligands can crosslink two or more bivalent receptors, forming aggregates (or oligomers) comprising multiple ligands and receptors. The monovalent ligands act to inhibit the immune response, since while a site on a bivalent receptor is occupied by a monovalent ligand, no crosslinking reaction can occur. The original Dembo and Goldstein model (Dembo and Goldstein 1978) for the dynamic equilibrium between complexes in this system made several key assumptions to facilitate analytical progress, among them: (1) bivalent ligand is present in the mixture in great excess, so that the amount of free (unbound) ligand present may be assumed to be the same as the total amount of ligand in the original mixture; and (2) the affinities of the monovalent and singly-bound bivalent ligands for the binding sites of bivalent receptors are identical. Both of these conditions are relaxed in our model.

The Dembo and Goldstein model has also been generalized in several different directions by these and other authors, to account for heterogeneous populations of bivalent receptors (Goldstein and Wofsy 1980), for asymmetrical bivalent ligands (Wofsy 1980), and for cooperativity in the binding (Wofsy and Goldstein 1987). More recently, Posner et al. (1995a, 1995b) studied a time-dependent model that predicts the concentrations of bivalent receptors and ligands in a mixture as functions of time, and Barisas (2003) proposed a model similar to that of Dembo and Goldstein that describes a population of bivalent receptors, and ligands with valences ≥ 2. Other approaches to modeling such complex multivalent systems include so-called "rule-based modeling"; see, e.g., Colvin et al. (2009), Faeder et al. (2005), and Hlavacek et al. (2006).

Using the original model of Dembo and Goldstein, Goldstein (1988) reported an exact result for the total concentration of bivalent ligand that maximizes the concentration of complexes containing two or more bivalent receptors. Such an explicit prediction is very useful, as an experiment may then be carried out and compared with the model prediction, enabling estimates to be made of various dissociation or binding constants. Mack et al. (2011) carried out a similar analysis for their extended model to reduce the system to three algebraic equations, which are readily solved numerically. Here, we consider the same extended system, but focus principally on specific asymptotic limits in which analytical progress can be made. We analyze how concentrations of key (experimentally-measurable) quantities vary as a function of the system parameters. Determining such functional relationships allows us to extract the values of system parameters—such as the dissociation constants, which are difficult to measure directly—from experimentally-measurable data (Hendrickson et al. 2002; Hlavacek et al. 1999; Posner et al. 2002; Sklar et al. 2002). Such information is useful as it provides a means of quantifying the response of various antibodies to given

pathogens. In contrast to previous approaches based on numerical calculations (e.g., Mack et al. 2011), we find analytical expressions that can be used to determine system parameters directly from experimental data (see also Mack et al. 2008). We expect the simple analytical expressions presented here to be of great interest to experimentalists who are unfamiliar with numerical techniques. Specific experiments are suggested that will allow the experimentalist to use the analytical formulae to determine dissociation constants for the system.

In order to facilitate the use of the paper by nonmathematicians, each section of mathematical analysis is followed by a separate subsection (identified by the title *Determining the dissociation constants*), suggesting how the results might be used to determine certain of the dissociation constants in the laboratory. Additionally, two tables summarize the results of this paper: Table 1 lists the definitions and Table 2 the main analytical expressions determined in this study.

2 Reaction Scheme and Mathematical Model

Following Mack et al. (2011), we consider a model system in which identical symmetric bivalent receptors, identical symmetric bivalent ligands, and identical monovalent ligands (inhibitor molecules) are present. The various possible complexes that may form in such a system, and the (dynamic) equilibria between them, are detailed in Scheme 1 of that paper, which appears in adapted form here as Fig. 1. Individual equilibria are depicted in Fig. 1 as pairs of arrows pointing in opposite directions: $\rightleftharpoons$. Each of the two binding sites of a bivalent receptor (depicted in Fig. 1 as $\sqcup$) can noncovalently bind to either one monovalent ligand (depicted as $\circ$) or to one of the two binding moieties of a bivalent ligand (depicted as $\bullet\!\!-\!\!\bullet$). The uppermost block of Fig. 1 shows the complete set of seven possible receptor-ligand complexes that can form with one bivalent receptor: the unbound (free) receptor (identified in Fig. 1 as Z_1); the bivalent receptor bound to one of the binding sites of one bivalent ligand (A_1); the bivalent receptor bound to one of the binding sites of two bivalent ligands simultaneously (B_1); the bivalent receptor bound to the two binding sites of one bivalent ligand (C_1); the bivalent receptor bound to one monovalent ligand (D_1); the bivalent receptor bound to two monovalent ligands (E_1); and the bivalent receptor bound to one of the binding sites of one bivalent ligand and to one monovalent ligand simultaneously (F_1). The subscript 1 indicates that these molecular complexes contain only one bivalent receptor. The central block in Fig. 1 shows the complexes containing exactly two bivalent receptors: accordingly, the subscript for the complexes in this block is 2. In general, the seven complexes with n bivalent receptors are described as A_n, B_n, C_n, D_n, E_n, and F_n (bottom block of Fig. 1).

The detailed description of the equilibria of the system studied here can be found elsewhere (Mack et al. 2011). Briefly, the equilibria between all the molecular species in this system are described through the product of stoichiometric factors (derived from the ratio of the number of binding moieties to the available number of binding sites) and four dissociation constants (Connors 1987; Mack et al. 2011). The *monovalent dissociation constant* $K_d^{*\mathrm{mono}}$ relates the concentration of free (unbound) monovalent ligand (identified as I in Fig. 1); the concentration of bivalent receptors (either free or bound) with at least one available binding site (A_n, D_n, Z_n in Fig. 1);

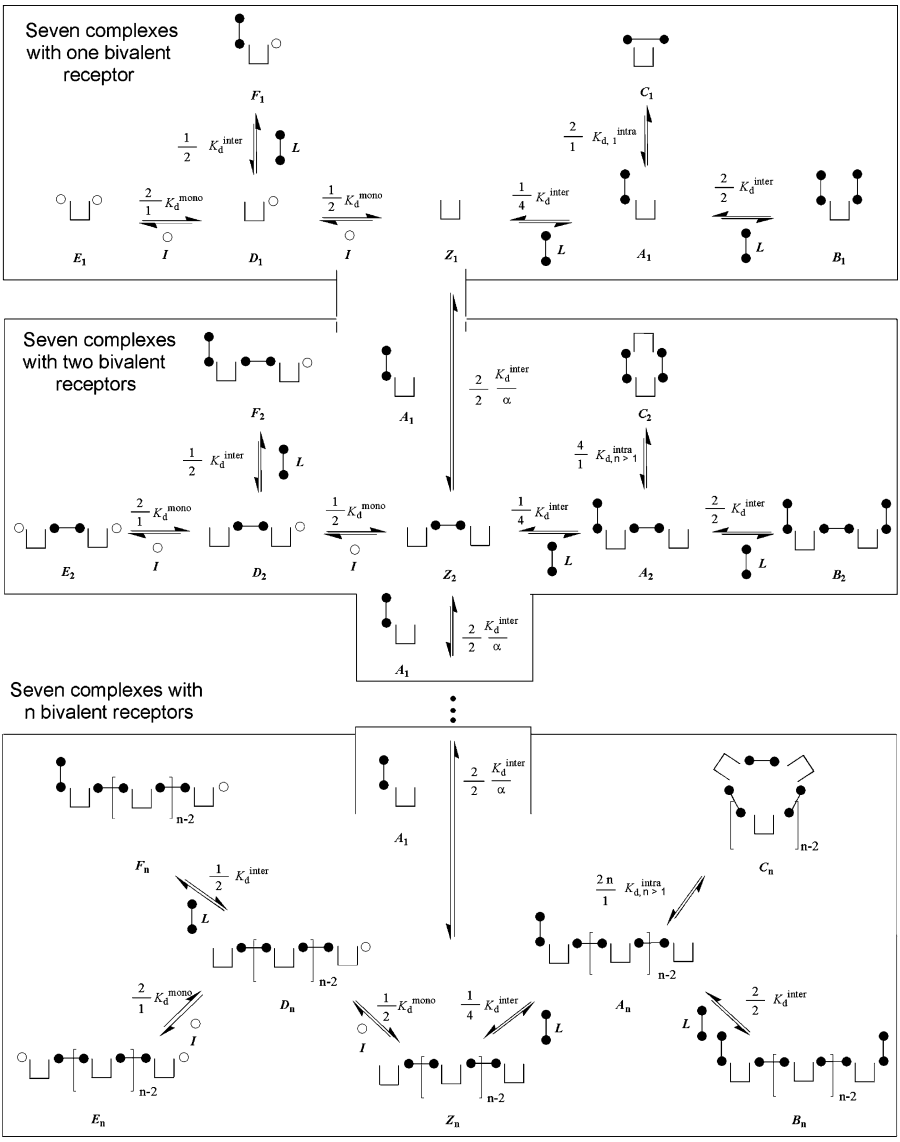


Fig. 1 Summary of all possible dynamic equilibria between symmetric bivalent ligands (●—●), symmetric bivalent receptors (⊔), and monovalent ligands (○), adapted from Mack et al. (2011)

and the concentration of the complex after the monovalent ligand is bound to the available receptor site (F_n, E_n, D_n, respectively, for the preceding list). The *intermolecular dissociation constant* $K_d^{*\text{inter}}$ relates the concentration of free (unbound) bivalent ligand (identified as L in Fig. 1); the concentration of bivalent receptors (either free or bound) with at least one available binding site (A_n, D_n, Z_n in Fig. 1); and the concentration of the resulting complex when the bivalent ligand is bound to the available receptor site (B_n, F_n, A_n, respectively, for the preceding list). The

intramolecular dissociation constant $K_d^{*\text{intra}}$ relates the concentration of complexes exhibiting a bivalent ligand with one free moiety, the other being bound to a complex presenting a bivalent receptor with an unoccupied binding site (A_n in Fig. 1); and the concentration of circular or cyclic complexes with all binding moieties of bivalent ligands and all binding sites of bivalent receptors bound to each other (C_n in Fig. 1). Finally, the *cooperativity parameter* α describes the tendency of complexes with $(n-1)$ bivalent receptors to form complexes with n receptors. Equations (1)–(6) describe each of the equilibria in the bottom block of Fig. 1 as a function of the dissociation constants introduced above. In this manuscript, the presence of an asterisk $(*)$ denotes that a quantity is dimensional (we nondimensionalize below and drop the $*$); in all cases, the dimensions are those of concentration, i.e., moles per volume. The overbrace in the equations denotes the "cyclic" complex, where a chain of alternating ligand-receptor pairs is joined up into a ring (intramolecular bonding) by a single bivalent ligand, which joins the two end receptors of the chain together.

In our model, the intramolecular dissociation constant may depend on the size n of the cyclic complex formed: $K_{d,n}^{*\text{intra}}$. This size-dependence is another important difference between our model and that of Dembo and Goldstein (1978). We consider two cases to encompass intramolecular binding: (i) The first case describes the possibility that the bivalent ligand is too short to span the distance between the two binding sites of a bivalent receptor, which ultimately prevents the formation of mono-receptor cyclic complexes (C_1^*). Mathematically, this case is described with $K_{d,1}^{*\text{intra}} = \infty$ while all $K_{d,n>1}^{*\text{intra}}$ are equal. (ii) The second case is that of bivalent ligands long enough to bind the two sites of one bivalent receptor simultaneously. Mathematically, this case has all $K_{d,n}^{*\text{intra}}$ equal.

The equilibrium between free bivalent receptors (Z_1^*) and complexes of *two* bivalent receptors with two available binding sites (Z_2^*) is described through the cooperativity parameter α as $Z_2^* = (\alpha/K_d^{*\text{inter}})Z_1^*A_1^* = (4\alpha/(K_d^{*\text{inter}})^2)(Z_1^*)^2L^*$ (the second equality on substitution for $A_1^* = (4/K_d^{*\text{inter}})Z_1^*L^*$). The general situation is summarized by the set of equations below, with the equilibrium between Z_{n-1}^* and Z_n^* given by (7):

$$\left[\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\right] = \frac{4}{K_d^{*\text{inter}}}\left[\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\right][\bullet\!\!-\!\!\bullet], \qquad A_n^* = \frac{4}{K_d^{*\text{inter}}}Z_n^*L^*, \tag{1}$$

$$\left[\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\right] = \frac{1}{K_d^{*\text{inter}}}\left[\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\right][\bullet\!\!-\!\!\bullet], \qquad B_n^* = \frac{1}{K_d^{*\text{inter}}}A_n^*L^*, \tag{2}$$

$$\overbrace{\left[\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\right]} = \frac{1}{2nK_{d,n}^{*\text{intra}}}\left[\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\right], \qquad C_n^* = \frac{1}{2nK_{d,n}^{*\text{intra}}}A_n^*, \tag{3}$$

$$\left[\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\circ\right] = \frac{2}{K_d^{*\text{mono}}}\left[\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\right][\circ], \qquad D_n^* = \frac{2}{K_d^{*\text{mono}}}Z_n^*I^*, \tag{4}$$

$$\left[\circ\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\circ\right] = \frac{1}{2K_d^{*\text{mono}}}\left[\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\circ\right][\circ], \qquad E_n^* = \frac{1}{2K_d^{*\text{mono}}}D_n^*I^*, \tag{5}$$

$$\left[\bullet\!\!-\!\!\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\circ\right] = \frac{2}{K_d^{*\text{inter}}}\left[\sqcup\!\!-\!\!\bullet\!\!-\!\!\bullet\cdots\bullet\!\!-\!\!\bullet\!\!-\!\!\sqcup\!\!-\!\!\circ\right][\bullet\!\!-\!\!\bullet], \qquad F_n^* = \frac{2}{K_d^{*\text{inter}}}D_n^*L^*, \tag{6}$$

$$\begin{aligned}\left[\sqcup^{\bullet\bullet}\cdots^{\bullet\bullet}\sqcup\right] &= \left(\frac{4\alpha}{(K_d^{*\mathrm{inter}})^2}\right)^{n-1}[\bullet\!\!-\!\!\bullet]^{n-1}[\sqcup]^n,\\ Z_n^* &= \left(\frac{4\alpha}{(K_d^{*\mathrm{inter}})^2}\right)^{n-1}(L^*)^{n-1}(Z_1^*)^n.\end{aligned} \tag{7}$$

This system of equilibria is augmented by mass conservation conditions, which state that the total amount of receptor (R_{tot}^*), ligand (L_{tot}^*), and inhibitor (I_{tot}^*) in the system must be conserved. Accounting for the amounts in complexes of all sizes, these conditions are

$$R_{\mathrm{tot}}^* = \sum_{n=1}^{\infty} n\left(Z_n^* + A_n^* + B_n^* + C_n^* + D_n^* + E_n^* + F_n^*\right), \tag{8}$$

$$\begin{aligned}L_{\mathrm{tot}}^* = L^* + \sum_{n=1}^{\infty}\big((n-1)Z_n^* + nA_n^* + (n+1)B_n^* + nC_n^* + (n-1)D_n^*\\ + (n-1)E_n^* + nF_n^*\big),\end{aligned} \tag{9}$$

$$I_{\mathrm{tot}}^* = I^* + \sum_{n=1}^{\infty}\left(D_n^* + 2E_n^* + F_n^*\right). \tag{10}$$

Normalizing all concentrations with $K_d^{*\mathrm{inter}}$, (1)–(7) become

$$A_n = 4Z_nL, \tag{11}$$

$$B_n = A_nL, \tag{12}$$

$$C_n = \frac{1}{2nK_n^{\mathrm{intra}}}A_n, \tag{13}$$

$$D_n = \frac{2}{K^{\mathrm{mono}}}Z_nI, \tag{14}$$

$$E_n = \frac{1}{2K^{\mathrm{mono}}}D_nI, \tag{15}$$

$$F_n = 2D_nL, \tag{16}$$

$$Z_n = (4\alpha LZ_1)^{n-1}Z_1, \tag{17}$$

where $K^{\mathrm{mono}} = K_d^{*\mathrm{mono}}/K_d^{*\mathrm{inter}}$, $K_n^{\mathrm{intra}} = K_{d,n}^{*\mathrm{intra}}/K_d^{*\mathrm{inter}}$ (we drop subscripts d in the dimensionless model), $A_n = A_n^*/K_d^{*\mathrm{inter}}$ and similarly for all new unstarred quantities introduced. The mass conservation equations (8)–(10) are unchanged except that $*$'s are dropped as quantities become dimensionless, and we can simplify by replacing (9) with ((8) minus (9)). In dimensionless form then, (8)–(10) are equivalent to

$$R_{\mathrm{tot}} = \sum_{n=1}^{\infty} n(Z_n + A_n + B_n + C_n + D_n + E_n + F_n), \tag{18}$$

$$R_{\mathrm{tot}} - L_{\mathrm{tot}} = -L + \sum_{n=1}^{\infty}(Z_n - B_n + D_n + E_n), \tag{19}$$

$$I_{\mathrm{tot}} = I + \sum_{n=1}^{\infty}(D_n + 2E_n + F_n). \tag{20}$$

The complete mathematical model of the equilibria shown in Fig. 1 consists of (11)–(20). In general, we must solve this system of 10 equations for quantities A_n, B_n, C_n, D_n, E_n, F_n, Z_n, Z_1, L, I, given the total amounts of receptor, ligand, and inhibitor, R_{tot}, L_{tot}, I_{tot} added to the system initially, and the values of the normalized dissociation constants K_n^{intra}, K^{mono}. We simplify matters by noting that we may substitute for Z_n from (17) into (11) and (14), and then for A_n from (11) into (12) and (13), and finally for D_n from (14) into (15) and (16). This sequence of operations gives expressions for all quantities A_n–F_n, and Z_n, in terms of Z_1, L and I. Moreover, on substitution of these expressions into the conservation equations (18)–(20), we are able to compute the required infinite sums explicitly, assuming $4\alpha L Z_1 < 1$ (this condition, which can be checked a posteriori, is mathematically necessary for convergence of the infinite sums; failure to satisfy this condition means that the sums do not converge, and would point physically to some kind of bifurcation in the system, where all the mass ends up in extremely large complexes). We have

$$\sum_1^\infty Z_n = Z_1 \sum_0^\infty (4\alpha L Z_1)^k = \frac{Z_1}{1-4\alpha L Z_1},$$

$$\sum_1^\infty A_n = 4L \sum_1^\infty Z_n = \frac{4LZ_1}{1-4\alpha L Z_1},$$

$$\sum_1^\infty B_n = L \sum_1^\infty A_n = \frac{4L^2 Z_1}{1-4\alpha L Z_1},$$

$$\sum_1^\infty D_n = \frac{2I}{K^{\text{mono}}} \sum_1^\infty Z_n = \frac{2IZ_1}{K^{\text{mono}}(1-4\alpha L Z_1)},$$

$$\sum_1^\infty E_n = \frac{I}{2K^{\text{mono}}} \sum_1^\infty D_n = \frac{I^2 Z_1}{(K^{\text{mono}})^2(1-4\alpha L Z_1)},$$

$$\sum_1^\infty F_n = 2L \sum_1^\infty D_n = \frac{4ILZ_1}{K^{\text{mono}}(1-4\alpha L Z_1)}$$

(we do not require $\sum C_n$, though this can be evaluated). For the receptor conservation law (18), we also need to evaluate $\sum nZ_n$ and similar quantities, for which we note, writing $x = 4\alpha L Z_1$, that

$$\sum_1^\infty nZ_n = Z_1 \sum_1^\infty n x^{n-1} = Z_1 \frac{d}{dx}\left(\sum_0^\infty x^n\right) = Z_1 \frac{d}{dx}\left((1-x)^{-1}\right) = \frac{Z_1}{(1-x)^2}.$$

Hence,

$$\sum_1^\infty nZ_n = \frac{Z_1}{(1-4\alpha L Z_1)^2},$$

$$\sum_1^\infty nA_n = 4L \sum_1^\infty nZ_n = \frac{4LZ_1}{(1-4\alpha L Z_1)^2},$$

$$\sum_1^\infty nB_n = L\sum_1^\infty nA_n = \frac{4L^2Z_1}{(1-4\alpha LZ_1)^2}.$$

For $\sum nC_n$, we have $\sum nC_n = (1/2)\sum(A_n/K_n^{\text{intra}})$, and as stated, we wish to consider the possibility that the dissociation constants K_n^{intra} vary with n. For general n-dependence, we cannot compute the sum explicitly, but we consider the two special stated cases of relevance:

- *Case* (i), where $K_1^{\text{intra}} = \infty$ and all other K_n^{intra} are equal (short bivalent ligand); and
- *Case* (ii), where all the K_n^{intra}, including K_1^{intra}, are equal (long bivalent ligand).

In case (i), the cyclic complex with just one receptor and one inhibitor, $\overset{\bullet\bullet}{\sqcup}$, is not formed (for example, if the ligand is too short to span the two sites of a single bivalent receptor), but all other cyclic complexes are equally likely to form. We have (dropping the subscript n from K_n^{intra} henceforth)

$$\sum_1^\infty nC_n = \frac{1}{2K^{\text{intra}}}\sum_2^\infty A_n = \frac{1}{2K^{\text{intra}}}\left(\frac{4LZ_1}{1-4\alpha LZ_1} - 4LZ_1\right)$$
$$= \frac{8\alpha L^2Z_1^2}{K^{\text{intra}}(1-4\alpha LZ_1)} \quad \big(\text{case (i)}\big),$$

and

$$\sum_1^\infty nC_n = \frac{1}{2K^{\text{intra}}}\sum_1^\infty A_n = \frac{2LZ_1}{K^{\text{intra}}(1-4\alpha LZ_1)} \quad \big(\text{case (ii)}\big).$$

We also have

$$\sum_1^\infty nD_n = \frac{2I}{K^{\text{mono}}}\sum_1^\infty nZ_n = \frac{2IZ_1}{K^{\text{mono}}(1-4\alpha LZ_1)^2},$$
$$\sum_1^\infty nE_n = \frac{I}{2K^{\text{mono}}}\sum_1^\infty nD_n = \frac{I^2Z_1}{(K^{\text{mono}})^2(1-4\alpha LZ_1)^2},$$
$$\sum_1^\infty nF_n = 2L\sum_1^\infty nD_n = \frac{4ILZ_1}{K^{\text{mono}}(1-4\alpha LZ_1)^2}.$$

Substitution of these expressions into the conservation equations (18)–(20) then reduces the whole system to three algebraic equations for Z_1, L and I, which after simplification take the form:

$$R_{\text{tot}} = \frac{Z_1}{(1-4\alpha LZ_1)^2}\left[\left(1+\frac{I}{K^{\text{mono}}}+2L\right)^2 + \frac{8\alpha L^2Z_1}{K^{\text{intra}}}(1-4\alpha LZ_1)\right]$$
in case (i), (21)

$$R_{\text{tot}} = \frac{Z_1}{(1-4\alpha LZ_1)^2}\left[\left(1+\frac{I}{K^{\text{mono}}}+2L\right)^2 + \frac{2L}{K^{\text{intra}}}(1-4\alpha LZ_1)\right]$$
in case (ii), (22)

$$R_{\text{tot}} = L_{\text{tot}} - L + \frac{Z_1}{(1 - 4\alpha L Z_1)}\left(1 + \frac{I}{K^{\text{mono}}} + 2L\right)\left(1 + \frac{I}{K^{\text{mono}}} - 2L\right), \quad (23)$$

$$I_{\text{tot}} = I + \frac{2IZ_1}{K^{\text{mono}}(1 - 4\alpha L Z_1)}\left(1 + \frac{I}{K^{\text{mono}}} + 2L\right), \quad (24)$$

where (23) and (24) apply in both cases (i) and (ii).

3 Predictive Analysis

We want to use our model to extract information such as the values of dissociation constants for the system. One approach is the following: A quantity that the experimentalist can determine in any given experiment is the concentration of complexes of size $n \geq 2$ (by which we mean, complexes containing at least two receptor molecules) in the mixture. Consider a titration experiment in which ligand is slowly added to known (fixed) amounts of receptor and inhibitor. If we monitor the concentration of complexes as a function of ligand concentration (methods for doing this are discussed by, for example, Hendrickson et al. 2002, Hlavacek et al. 1999, Posner et al. 2002, Sklar et al. 2002, and references therein), then we should be able to compare this concentration profile, or at least key features of it, with predictions from our model. Easy features to compare could be the value of the ligand concentration at which the concentration of complexes is maximized (and/or the value of this maximum concentration), or we might measure the initial gradient of the titration concentration curve, and compare this with predictions from the model.

In our notation, the relative concentration of complexes of size $n \geq 2$, which we call $\tilde{f}_1$, is

$$\tilde{f}_1 = \frac{1}{R_{\text{tot}}}\big(R_{\text{tot}} - (Z_1 + A_1 + B_1 + C_1 + D_1 + E_1 + F_1)\big), \quad (25)$$

so that in case (i)

$$\tilde{f}_1 = 1 - \frac{Z_1}{R_{\text{tot}}}\left(1 + \frac{I}{K^{\text{mono}}} + 2L\right)^2, \quad (26)$$

while in case (ii)

$$\tilde{f}_1 = 1 - \frac{Z_1}{R_{\text{tot}}}\left[\left(1 + \frac{I}{K^{\text{mono}}} + 2L\right)^2 + \frac{2L}{K^{\text{intra}}}\right]. \quad (27)$$

Although in principle any required information (a turning-point, or a gradient of $\tilde{f}_1$ at a given point) may be computed numerically for *given* values of the system parameters, determining such information analytically, in terms of arbitrary system parameter values, using (21)–(24), is very difficult. In the following sections, we discuss certain simplified cases in which analytical progress may be made, and the predictive powers of the reduced models.

4 Case Without Monovalent Inhibitor

The main simplification of relevance is when no inhibitor (monovalent ligand) is present. In this case, (24) reduces to an identity, and (21) (or (22)) and (23) simplify:

$$R_{\text{tot}} = \frac{Z_1}{(1-4\alpha L Z_1)^2}\left[(1+2L)^2 + \frac{8\alpha L^2 Z_1}{K^{\text{intra}}}(1-4\alpha L Z_1)\right]$$
in case (i), or (28)

$$R_{\text{tot}} = \frac{Z_1}{(1-4\alpha L Z_1)^2}\left[(1+2L)^2 + \frac{2L}{K^{\text{intra}}}(1-4\alpha L Z_1)\right]$$
in case (ii), and (29)

$$R_{\text{tot}} = L_{\text{tot}} - L + \frac{Z_1(1-4L^2)}{(1-4\alpha L Z_1)}. \tag{30}$$

Solving (30) for Z_1,

$$Z_1 = \frac{L + R_{\text{tot}} - L_{\text{tot}}}{1-4(1-\alpha)L^2 + 4\alpha L(R_{\text{tot}} - L_{\text{tot}})} \tag{31}$$

and substituting in (28) or (29) gives a sixth-order polynomial equation for the free ligand concentration L in case (i), and a quartic in case (ii). Neither have general analytical solution, but special cases may be solved exactly.

4.1 Cooperativity $\alpha = 1$. Case (ii), Long Bivalent Ligand

Setting $\alpha = 1$ lowers (by one) the degree of the polynomial satisfied by L, so that case (ii) reduces to a cubic for L, with analytical solution. Case (i) is governed by a quintic and is still intractable in general. So, we first consider case (ii) with $\alpha = 1$ which, as we shall see, provides insight into the short bivalent ligand case (i). In case (ii) ($\alpha = 1$), L satisfies

$$\begin{aligned}
&4\big(1+2K^{\text{intra}}L_{\text{tot}}\big)L^3 \\
&\quad - 2L^2\big(1-2R_{\text{tot}}+2L_{\text{tot}}+K^{\text{intra}}\big(4L_{\text{tot}}^2+(1+2R_{\text{tot}})^2-2L_{\text{tot}}(1+4R_{\text{tot}})\big)\big) \\
&\quad + L\big(2L_{\text{tot}}-2R_{\text{tot}}-K^{\text{intra}}\big(4L_{\text{tot}}^2+(1+2R_{\text{tot}})^2-2L_{\text{tot}}(1+4R_{\text{tot}})\big)\big) \\
&\quad + K^{\text{intra}}L_{\text{tot}} = 0
\end{aligned} \tag{32}$$

or, introducing convenient shorthand notation for the coefficients in (32) (considering R_{tot} and K^{intra} fixed but allowing L_{tot} to vary)

$$g(L, L_{\text{tot}}) := b_0(L_{\text{tot}})L^3 + b_1(L_{\text{tot}})L^2 + b_2(L_{\text{tot}})L + b_3(L_{\text{tot}}) = 0. \tag{33}$$

The cubic (33) has three solutions, given in terms of the following parameters:

$$Q = \frac{3b_2b_0 - b_1^2}{9b_0^2}, \qquad R = \frac{9b_0b_1b_2 - 27b_0^2b_3 - 2b_1^3}{54b_0^3},$$
$$D = Q^3 + R^2, \qquad S = \big(R + \sqrt{D}\big)^{1/3}, \qquad T = \big(R - \sqrt{D}\big)^{1/3},$$

with $ST = -Q$. The solutions are

$$L_1(L_{\text{tot}}) = S + T - \frac{b_1}{3b_0}, \tag{34}$$

$$L_2(L_{\text{tot}}) = -\frac{1}{2}(S+T) - \frac{b_1}{3b_0} + \frac{i\sqrt{3}}{2}(S-T), \tag{35}$$

$$L_3(L_{\text{tot}}) = -\frac{1}{2}(S+T) - \frac{b_1}{3b_0} - \frac{i\sqrt{3}}{2}(S-T), \tag{36}$$

and here the discriminant $D \leq 0$ always, which leads to real (and in general, distinct) roots (S and T are complex conjugates). Hence, $L(L_{\text{tot}})$ is given by the relevant root (real, positive and, to guarantee convergence of the infinite sums, such that $0 < 4LZ_1 < 1$), with Z_1 then given by

$$Z_1 = \frac{L + R_{\text{tot}} - L_{\text{tot}}}{1 + 4L(R_{\text{tot}} - L_{\text{tot}})} \tag{37}$$

from (31). It is not difficult to check that there is always exactly one relevant solution for L: although two solutions are positive and, therefore, possible candidates, one of these always leads to $4LZ_1 > 1$ and so is unacceptable. The relevant solution is the smaller positive root (though which of the three expressions (34), (35), or (36) this is given by varies depending on the values of the parameters, as we shall see explicitly below).

A special case of the system (32), (37) arises when $L_{\text{tot}} = R_{\text{tot}} + 1/2$. When this happens, the cubic equation (32) has a simple factorization:

$$(1-2L)^2\big(L\big(1+K^{\text{intra}}\big) + K^{\text{intra}}(R_{\text{tot}} + 1/2)\big) = 0,$$

and the only relevant root is the repeated one, $L = 1/2$.[1] This situation corresponds to a maximum value (with respect to L_{tot}) of the quantity $\tilde{f}_1$ defined in (27), as we shall now see.

With $L_{\text{tot}} = R_{\text{tot}} + 1/2$ and $L = 1/2$, the solution (37) for Z_1 is undefined, and careful local analysis is required to determine its value. This we do by examining the cubic equation (32) when $L_{\text{tot}} = R_{\text{tot}} + 1/2 + \epsilon$ for $|\epsilon| \ll 1$, and determining the appropriate root L, correct to order ϵ. For ϵ of either sign (but small), we have

$$L = \frac{1}{2} + \epsilon L_1 + O\big(\epsilon^2\big), \qquad Z_1 = \frac{1 - L_1}{2(1+L_1)} + O(\epsilon), \tag{38}$$

$$\text{where } L_1 = \frac{1}{2\lambda}\big[1 + \big(1 + 8\lambda K^{\text{intra}}\big)^{1/2}\big], \qquad \lambda = 1 + 2K^{\text{intra}}(1 + 2R_{\text{tot}}). \tag{39}$$

Further asymptotic analysis enables us to determine higher-order corrections to Z_1 and L. Substituting these asymptotic expressions for Z_1 and L into our expression (27) (setting $I = 0$) for $\tilde{f}_1$, we find that $\tilde{f}_1$ has no order-ϵ contribution, and that its order ϵ^2 correction term is strictly negative—that is, considered as a function of L_{tot}, it has a local maximum at the value $L_{\text{tot}} = R_{\text{tot}} + 1/2$. The value of $\tilde{f}_1$

[1] Mathematically, L_{tot} increasing through the value $R_{\text{tot}} + 1/2$ corresponds to the "crossing over" of the two positive roots mentioned above: the smaller root increases through the value $1/2$ and then ceases to be relevant, while the larger root decreases through $1/2$ and becomes the relevant one. Moreover, at this value of L_{tot}, examination of (37) gives $Z_1 = -1/2$ (an unphysical result) except in the special case $L = 1/2$. Thus for $L_{\text{tot}} = R_{\text{tot}} + 1/2$ we always require $L = 1/2$.

at this local maximum is found by setting $L_{\text{tot}} = R_{\text{tot}} + 1/2$, $I = 0$, $L = 1/2$, and $Z_1 = (1 - L_1)/(2(1 + L_1))$ in (27), with L_1 given by (39). This procedure gives (after simplification)

$$\tilde{f}_{1,\max} = 1 - \frac{(1 + 4K^{\text{intra}})[2\lambda - 1 - \sqrt{1 + 8\lambda K^{\text{intra}}}]}{2K^{\text{intra}} R_{\text{tot}}[2\lambda + 1 + \sqrt{1 + 8\lambda K^{\text{intra}}}]} \tag{40}$$

with λ as given in (39) above.

4.1.1 Determining the Dissociation Constants

If the value of the cooperativity parameter α is close to unity, then these results can be used to determine the dissociation constants $K_d^{*\text{inter}}$, $K_d^{*\text{intra}}$, as follows. Note that, since the model is made dimensionless by scaling concentrations with the unknown $K_d^{*\text{inter}}$, we must here work with dimensional quantities that the experimentalist would know. Carry out a titration with no inhibitor, and a known concentration of receptor, R_{tot}^*. At regular points throughout the experiment, measure the concentration C^* of complexes of size $n \geq 2$ which, in the notation above, is given by $C^* = R_{\text{tot}}^* \tilde{f}_1$ (e.g. Hendrickson et al. 2002; Hlavacek et al. 1999; Posner et al. 2002; Sklar et al. 2002). Determine, as closely as possible (perhaps using a curve-fit to extrapolate if only a few data points are taken) the value of added ligand, L_{tot}^*, at which C^* is maximized, and the corresponding maximal value, $C_{\max}^* = R_{\text{tot}}^* \tilde{f}_{1,\max}$. We know, from the analysis above that the maximum is obtained when $L_{\text{tot}} = R_{\text{tot}} + 1/2$ or, in dimensional terms, when

$$L_{\text{tot}}^* = R_{\text{tot}}^* + \frac{K_d^{*\text{inter}}}{2}.$$

Hence, from the recorded maximizing value of L_{tot}^*, we obtain $K_d^{*\text{inter}}$.

Next, we know that at the maximum, (40) holds. With $K_d^{*\text{inter}}$ now determined, we know all quantities in (40) except for K^{intra} ($R_{\text{tot}} = R_{\text{tot}}^*/K_d^{*\text{inter}}$, and $\tilde{f}_{1,\max} = C_{\max}^*/R_{\text{tot}}^*$). Hence, (40) is a nonlinear equation to be solved for K^{intra}. After rearrangement, this reduces to a cubic, one root of which is the physically-relevant one (it is possible that ambiguity will arise at this stage). Finally, then $K_d^{*\text{intra}} = K_d^{*\text{inter}} K^{\text{intra}}$.

4.2 Cooperativity $\alpha = 1$. Case (i), Short Bivalent Ligand

Remarkably, although case (i) is governed by a different, more complicated (quintic, rather than quartic) equation for L in the case $I = 0$, $\alpha = 1$, a similar simplification occurs at the same value $L_{\text{tot}} = R_{\text{tot}} + 1/2$ as in case (ii). The system is governed by coupled equations

$$R_{\text{tot}} = \frac{Z_1}{(1 - 4LZ_1)^2}\left[(1 + 2L)^2 + \frac{8L^2 Z_1}{K^{\text{intra}}}(1 - 4LZ_1)\right], \tag{41}$$

$$Z_1 = \frac{L - L_{\text{tot}} + R_{\text{tot}}}{1 + 4L(R_{\text{tot}} - L_{\text{tot}})}, \tag{42}$$

(set $\alpha = 1$ in (28) and (31)). Substitution of Z_1 from (42) into (41) leads to a quintic for L, which factorizes in the special case $L_{\text{tot}} = R_{\text{tot}} + 1/2$, with a triple factor $(2L - 1)$ emerging:

$$0 = (2L-1)^3\big(4L^2 - 2LK^{\text{intra}}(1+2R_{\text{tot}}) + K^{\text{intra}}(1+2R_{\text{tot}})\big). \tag{43}$$

This equation has five real roots for L: $L = 1/2$ (with multiplicity 3), and two others, neither of which are physically relevant (they violate the convergence condition $0 < 4LZ_1 < 1$, leading to $Z_1 = -1/2$ in (42)). Again, with $L_{\text{tot}} = R_{\text{tot}} + 1/2$ and $L = 1/2$, Z_1 is undefined in (42) and we need a local analysis. Setting $L_{\text{tot}} = R_{\text{tot}} + 1/2 + \epsilon$, $L = 1/2 + \epsilon L_1 + \epsilon^2 L_2 + \cdots$, $|\epsilon| \ll 1$ in (43), we can determine L_1, L_2, successively; substitution in (42) then gives Z_1 correct to order ϵ (just as in case (ii), $Z_1 = (1 - L_1)/(2(1 + L_1)) + O(\epsilon)$). The correction L_1 is found to satisfy a cubic equation,

$$\begin{aligned}&\big(1 - 2K^{\text{intra}}(1+2R_{\text{tot}})\big)L_1^3 - 2\big(1 + K^{\text{intra}}(1+2R_{\text{tot}})\big)L_1^2\\ &\quad + \big(1 + 2K^{\text{intra}}\big)L_1 + 2K^{\text{intra}} = 0.\end{aligned} \tag{44}$$

With $L = 1/2 + \epsilon L_1 + O(\epsilon^2)$, $Z_1 = (1 - L_1)/(2(1 + L_1)) + O(\epsilon)$, only one of the three roots L_1 satisfies the convergence criterion $0 < 4LZ_1 < 1$; this is the real root of (44) that lies in the interval $(0, 1)$. We substitute the physically-relevant expressions in

$$\tilde{f}_1 = 1 - \frac{Z_1}{R_{\text{tot}}}(1+2L)^2 \tag{45}$$

(which follows from (26) with $I = 0$ and $\alpha = 1$). Again explicit calculation reveals a local maximum in $\tilde{f}_1$ at $\epsilon = 0$ ($L = 1/2$). The value of $\tilde{f}_1$ at this maximum is obtained by setting $L_{\text{tot}} = R_{\text{tot}} + 1/2$, $L = 1/2$, $Z_1 = (1 - L_1)/(2(1 + L_1))$ in (45), giving

$$\tilde{f}_{1,\max} = 1 - \frac{2(1-L_1)}{R_{\text{tot}}(1+L_1)}, \tag{46}$$

with L_1 the unique root of (44) lying in $(0, 1)$. Identifying the coefficients in (44) by

$$\begin{aligned}&c_0 = 1 - 2K^{\text{intra}}(1+2R_{\text{tot}}), \qquad c_1 = -2\big(1 + K^{\text{intra}}(1+2R_{\text{tot}})\big),\\ &c_2 = 1 + 2K^{\text{intra}}, \qquad c_3 = 2K^{\text{intra}},\end{aligned}$$

the roots are found in terms of parameters

$$\begin{aligned}&Q = \frac{3c_2c_0 - c_1^2}{9c_0^2}, \qquad R = \frac{9c_0c_1c_2 - 27c_0^2c_3 - 2c_1^3}{54c_0^3},\\ &D = Q^3 + R^2, \qquad S = \big(R + \sqrt{D}\big)^{1/3}, \qquad T = \big(R - \sqrt{D}\big)^{1/3},\end{aligned}$$

(with $ST = -Q$). The three solutions are

$$\begin{aligned}L_{1,1} &= S + T - \frac{c_1}{3c_0},\\ L_{1,2} &= -\frac{1}{2}(S+T) - \frac{c_1}{3c_0} + \frac{i\sqrt{3}}{2}(S-T),\\ L_{1,3} &= -\frac{1}{2}(S+T) - \frac{c_1}{3c_0} - \frac{i\sqrt{3}}{2}(S-T),\end{aligned}$$

and here $D \leq 0$ always, so all roots are real. Which root lies in (0, 1) varies according to the values of K^{intra} and R_{tot}. The results may be summarized as follows: Considered as a function of total ligand concentration L_{tot}, the maximum value of $\tilde{f}_1$ occurs for $L_{\text{tot}} = R_{\text{tot}} + 1/2$. The maximal value at this value of L_{tot} is given by the following expressions (from (46)):

$$0 < K^{\text{intra}} < \frac{1}{2(1+2R_{\text{tot}})}: \quad \tilde{f}_{1,\max} = 1 - \frac{2(1-L_{1,3})}{R_{\text{tot}}(1+L_{1,3})}, \tag{47}$$

$$K^{\text{intra}} > \frac{1}{2(1+2R_{\text{tot}})}: \quad \tilde{f}_{1,\max} = 1 - \frac{2(1-L_{1,1})}{R_{\text{tot}}(1+L_{1,1})}. \tag{48}$$

4.2.1 Determining the Dissociation Constants

As explained for case (ii) above, if cooperativity is close to unity, these results can be used to estimate the dissociation constants $K_d^{*\text{inter}}$ and $K_d^{*\text{intra}}$. The value of $K_d^{*\text{inter}}$ is obtained exactly as explained at the end of Sect. 4.1, using the relation

$$L_{\text{tot}}^* = R_{\text{tot}}^* + \frac{K_d^{*\text{inter}}}{2}$$

that holds when the concentration of complexes of size $n \geq 2$, C^*, is maximized.

The measured value of $C_{\max}^*$ must equal $R_{\text{tot}}^* \tilde{f}_{1,\max}$, where $\tilde{f}_{1,\max} = C_{\max}^*/R_{\text{tot}}^*$ is given by (47) or (48). Either of these relations gives the value of L_1 at the maximum (remember that $R_{\text{tot}} = R_{\text{tot}}^*/K_d^{*\text{inter}}$). It is known that L_1 satisfies (44); with L_1 and R_{tot} known, (44) is a linear equation for the remaining unknown, K^{intra}, giving

$$K^{\text{intra}} = \frac{L_1(1-L_1)^2}{2(1+L_1)[L_1^2(1+2R_{\text{tot}})-1]}.$$

Hence, finally, $K_d^{*\text{intra}} = K_d^{*\text{inter}} K^{\text{intra}}$.

4.3 A Posteriori Checking

Without prior information, it is difficult to know whether or not the cooperativity $\alpha \approx 1$. Some form of check may be provided by carrying out more than one experiment of the kind described in Sects. 4.1.1, 4.2.1, with different values of R_{tot}^*. Each such experiment should yield the same (or approximately the same) values for the dissociation constants $K_d^{*\text{inter}}$, $K_d^{*\text{intra}}$ (the first is easily checked by looking at the values of $(L_{\text{tot}}^* - R_{\text{tot}}^*)$ at the maximum of $\tilde{f}_1$ for all experiments; the values should be the same). Large deviations in the values obtained for $K_d^{*\text{inter}}$ and $K_d^{*\text{intra}}$ would suggest that α is not close to unity. However, if the values are close for all experiments, then the approximation is likely good.

5 General Case—Numerical Simulations

Before considering asymptotic limits that permit analytical progress (enabling explicit predictions to be made) we first present sample numerical computations that

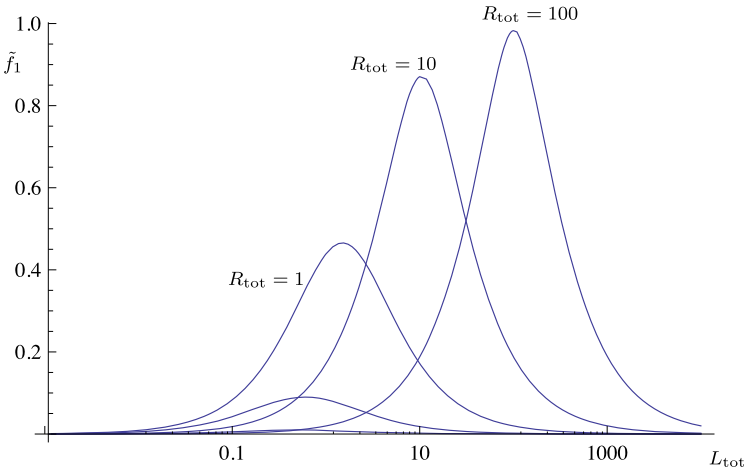


Fig. 2 Fraction of complexes with more than one bivalent receptor ($\tilde{f}_1$) as a function of the total concentration of bivalent ligand $L_{\rm tot}$. *Each curve* corresponds to a different value of the total concentration of bivalent receptor $R_{\rm tot}$; *from the bottom curve upward* the values are: $R_{\rm tot} = 0.01, 0.1, 1, 10, 100$

allow us to investigate the effect of individual system parameters for the general case in which (21)–(24) hold. Such simulations corroborate the analytical results of the preceding section, and provide guidance as to which asymptotic limits might usefully be investigated.

5.1 Dependence on Normalized Total Receptor Concentration $R_{\rm tot}$

Figure 2 shows the fraction of complexes with more than one bivalent receptor ($\tilde{f}_1$) as a function of the total concentration of bivalent ligand $L_{\rm tot}$, at fixed values of the intramolecular dissociation constant $K_n^{\rm intra}$ and the cooperativity parameter α. This simulation corresponds to the analysis of Sect. 4.2, since we simulate the simple case in which total monovalent ligand (inhibitor) concentration $I_{\rm tot} = 0$; cooperativity $\alpha = 1$, and $K_1^{\rm intra} = \infty$, with all other $K_n^{\rm intra} = K^{\rm intra}$ constant for $n \geq 2$ (short bivalent ligand). For this figure, we set $K^{\rm intra} = 0.1$, and used five different values of normalized bivalent receptor concentration $R_{\rm tot}$ as detailed in the caption. The figure bears out the analytical finding of a maximum in $\tilde{f}_1$ at $L_{\rm tot} = R_{\rm tot} + 1/2$.

5.2 Dependence on Cooperativity Parameter α

Figure 3 shows $\tilde{f}_1$ as a function of total bivalent ligand concentration $L_{\rm tot}$, at fixed values of intramolecular dissociation constant $K^{\rm intra}$ and total bivalent receptor concentration $R_{\rm tot}$, and total inhibitor concentration $I_{\rm tot} = 0$. We again consider case (i) (short bivalent ligand), with $K_1^{\rm intra} = \infty$, and all other $K_n^{\rm intra} = K^{\rm intra} = 0.1$ ($n \geq 2$), and $R_{\rm tot} = 0.1$. The plot shows curves for six different values of the cooperativity α; from the bottom curve upwards the values are: $\alpha = 0.1, 1, 10, 100, 1000, 10000$. We observe that the curves become left–right asymmetric as α becomes large. This effect is not observed with the model of Dembo and Goldstein (1978), and is attributable

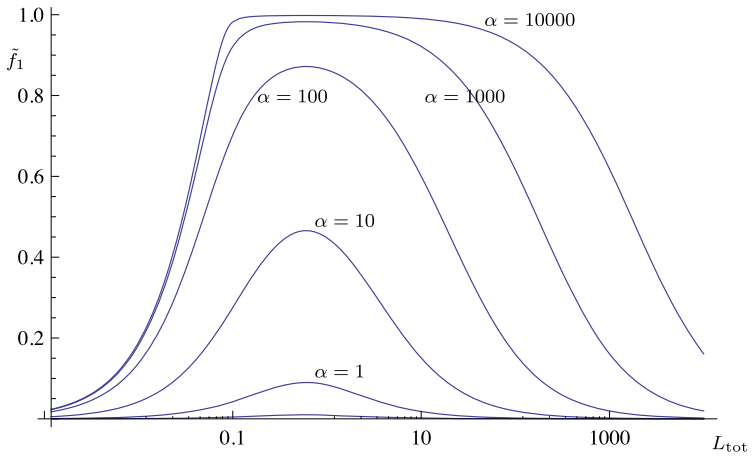


Fig. 3 Fraction of complexes with more than one bivalent receptor ($\tilde{f}_1$) as a function of the total concentration of bivalent ligand L_{tot}. *Each curve* corresponds to a different value of cooperativity α; *from the bottom curve upward* the values are: $\alpha = 0.1$, 1, 10, 100, 1000, 10000

to our dealing explicitly with the concentration of free ligand L, which those authors assumed fixed.

5.3 Dependence on Intramolecular Dissociation Constant K^{intra}

Figures 4, 5 show $\tilde{f}_1$ as a function of total bivalent ligand concentration L_{tot}, at fixed values of α and R_{tot}. Total receptor concentration $R_{\text{tot}} = 0.1$; total inhibitor concentration $I_{\text{tot}} = 0$, and cooperativity $\alpha = 1$ in both plots. For Fig. 4, we consider case (i), with intramolecular dissociation constants $K_1^{\text{intra}} = \infty$, and all other $K_n^{\text{intra}} = K^{\text{intra}}$, constant for $n \geq 2$; thus we are in the situation of Sect. 4.2 (short bivalent ligand). The plot shows curves for seven different values of K^{intra} as detailed in the caption.

For Fig. 5, we consider case (ii), with all K_n^{intra} equal for $n \geq 1$; thus we are in the situation of Sect. 4.1 (long bivalent ligand). The plot shows curves for six different values of K^{intra} as detailed in the caption. Again, both Figs. 4 and 5 corroborate the analytical result of a maximum in $\tilde{f}_1$ at $L_{\text{tot}} = R_{\text{tot}} + 1/2$ with no inhibitor and $\alpha = 1$. The dependence on K^{intra} varies dramatically in the two cases (i) and (ii), with opposite trends being observed. In case (i), as we decrease the dissociation constant K^{intra}, the cyclic complexes are much more likely to form, and here, since the singleton cyclic complex (C_1) cannot form, cyclic complexes with size $n \geq 2$ predominate (in fact, the $n = 2$ cyclic complex will dominate all others). Hence, $\tilde{f}_1$ increases. In case (ii), as we decrease K^{intra} it is easier for all cyclic complexes, including the singleton, to form. Since the singleton forms first, and is unlikely to dissociate at small K^{intra}, this will be the dominant complex, meaning that $\tilde{f}_1$ (which measures complexes containing two or more receptors) goes to zero. This is discussed in detail in Sects. 6.1 and 6.2 below.

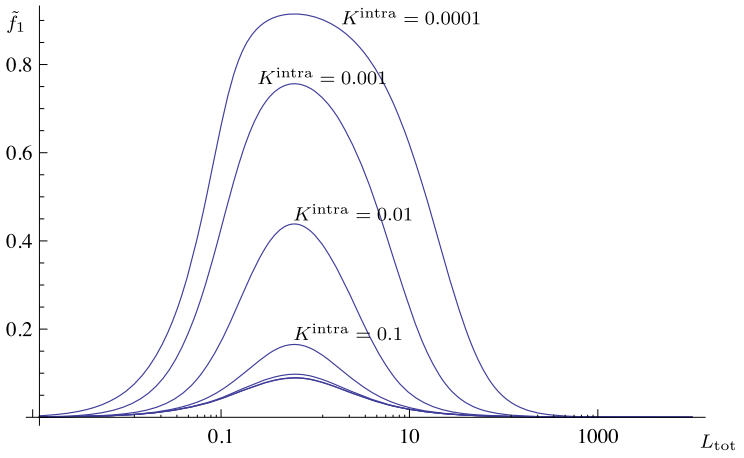


Fig. 4 Fraction of complexes with more than one bivalent receptor ($\tilde{f}_1$) as a function of the total concentration of bivalent ligand L_{tot}. Here, the intramolecular dissociation parameter for mono-receptor complexes $K_1^{\text{intra}} = \infty$, while the intramolecular dissociation constant for complexes with more than one bivalent receptor are all equal, $K_{n>1}^{\text{intra}} = K^{\text{intra}}$ (case (i)). *Each curve* corresponds to a different value of K^{intra}; *from the bottom curve upward* the values are: $K^{\text{intra}} = 100, 10, 1, 0.1, 0.01, 0.001, 0.0001$

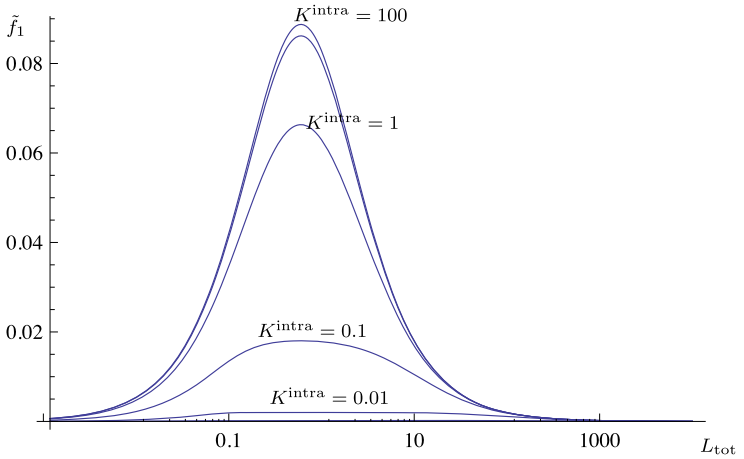


Fig. 5 Fraction of complexes with more than one bivalent receptor ($\tilde{f}_1$) as a function of the total concentration of bivalent ligand L_{tot}. Here, all the intramolecular dissociation constants K^{intra} are equal (case (ii)), and *each curve* corresponds to a different value; *from the bottom curve upward* the values are: $K^{\text{intra}} = 0.001, 0.01, 0.1, 1, 10, 100$

5.4 Dependence on Normalized Total Inhibitor Concentration I_{tot}

Figure 6 shows $\tilde{f}_1$ as a function of total bivalent ligand concentration L_{tot}, at fixed values of K^{intra}, K^{mono} (the dimensionless form of the dissociation constant relating to complexes between monovalent inhibitor and bivalent receptor, as described in Sect. 2), and R_{tot}. Total inhibitor concentration I_{tot} differs for each curve in the plot.

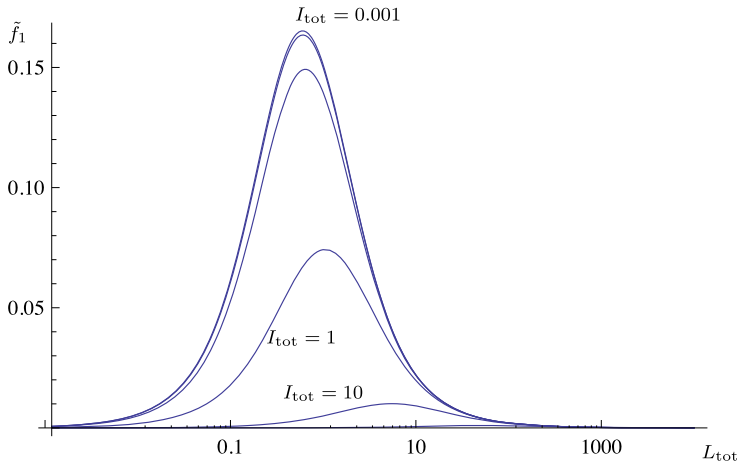


Fig. 6 Fraction of complexes with more than one bivalent receptor ($\tilde{f}_1$) as a function of the total concentration of bivalent ligand $L_{\rm tot}$. *Each curve* corresponds to a different value of the total concentration of monovalent ligand (inhibitor) $I_{\rm tot}$; *from bottom curve upward* the values are: $I_{\rm tot} = 100$, 10, 1, 0.1, 0.01, 0.001

We assume case (i), short bivalent ligands, with $K_1^{\rm intra} = \infty$, and all other $K_n^{\rm intra} = 0.1$ for $n \geq 2$; the normalized inhibitor dissociation constant $K^{\rm mono} = 1$, and $R_{\rm tot} = 0.1$. The plot shows curves for five different values of $I_{\rm tot}$ as detailed in the caption. Strong inhibition suppresses the number of large complexes formed, as we would expect, and also shifts the value of the maximum complex concentration to larger values of added ligand.

6 Asymptotic Analysis

The general case governed by (21)–(24) is highly nonlinear, but considerable analytical progress may be made usefully in certain asymptotic limits. We explore these below, noting the physical interpretation of the limits, and comparing results to the numerical computations of the previous section.

6.1 $K^{\rm intra} = \epsilon$ Asymptotically Small, Case (i) (Short Bivalent Ligand)

If the parameter $K^{\rm intra}$ is small cyclic complexes are favorable and form preferentially compared with linear complexes. In case (i), ligands are short, and the singleton cyclic complex cannot form, so the dominant complex is the cyclic 2-form (C_2). Figure 4 suggests that this asymptotic limit is nontrivial and might yield a useful predictive result.[2] We consider $K^{\rm intra} = \epsilon \ll 1$ in (21), (23), and (24). Solutions depend on the

[2] In case (ii), considered next, the ligand is long enough to form cyclic singletons, and this is the dominant complex in the equilibrium mixture; Fig. 5 suggests that in this limit $\tilde{f}_1 \to 0$ uniformly in $K^{\rm intra}$ and that therefore this limit may be difficult to use predictively.

relative proportions of receptor and ligand present in the original mixture, since one of these will be entirely used up (at leading order), so that either $L \ll 1$ or $Z_1 \ll 1$.

6.1.1 $L_{\text{tot}} < R_{\text{tot}}$

In this case, all ligand is bound up in cyclic complexes, leaving only asymptotically small amounts of free ligand: $L \ll 1$, $Z_1 = O(1)$, $I = O(1)$, so that the asymptotic expansions proceed as

$$Z_1 = Z_{10} + o(1), \qquad L = o(1), \qquad I = I_0 + o(1).$$

Equations (23) and (24) are simple at leading order,

$$R_{\text{tot}} - L_{\text{tot}} = Z_{10}\left(1 + \frac{I_0}{K^{\text{mono}}}\right)^2,$$
$$I_{\text{tot}} = I_0 + \frac{2I_0 Z_{10}}{K^{\text{mono}}}\left(1 + \frac{I_0}{K^{\text{mono}}}\right).$$

Eliminating Z_{10}, I_0 satisfies a quadratic equation,

$$I_0^2 + I_0\big(2(R_{\text{tot}} - L_{\text{tot}}) - I_{\text{tot}} + K^{\text{mono}}\big) - I_{\text{tot}}K^{\text{mono}} = 0, \tag{49}$$

with unique positive solution I_0. Hence,

$$Z_{10} = \frac{R_{\text{tot}} - L_{\text{tot}}}{(1 + \frac{I_0}{K^{\text{mono}}})^2}. \tag{50}$$

Study of (21) suggests that ligand concentration L scales with $\epsilon^{1/2}$: $L = \epsilon^{1/2}L_0 + O(\epsilon)$, and $\tilde{f}_{10}$ is given by the leading-order terms in (26). Since $L \ll 1$, it is clear that in this case $\tilde{f}_{10}$ is given by

$$\tilde{f}_{10} = \frac{L_{\text{tot}}}{R_{\text{tot}}} \in (0, 1),$$

and we do not need to calculate L_0 unless we wish to determine higher-order terms. This expression for $\tilde{f}_{10}$ makes intuitive sense: with short ligands, but cyclic complexes favored, the dominant reaction will be the formation of the cyclic 2-complex, limited only by availability of ligand when $L_{\text{tot}} < R_{\text{tot}}$. So, if all available ligand ends up in cyclic 2-complexes then these will constitute all complexes of size $n \geq 2$, and hence account for the entirety of $\tilde{f}_{10}$.

To find the next order correction, we expand variables in powers of $\epsilon^{1/2}$:

$$Z_1 = Z_{10} + \epsilon^{1/2}Z_{11} + \cdots, \qquad L = \epsilon^{1/2}L_0 + \cdots, \qquad I = I_0 + \epsilon^{1/2}I_1 + \cdots,$$

in (21), (23), (24), and in the expression (26) for $\tilde{f}_1$. After solving for Z_{11}, L_0, and I_1 in terms of (known) leading order quantities, substitution in (26) gives

$$\tilde{f}_1 = \frac{L_{\text{tot}}}{R_{\text{tot}}} + \frac{\epsilon^{1/2}}{2\sqrt{2}R_{\text{tot}}(K^{\text{mono}})^2(L_{\text{tot}} - R_{\text{tot}})}\sqrt{\frac{L_{\text{tot}}}{\alpha}}$$
$$\times \big[I_0\big(2(R_{\text{tot}} - L_{\text{tot}})\big(1 - 2K^{\text{mono}}\big) - I_{\text{tot}} - K^{\text{mono}}\big) + \big(K^{\text{mono}}\big)^2$$
$$\times \big(4\alpha(R_{\text{tot}} - L_{\text{tot}})^2 - 4(R_{\text{tot}} - L_{\text{tot}}) - 1\big) - I_{\text{tot}}K^{\text{mono}}\big] + O(\epsilon), \tag{51}$$

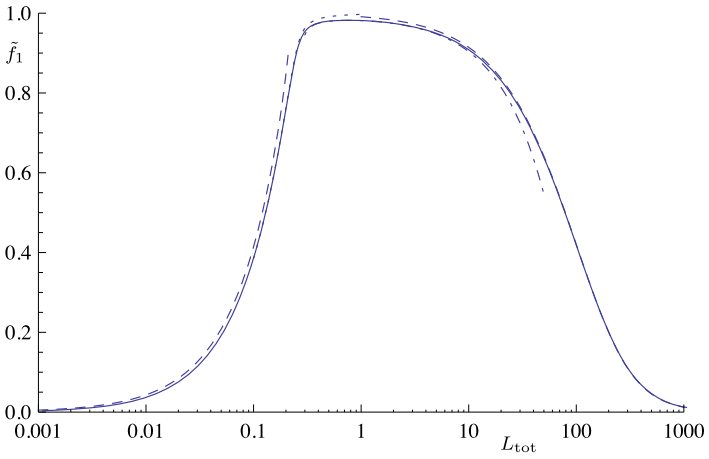


Fig. 7 Comparison of numerical (*solid lines*) and asymptotic solutions for $\tilde{f}_1$ as a function of total concentration of bivalent ligand $L_{\rm tot}$. The four different asymptotic regions are distinguished as (*from left to right*) $L_{\rm tot} < R_{\rm tot}$: *dashed*; $L_{\rm tot} \approx R_{\rm tot}$: *dotted*; $L_{\rm tot} > R_{\rm tot}$: *dash-dotted*; $L_{\rm tot} \gg R_{\rm tot}$: *dashed*. Parameter values are: total concentration of bivalent receptor $R_{\rm tot} = 0.25$; total concentration of monovalent inhibitor $I_{\rm tot} = 0$; intramolecular dissociation constant $K^{\rm intra} = 10^{-5}$ (independent of the complex size, case (ii)); monovalent dissociation constant $K^{\rm mono} = 0.25$; and cooperativity parameter $\alpha = 1$

with I_0 as given by (49). For $\epsilon = K^{\rm intra} \ll 1$, this expression gives very good agreement with the full numerical solution in the region $L_{\rm tot} < R_{\rm tot}$ (see the whole range of asymptotic approximations constructed in Fig. 7).

6.1.2 $L_{\rm tot} > R_{\rm tot}$

Here, ligand is in excess, so (to leading order) all receptor is bound up in cyclic complexes, leaving only asymptotically small amounts: $Z_1 \ll 1$, $L = O(1)$, $I = O(1)$. Leading-order behavior is trivial, (23) and (24) giving

$$L_0 = L_{\rm tot} - R_{\rm tot}, \qquad I_0 = I_{\rm tot} \tag{52}$$

(the only free ligand is the excess ligand; and since cyclic complexes are favored, inhibitor is left with nothing to bind to). The leading-order balance in (21) is $R_{\rm tot} \sim 8\alpha L^2 Z_1^2/\epsilon$, suggesting that Z_1 scales as $\epsilon^{1/2}$: $Z_1 = \epsilon^{1/2} Z_{10} + O(\epsilon)$, and $L = L_0 + \epsilon^{1/2} L_1 + O(\epsilon)$, $I = I_0 + \epsilon^{1/2} I_1 + O(\epsilon)$. This leading-order balance, together with (52) gives

$$Z_{10} = \sqrt{\frac{R_{\rm tot}}{8\alpha}} \frac{1}{(L_{\rm tot} - R_{\rm tot})}.$$

Equations (23) and (24) at order $\epsilon^{1/2}$ give expressions for the corrections L_1, I_1, and substitution of the expansions into (26) gives the fraction of receptor bound up in complexes of size $n \geq 2$ (correct to order $\epsilon^{1/2}$) as

$$\tilde{f}_1 = 1 - \frac{\epsilon^{1/2}}{\sqrt{8\alpha R_{\rm tot}}} \frac{(2(L_{\rm tot} - R_{\rm tot}) + 1 + \frac{I_{\rm tot}}{K^{\rm mono}})^2}{(L_{\rm tot} - R_{\rm tot})} + O(\epsilon). \tag{53}$$

6.1.3 $L_{\text{tot}} \sim R_{\text{tot}}$

The asymptotic expansions constructed in the regions $L_{\text{tot}} < R_{\text{tot}}$ and $L_{\text{tot}} > R_{\text{tot}}$ break down when $L_{\text{tot}} \approx R_{\text{tot}}$. In this case, at leading order, all free receptor and ligand are used up in making the cyclic complex, so both Z_1 and L are asymptotically small; and no inhibitor can bind to receptors. While many possible balances could be explored, the "crossover" region between $L_{\text{tot}} < R_{\text{tot}}$ and $L_{\text{tot}} > R_{\text{tot}}$ is adequately described by studying the asymptotic regime

$$L_{\text{tot}} = R_{\text{tot}} + \epsilon^{1/4} l_{\text{tot}}, \qquad Z_1 = \epsilon^{1/4} Z_{10} + \cdots,$$
$$L = \epsilon^{1/4} L_0 + \cdots, \qquad I = I_{\text{tot}} + \cdots.$$

Equations (21) and (23) lead to

$$L_0 = \frac{1}{Z_{10}} \left(\frac{R_{\text{tot}}}{8\alpha} \right)^{1/2}, \qquad L_0 = l_{\text{tot}} + Z_{10} \left(1 + \frac{I_{\text{tot}}}{K^{\text{mono}}} \right)^2,$$

which together yield

$$Z_{10} = \frac{1}{2(1 + \frac{I_{\text{tot}}}{K_{\text{tot}}})^2} \left\{ -l_{\text{tot}} + \left[l_{\text{tot}}^2 + 4 \left(\frac{R_{\text{tot}}}{8\alpha} \right)^{1/2} \left(1 + \frac{I_{\text{tot}}}{K^{\text{mono}}} \right)^2 \right]^{1/2} \right\}, \tag{54}$$

where recall $l_{\text{tot}} = (L_{\text{tot}} - R_{\text{tot}})/(K^{\text{intra}})^{1/4} = O(1)$. The expression (26) for $\tilde{f}_1$ then gives

$$\tilde{f}_1 = 1 - \epsilon^{1/4} \frac{Z_{10}}{R_{\text{tot}}} \left(1 + \frac{I_{\text{tot}}}{K^{\text{mono}}} \left(2 + \frac{I_{\text{tot}}}{K^{\text{mono}}} \right) \right) + O\left(\epsilon^{1/2}\right), \tag{55}$$

with Z_{10} as given by (54) above.

6.1.4 $L_{\text{tot}} \gg R_{\text{tot}}$

When L_{tot} is asymptotically large, the scaling for Z_1 changes. With $L_{\text{tot}} = O(1/\epsilon^{1/2})$, leading order in (21) gives

$$R_{\text{tot}} \sim \frac{Z_1}{(1 - 4\alpha L Z_1)^2} \left(4L^2 + \frac{8\alpha L^2 Z_1}{\epsilon} (1 - 4\alpha L Z_1) \right).$$

The exact balance of terms here depends on the size of LZ_1 (which, recall, must always be less than $1/(4\alpha)$). Note first that $(1 - 4\alpha L Z_1)$ cannot be asymptotically small, since this can give no balance of terms. Neither can we have both LZ_1 and $(1 - 4\alpha L Z_1)$ order-one, since then $4L^2 Z_1/(1 - 4\alpha L Z_1)^2 \gg 1$ with nothing to balance it. So, we must have $LZ_1 \ll 1$, and

$$R_{\text{tot}} \sim 4L^2 Z_1 + \frac{8\alpha L^2 Z_1^2}{\epsilon},$$

(this new balance is where we will first depart from the previous case, where $R_{\text{tot}} \sim 8\alpha L^2 Z_1^2/\epsilon \gg 4L^2 Z_1$). The asymptotic expansions now proceed as

$$L = \frac{L_0}{\epsilon^{1/2}} + L_1 + \epsilon^{1/2} L_2 + O(\epsilon), \qquad Z_1 = \epsilon Z_{10} + O\left(\epsilon^{3/2}\right),$$
$$I = I_0 + \epsilon^{1/2} I_1 + O(\epsilon),$$

and also $L_{\text{tot}} = \tilde{L}_{\text{tot}}/\epsilon^{1/2}$, with $\tilde{L}_{\text{tot}} \geq O(1)$. Leading-order analysis of (21)–(24) yields

$$L_0 = \tilde{L}_{\text{tot}}, \qquad I_0 = I_{\text{tot}}, \qquad Z_{10} = -\frac{1}{4\alpha} + \frac{1}{4\alpha}\left(1 + \frac{2\alpha R_{\text{tot}}}{\tilde{L}_{\text{tot}}^2}\right)^{1/2},$$

and substitution in (26) gives the leading-order expression for $\tilde{f}_1$ as

$$\tilde{f}_1 = 1 + \frac{\tilde{L}_{\text{tot}}^2}{\alpha R_{\text{tot}}}\left(1 - \left(1 + \frac{2\alpha R_{\text{tot}}}{\tilde{L}_{\text{tot}}^2}\right)^{1/2}\right) + o(1). \tag{56}$$

Figure 7 shows all four asymptotic expressions for $\tilde{f}_1$, plotted (as functions of L_{tot}) against the full numerical solution, on the appropriate domains of L_{tot}. As the figure shows, to the orders obtained, the asymptotic expressions are quite accurate on their regions of applicability.

6.1.5 Determining the Dissociation Constants

Since a primary goal of our analysis is to derive results that experimentalists might use to extract information about a given system, we now consider how the expressions derived above can be used if it is suspected that $K^{\text{intra}} = K_d^{*\text{intra}}/K_d^{*\text{inter}} \ll 1$. The most easily-identified experimental regimes in Fig. 7 appear to be $L_{\text{tot}} < R_{\text{tot}}$, and $L_{\text{tot}} \gg R_{\text{tot}}$; accordingly, we focus on these regimes. We again consider a titration experiment in which ligand is added to a receptor/inhibitor mixture, and the concentration, $C^* = R_{\text{tot}}^* \tilde{f}_1$, of complexes of size $n \geq 2$, is measured at selected stages. In the regime $L_{\text{tot}} \gg R_{\text{tot}}$, the late stages of a titration, the expression (56) is valid, and thus in the dimensional variables

$$C^* = R_{\text{tot}}^* + \frac{K_d^{*\text{intra}} L_{\text{tot}}^{*2}}{\alpha (K_d^{*\text{inter}})^2}\left[1 - \left(1 + \frac{2\alpha K_d^{*\text{inter}} R_{\text{tot}}^*}{K_d^{*\text{intra}} L_{\text{tot}}^*}\right)^{1/2}\right], \tag{57}$$

or, rearranging to make $\alpha/K_d^{*\text{intra}}$ the subject,

$$\frac{\alpha}{K_d^{*\text{intra}}} = \frac{2L_{\text{tot}}^{*3}}{(K_d^{*\text{inter}})^3 (C^* - R_{\text{tot}}^*)^2}\left(R_{\text{tot}}^* + \frac{(C^* - R_{\text{tot}}^*)}{L_{\text{tot}}^*} K_d^{*\text{inter}}\right). \tag{58}$$

In a given experiment, the quantities C^*, R_{tot}^*, and L_{tot}^* can be measured at any stage, and we would like to extract the unknowns $K_d^{*\text{inter}}$, $K_d^{*\text{intra}}$, and α that appear in (57), (58). Suppose we carry out an experiment with a fixed amount of receptor R_{tot}^*, and make measurements at two different concentrations of added ligand, L_1^* and L_2^*. We measure the two corresponding values of complex concentration, C_1^* and C_2^*. The expression (58) applies to each measurement: if we take the ratio of the two expressions for the two measurements, we obtain a relation that contains only one unknown, $K_d^{*\text{inter}}$. Solving this relation for $K_d^{*\text{inter}}$ and simplifying, we find

$$K_d^{*\mathrm{inter}} = \frac{R_{\mathrm{tot}}^*[L_1^{*3}(C_2^* - R_{\mathrm{tot}}^*)^2 - L_2^{*3}(C_1^* - R_{\mathrm{tot}}^*)^2]}{(C_1^* - R_{\mathrm{tot}}^*)(C_2^* - R_{\mathrm{tot}}^*)[L_2^{*2}(C_1^* - R_{\mathrm{tot}}^*) - L_1^{*2}(C_2^* - R_{\mathrm{tot}}^*)]}. \tag{59}$$

With $K_d^{*\mathrm{inter}}$ determined, we can now return to (58) and use either of the experimental measurements to determine the ratio $\alpha/K_d^{*\mathrm{intra}}$. We cannot, however, determine the two constants separately using these results.

To make further progress we consider another experiment with less ligand than receptor, $L_{\mathrm{tot}}^* < R_{\mathrm{tot}}^*$, so that the expression (51) is valid (this could simply be a measurement taken from the earlier stages of the same experiment discussed above). Consider first an experiment in which no inhibitor is present. Equation (51) then simplifies, and in terms of dimensional quantities takes the form

$$C^* = L_{\mathrm{tot}}^* - \frac{1}{2}\left(\frac{K_d^{*\mathrm{intra}}}{2\alpha}\right)^{1/2} \frac{(L_{\mathrm{tot}}^*)^{1/2}}{K_d^{*\mathrm{inter}}(R_{\mathrm{tot}}^* - L_{\mathrm{tot}}^*)} \times \left[4\alpha\left(R_{\mathrm{tot}}^* - L_{\mathrm{tot}}^*\right)^2 - 4K_d^{*\mathrm{inter}}\left(R_{\mathrm{tot}}^* - L_{\mathrm{tot}}^*\right) - \left(K_d^{*\mathrm{inter}}\right)^2\right]. \tag{60}$$

For a given measurement of C^*, at a ligand concentration $L_{\mathrm{tot}}^* < R_{\mathrm{tot}}^*$, since we already know $K_d^{*\mathrm{inter}}$ and $K_d^{*\mathrm{intra}}/\alpha$ by the procedure outlined above, this relation (60) is readily solved for α.

Finally, if we wish also to determine the dissociation constant $K_d^{*\mathrm{mono}}$, we must carry out a further experiment with nonzero concentration of monovalent ligand (inhibitor), $I_{\mathrm{tot}}^* \neq 0$. Rewriting (51) in dimensional form, the only unknown is now $K_d^{*\mathrm{mono}}$. The problem reduces to solving a cubic equation for $K_d^{*\mathrm{mono}}$, the physically-relevant root of which must be selected.

6.2 $K^{\mathrm{intra}} = \epsilon$ Asymptotically Small, Case (ii) (Long Bivalent Ligand)

In this case, regardless of the relative sizes of L_{tot} and R_{tot}, the fraction of receptor bound up in complexes of size $n \geq 2$, $\tilde{f}_1$, is always asymptotically small. This makes intuitive sense because if there is less ligand than receptor in the initial mixture then all available ligand is immediately taken up to form the cyclic 1-complex, leaving none free to form larger complexes. Available inhibitor can bind to remaining receptor that is not taken up in singleton cyclic complexes (C_1). On the other hand, if there is more ligand than receptor in the initial mix then it will bind all available receptor into singleton cyclic complexes; there will be excess free ligand, and no inhibitor will be able to bind to receptor. No receptor is left over that can form any of the larger complexes.

Since $\tilde{f}_1$ is always asymptotically small (and reliable estimates of dissociation constants would therefore be difficult to obtain), we do not pursue this case in detail; but we briefly demonstrate the above statements in the cases $L_{\mathrm{tot}} < R_{\mathrm{tot}}$, $L_{\mathrm{tot}} > R_{\mathrm{tot}}$, below.

6.2.1 $L_{\mathrm{tot}} < R_{\mathrm{tot}}$

Here, study of (22) reveals ligand concentration L to scale with ϵ: $L = \epsilon L_0 + O(\epsilon^2)$, and $\tilde{f}_{10}$ is given by the leading-order terms in (27). In this case, we cannot simply neglect all terms in L at leading order, since (27) contains a term in L/ϵ; so we need to find L_0. This we do from (22), which gives

$$R_{\text{tot}} = Z_{10}\left[1 + 2L_0 + \frac{I_0}{K^{\text{mono}}}\left(2 + \frac{I_0}{K^{\text{mono}}}\right)\right]. \tag{61}$$

The fraction of receptor bound up in complexes of size $n \geq 2$, $\tilde{f}_{10}$, is then given (to leading order) by

$$\tilde{f}_{10} = 1 - \frac{Z_{10}}{R_{\text{tot}}}\left[1 + 2L_0 + \frac{I_0}{K^{\text{mono}}}\left(2 + \frac{I_0}{K^{\text{mono}}}\right)\right] = 0,$$

using (61) in the final step. The dominant complex formed is the 1-cyclic complex; no complexes of size $n \geq 2$ are formed (to leading order).

6.2.2 $L_{\text{tot}} > R_{\text{tot}}$

In this case, ligand is present in excess and free-receptor concentration is asymptotically small. The leading-order balance in (22) is $R_{\text{tot}} \sim 2LZ_1/\epsilon$, suggesting that Z_1 scales as ϵ. It is then easily deduced from (23) and (24) that $Z_1 = \epsilon Z_{10} + O(\epsilon^2)$, $L = L_{\text{tot}} - R_{\text{tot}} + O(\epsilon)$ and $I = I_{\text{tot}} + O(\epsilon)$; (22) then gives $Z_{10} = R_{\text{tot}}/(2(R_{\text{tot}} - L_{\text{tot}}))$. Hence, in (27), retaining only leading order terms, $\tilde{f}_{10} = 0$.

6.3 High Cooperativity $\alpha \gg 1$, Case (i) (Short Bivalent Ligand)

Here, the formation of large complexes is favored. Figure 3 suggests that this is a non-trivial asymptotic limit worth exploring, which may yield useful predictions. In this case, the condition $4\alpha LZ_1 < 1$ shows that one or both of L or Z_1 must be asymptotically small. Again, the asymptotic scalings depend on the relative quantities of receptor and ligand in the system.

6.3.1 $L_{\text{tot}} < R_{\text{tot}}$

With $L_{\text{tot}} < R_{\text{tot}}$ receptor is in excess, and all free ligand is taken up to form larger complexes. In this regime, L is asymptotically small, while $Z_1 = O(1)$. We know that $0 < 1 - 4\alpha LZ_1 < 1$, but we do not know whether this quantity is $O(1)$ or asymptotically small. However, the dominant balance in (21) and (23) (noting that $\alpha L^2 Z_1$ must be asymptotically small because αLZ_1 is at most $O(1)$) is

$$R_{\text{tot}} \sim \frac{Z_1}{(1 - 4\alpha LZ_1)^2}\left(1 + \frac{I}{K^{\text{mono}}}\right)^2, \tag{62}$$

$$R_{\text{tot}} - L_{\text{tot}} \sim \frac{Z_1}{(1 - 4\alpha LZ_1)}\left(1 + \frac{I}{K^{\text{mono}}}\right)^2, \tag{63}$$

showing that (take the ratio of (63) and (62))

$$4\alpha LZ_1 \sim \frac{L_{\text{tot}}}{R_{\text{tot}}}.$$

So, with $Z_1 = O(1)$, L is of order $1/\alpha$, and asymptotic expansions proceed as

$$Z_1 = Z_{10} + \frac{1}{\alpha}Z_{11} + \cdots, \qquad L = \frac{1}{\alpha}L_0 + \cdots, \qquad I = I_0 + \frac{1}{\alpha}I_1 + \cdots.$$

Substitution into (21), (23), (24), and retaining only leading-order terms gives

$$Z_{10} = \frac{x^2}{R_{\rm tot}}, \qquad L_0 = \frac{L_{\rm tot}}{4x^2}, \qquad I_0 = K^{\rm mono}\left(\frac{R_{\rm tot} - L_{\rm tot}}{x} - 1\right), \tag{64}$$

where x is the unique positive root of

$$\frac{2}{K^{\rm mono}}x^2 + x\left(\frac{I_{\rm tot}}{K^{\rm mono}} - \frac{2}{K^{\rm mono}}(R_{\rm tot} - L_{\rm tot}) + 1\right) - (R_{\rm tot} - L_{\rm tot}) = 0.$$

The concentration of nontrivial complexes, $\tilde{f}_1$, defined in (26), then has a simple leading-order expression in terms of $L_{\rm tot}$:

$$\tilde{f}_1 = 1 - \left(1 - \frac{L_{\rm tot}}{R_{\rm tot}}\right)^2 + o(1). \tag{65}$$

6.3.2 $L_{\rm tot} > R_{\rm tot}$

In this case, ligand is in excess, and all available free receptor is bound, leaving only asymptotically small amounts. Free ligand and inhibitor are present in $O(1)$ concentrations, however. With $R_{\rm tot} = O(1)$, (21) shows that $1 - 4\alpha L Z_1$ must also be asymptotically small. Careful consideration of (21) and (23) reveals the correct scalings to be

$$\begin{aligned} Z_1 &= \frac{1}{\alpha}Z_{10} + \frac{1}{\alpha^{3/2}}Z_{11} + \cdots, \qquad L = L_0 + \frac{1}{\alpha^{1/2}}L_1 + \cdots, \\ I &= I_0 + \frac{1}{\alpha^{1/2}}I_1 + \cdots, \end{aligned} \tag{66}$$

and substitution of these expansions into (21) and (23) leads to

$$\begin{aligned} Z_1 &= \frac{1}{4\alpha(L_{\rm tot} - R_{\rm tot})} + O\left(\frac{1}{\alpha^{3/2}}\right), \qquad L = L_{\rm tot} - R_{\rm tot} + O\left(\frac{1}{\alpha^{1/2}}\right), \\ I &= I_{\rm tot} + O\left(\frac{1}{\alpha^{1/2}}\right). \end{aligned} \tag{67}$$

Equation (26) then gives $\tilde{f}_1$ correct to $O(1/\alpha)$ as

$$\tilde{f}_1 = 1 - \frac{1}{4\alpha R_{\rm tot}(L_{\rm tot} - R_{\rm tot})}\left(1 + 2(L_{\rm tot} - R_{\rm tot}) + \frac{I_{\rm tot}}{K^{\rm mono}}\right)^2 + \cdots. \tag{68}$$

This expression is clearly not valid as $L_{\rm tot} \to R_{\rm tot}$, or as $L_{\rm tot}$ becomes asymptotically large. In these cases, a separate analysis is required (below).

6.3.3 $L_{\rm tot} \approx R_{\rm tot}$

When total ligand and receptor concentrations are approximately equal, the concentrations of free ligand and receptor are both asymptotically small, and the scalings differ from the cases $L_{\rm tot} < R_{\rm tot}$ and $L_{\rm tot} > R_{\rm tot}$ considered in Sects. 6.3.1 and 6.3.2 above. Study of (23) shows that, as we transition between these cases, we require

$Z_1 \ll 1 - 4\alpha L Z_1 \ll 1$. The transition region may be adequately described by the distinguished limit in which $|R_{\text{tot}} - L_{\text{tot}}| = O(\alpha^{-1/4})$, $L \sim O(\alpha^{-1/2})$, $Z_1 \sim O(\alpha^{-1/2})$, $1 - 4\alpha L Z_1 \sim O(\alpha^{-1/4})$, and $I \sim O(1)$. With expansions proceeding as

$$Z_1 = \frac{Z_{10}}{\alpha^{1/2}} + \frac{Z_{11}}{\alpha^{3/4}} + \cdots, \qquad L = \frac{L_0}{\alpha^{1/2}} + \frac{L_1}{\alpha^{3/4}} + \cdots, \qquad I = I_0 + \frac{I_1}{\alpha^{1/4}}, \quad (69)$$

and

$$1 - 4\alpha L Z_1 = O\left(\alpha^{-1/4}\right), \quad (70)$$

the condition (70) gives $L_0 Z_{10} = 1/4$; (24) trivially gives $I_0 = I_{\text{tot}}$ at leading order, while (21) and (23) give, respectively,

$$R_{\text{tot}} = \frac{Z_{10}}{16(L_0 Z_{11} + L_1 Z_{10})^2}\left(1 + \frac{I_{\text{tot}}}{K^{\text{mono}}}\right)^2,$$
$$R_{\text{tot}} - L_{\text{tot}} = -\frac{Z_{10}\alpha^{-1/4}}{4(L_0 Z_{11} + L_1 Z_{10})}\left(1 + \frac{I_{\text{tot}}}{K^{\text{mono}}}\right)^2.$$

Together these results give

$$Z_{10} = -\frac{\alpha^{1/2}(R_{\text{tot}} - L_{\text{tot}})^2}{R_{\text{tot}}(1 + \frac{I_{\text{tot}}}{K^{\text{mono}}})^2}, \qquad L_0 = \frac{1}{4Z_{10}}, \qquad I_0 = I_{\text{tot}}. \quad (71)$$

While these asymptotic solutions for Z_1, L, and I are clearly different to those found in regions $L_{\text{tot}} < R_{\text{tot}}$ and $L_{\text{tot}} > R_{\text{tot}}$, we nonetheless find (on substitution in (26)) that

$$\tilde{f}_1 = 1 - \left(1 - \frac{L_{\text{tot}}}{R_{\text{tot}}}\right)^2 + o(1) \quad \text{valid when } |L_{\text{tot}} - R_{\text{tot}}| \sim O\left(\alpha^{-1/4}\right), \quad (72)$$

an expression identical to (65) from the region $L_{\text{tot}} < R_{\text{tot}}$.

6.3.4 $L_{\text{tot}} \gg R_{\text{tot}}$

The expressions derived in Sect. 6.3.2 for $L_{\text{tot}} > R_{\text{tot}}$ cease to be valid when $L_{\text{tot}} \sim O(\alpha)$. Since ligand is greatly in excess (assuming $R_{\text{tot}} = O(1)$), $L = O(\alpha)$ also, and writing $L_{\text{tot}} = \alpha \tilde{L}_{\text{tot}}$ (where $\tilde{L}_{\text{tot}} \geq O(1)$) we assume asymptotic expansions

$$Z_1 = \frac{Z_{10}}{\alpha^2} + \frac{Z_{11}}{\alpha^3} + \cdots, \qquad L = \alpha L_0 + L_1 + \frac{L_2}{\alpha} + \cdots, \qquad I = I_0 + \frac{I_1}{\alpha} + \cdots$$

(the scaling for Z_1 comes from the requirement that $1 - 4\alpha L Z_1 \leq O(1)$). Leading order in (21) then gives

$$R_{\text{tot}} = \frac{4 Z_{10} L_0^2}{(1 - 4 L_0 Z_{10})^2}, \quad (73)$$

while $O(\alpha)$ and $O(1)$ in (23) give

$$L_0 = \tilde{L}_{\text{tot}}, \qquad R_{\text{tot}} = -\frac{4 L_0^2 Z_{10}}{1 - 4 L_0 Z_{10}} - L_1$$

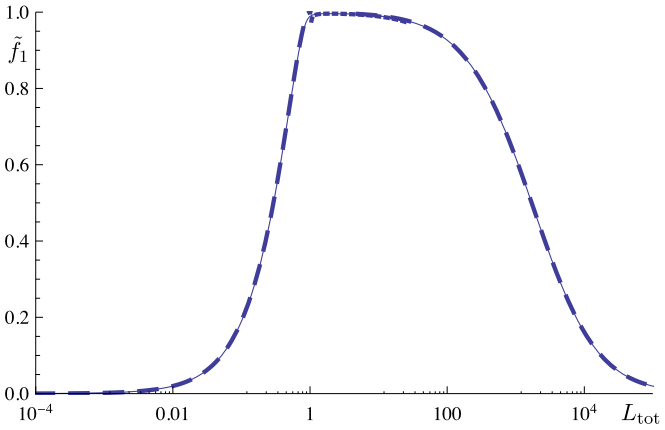


Fig. 8 Comparison of asymptotic (*dashed curves*) and full numerical solutions (*solid line*) in the different asymptotic regions. The parameter values used are: total concentration of bivalent ligand $R_{\rm tot} = 1.0$; total concentration of monovalent inhibitor $I_{\rm tot} = 1.0$; intramolecular dissociation constant $K^{\rm intra} = 1.0$ (independent of the complex size, case (ii)); monovalent dissociation constant $K^{\rm mono} = 1.0$; and cooperativity $\alpha = 1000$

(the final (24) gives $I_0 = I_{\rm tot}$ at leading order). Solving (73) for Z_{10}, the appropriate root (that which vanishes when $R_{\rm tot} = 0$) is given by

$$8R_{\rm tot}Z_{10} = 1 + \frac{2R_{\rm tot}}{\tilde{L}_{\rm tot}} - \left[1 + \frac{4R_{\rm tot}}{\tilde{L}_{\rm tot}}\right]^{1/2}.$$

Finally, substituting these expressions into (26), we obtain

$$\tilde{f}_1 = 1 - \frac{L_{\rm tot}^2}{2\alpha^2 R_{\rm tot}^2}\left(1 + \frac{2\alpha R_{\rm tot}}{L_{\rm tot}} - \left[1 + \frac{4\alpha R_{\rm tot}}{L_{\rm tot}}\right]^{1/2}\right) + O\left(\frac{1}{\alpha}\right). \tag{74}$$

The three asymptotic expressions for $\tilde{f}_1$ obtained, covering four different asymptotic regions, are illustrated against a full numerical solution at large α in Fig. 8.

6.4 High Cooperativity $\alpha \gg 1$, Case (ii) (Long Bivalent Ligand)

Despite the difference in the governing equations in case (ii) ((22) replaces (21), and the expression (26) for $\tilde{f}_1$ is replaced by (27)) the results for asymptotically-large α are essentially the same. Since the formation of large complexes is favored, and the two models differ in the numbers of small cyclic complexes they create, the differences are not apparent in this limit of large cooperativity. Thus, from a practical viewpoint, it is difficult to distinguish the two cases if $\alpha \gg 1$. We outline the analysis below. As in case (i), different values of $L_{\rm tot}$ require different analyses.

6.4.1 $L_{\rm tot} < R_{\rm tot}$

The analysis is much as in Sect. 6.3.1. Receptor is in excess, and any free ligand is taken up to form larger complexes in this highly cooperative system. L is asymptotically small, while Z_1 is $O(1)$. The dominant balance in (22) leads to (62), the same

result as the dominant balance in (21) in case (i); since this was the only possible difference between the two systems, the analysis then proceeds as before, with solution (64) for the leading-order variables. Though the expression (27) for $\tilde{f}_1$ differs from that of case (i), to leading order they are the same, and we again obtain the result (65).

6.4.2 $L_{\text{tot}} > R_{\text{tot}}$

Ligand is now in excess, and the quantity of free receptor is asymptotically small. Analysis of (22) and (23) reveal the correct form of the asymptotic expansions to be exactly as in (66). Again, after substitution in the governing equations (22)–(24), the same leading-order results are obtained as in case (i), (67), with the same expression (68) for $\tilde{f}_1$.

6.4.3 $L_{\text{tot}} \approx R_{\text{tot}}$

As described in Sects. 6.3.3, the above expansions are not valid when $L_{\text{tot}} \approx R_{\text{tot}}$, and a separate analysis is needed to describe the transition between the two cases in Sects. 6.4.1 and 6.4.2 above. The same scalings as proposed in (69) are also appropriate here, and with these scalings (22) is again the same as (21) to leading order, so we obtain leading-order solutions (71) as in case (i). Once more, no difference is apparent in $\tilde{f}_1$ at this order, so that it is given by (72), as in case (i).

6.4.4 $L_{\text{tot}} \gg R_{\text{tot}}$

With the same scalings as for case (i), the leading-order solution of (22), (23), and (24) is exactly as for case (i), as is the leading-order expression for $\tilde{f}_1$, (74).

6.5 Determining the Dissociation Constants: High Cooperativity $\alpha \gg 1$ (Both Cases)

We would like to use such analysis to determine various dissociation constants in the system. Since to the orders considered the large-α results are the same in cases (i) and (ii), the following applies equally to both cases, and we cannot extract information about the constant $K_d^{*\text{intra}}$ that distinguishes the cases. Again, we assume that in a titration where ligand is added to a mixture with known (fixed) concentrations of receptor and inhibitor, the concentration $C^* = \tilde{f}_1 R_{\text{tot}}^*$ of complexes of size $n \geq 2$ can be measured at selected points.

To the order obtained, the results from $L_{\text{tot}} < R_{\text{tot}}$ do not yield useful information about the dissociation constants, since in dimensional form (65) states

$$C^* = R_{\text{tot}}^* \left[1 - \left(1 - \frac{L_{\text{tot}}^*}{R_{\text{tot}}^*} \right)^2 \right], \tag{75}$$

an identity in which all quantities are measurable. However, this relation should be verified in the early stages of the experiment, since if it does not hold to a good level of approximation then α cannot be asymptotically large. (Note that this may also provide us with a means of distinguishing this case from the previously-considered case (small K^{intra3}, Sect. 6.1), which may look superficially similar with only a few experimental points.)

The most useful stage of the titration experiment is when ligand concentration is larger than receptor concentration, $L^*_{\rm tot} > R^*_{\rm tot}$, but not greatly in excess. Equation (68), in dimensional form, then gives the concentration of complexes, $C^* = \tilde{f}_1 R^*_{\rm tot}$, as

$$C^* = R^*_{\rm tot} - \frac{1}{4\alpha(L^*_{\rm tot} - R^*_{\rm tot})}\left(K_d^{*\rm inter} + 2\big(L^*_{\rm tot} - R^*_{\rm tot}\big) + \frac{K_d^{*\rm inter}}{K_d^{*\rm mono}} I^*_{\rm tot}\right)^2. \quad (76)$$

First consider a titration with no inhibitor, $I^*_{\rm tot} = 0$, for which, in this regime (76) gives

$$\big(K_d^{*\rm inter} + 2\big(L^*_{\rm tot} - R^*_{\rm tot}\big)\big)^2 = 4\alpha\big(L^*_{\rm tot} - R^*_{\rm tot}\big)\big(R^*_{\rm tot} - C^*\big). \quad (77)$$

Take two data points from the experiment, with ligand concentrations L^*_1 and L^*_2, and corresponding complex concentrations C^*_1 and C^*_2. Equation (77) applies equally to the two data points, so take the ratio of the two expressions to eliminate α and obtain an expression for the unknown $K_d^{*\rm inter}$:

$$K_d^{*\rm inter} = \frac{2(L^*_1 - R^*_{\rm tot})[(L^*_2 - R^*_{\rm tot})(R^*_{\rm tot} - C^*_2)]^{1/2} - 2(L^*_2 - R^*_{\rm tot})[(L^*_1 - R^*_{\rm tot})(R^*_{\rm tot} - C^*_1)]^{1/2}}{[(L^*_1 - R^*_{\rm tot})(R^*_{\rm tot} - C^*_1)]^{1/2} - [(L^*_2 - R^*_{\rm tot})(R^*_{\rm tot} - C^*_2)]^{1/2}}.$$

We can then substitute back in the expression (77), using values from either of the two data-points, to obtain an estimated value for α.

With $K_d^{*\rm inter}$ and α determined, an experiment with known inhibitor concentration $I^*_{\rm tot}$ can now be performed, and the expression (76) used to extract a value for the remaining unknown $K_d^{*\rm mono}$.

As discussed, in this asymptotic limit of large cooperativity α, it is not possible to determine $K_d^{*\rm intra}$ without going to higher order in the asymptotic analysis. Since the effect of $K_d^{*\rm intra}$ is asymptotically very small when α is large, we would not expect a reliable estimate for this parameter to result from such analysis.

6.6 Small-$L_{\rm tot}$ Analysis

In the case that no system parameters are asymptotically small or large, analytical progress appears daunting. Nonetheless, we can conceive of an experimental setup that will allow us to estimate the dissociation constants in the system. We consider the early stages of a titration experiment with ligand added slowly, during which the total ligand concentration $L_{\rm tot}$ may be considered asymptotically small (and free ligand concentration L is also necessarily small) so that we may use $L_{\rm tot} = \epsilon \ll 1$ as the asymptotic parameter. From the point of view of an experimentalist, the following analysis might reasonably be expected to hold in the range where $L^*_{\rm tot}/R^*_{\rm tot} \leq 0.1$, say.

If both L and $L_{\rm tot}$ are asymptotically small but other quantities are assumed $O(1)$, then

$$\begin{aligned} &Z_1 = Z_{10} + \epsilon Z_{11} + O\big(\epsilon^2\big), \qquad I = I_0 + \epsilon I_1 + O\big(\epsilon^2\big), \\ &L = \epsilon L_0 + O\big(\epsilon^2\big). \end{aligned} \quad (78)$$

The governing equations (21) (or (22)) and (23) are identical to leading order in ϵ in this case:

$$R_{\text{tot}} = Z_{10}\left(1 + \frac{I_0}{K^{\text{mono}}}\right)^2, \tag{79}$$

while (24) becomes

$$I_{\text{tot}} = I_0 + \frac{2I_0 Z_{10}}{K^{\text{mono}}}\left(1 + \frac{I_0}{K^{\text{mono}}}\right). \tag{80}$$

Equation (79) ensures that $\tilde{f}_1$, as defined by (26) or (27), vanishes to leading order (as we would expect). Equations (79) and (80) may be solved for Z_{10} and I_0, giving

$$Z_{10} = \frac{1}{8R_{\text{tot}}}\left(\left(I_{\text{tot}} + K^{\text{mono}}\right)^2 + (2R_{\text{tot}} - I_{\text{tot}})^2 - I_{\text{tot}}^2 + \nu\mu\right), \tag{81}$$

$$I_0 = \frac{1}{2}\left(I_{\text{tot}} - K^{\text{mono}} - 2R_{\text{tot}} + \mu\right), \tag{82}$$

where

$$\nu = 2R_{\text{tot}} - I_{\text{tot}} - K^{\text{mono}}, \qquad \mu = \sqrt{\left(2R_{\text{tot}} - I_{\text{tot}} + K^{\text{mono}}\right)^2 + 4K^{\text{mono}} I_{\text{tot}}}. \tag{83}$$

To find the first nonvanishing contribution to $\tilde{f}_1$ we need to solve the system at $O(\epsilon)$. At this stage, cases (i) and (ii) differ, and we first consider case (i).

Case (i), Short Bivalent Ligand Substituting the asymptotic expansions (78) into (21), (23), and (24), we obtain three linear equations for L_0, $Z_{11,}$ and I_1, which are easily solved in terms of leading order quantities (81), (82). Substitution of the expansions into (26) then gives

$$\tilde{f}_1 = \epsilon\tilde{f}_{11} + O\left(\epsilon^2\right), \tag{84}$$

where

$$\tilde{f}_{11} = \frac{2\alpha(\mu^2 + \nu\mu - 4K^{\text{mono}}R_{\text{tot}})}{R_{\text{tot}}(2(1+\nu+\mu) + \alpha(\mu^2 + \nu\mu - 4K^{\text{mono}}R_{\text{tot}}))}. \tag{85}$$

Recalling that $\epsilon = L_{\text{tot}}$, the expression (84) gives the approximate (relative) equilibrium concentration of complexes of size $n \geq 2$ in a mixture where a small quantity of ligand is present. Note that the expression simplifies further if no inhibitor is present in the mixture; in this case

$$\tilde{f}_{11} = \frac{8\alpha R_{\text{tot}}}{1 + 4R_{\text{tot}}(1 + \alpha R_{\text{tot}})}. \tag{86}$$

Case (ii), Long Bivalent Ligand Proceeding as above, we substitute the expansions (78) into (22), (23), and (24), and solve for L_0, $Z_{11,}$ and I_1. Substitution of the expansions into (27) then gives $\tilde{f}_1$ correct to $O(\epsilon)$ as in (84), where

$$\tilde{f}_{11} = \frac{16\alpha(K^{\text{mono}})^2 K^{\text{intra}} R_{\text{tot}}}{K^{\text{intra}}\{\mu^2 - \mu\nu - 4R_{\text{tot}}K^{\text{mono}}(1 + \nu - \mu - 2\alpha R_{\text{tot}}K^{\text{mono}})\} + 4R_{\text{tot}}(K^{\text{mono}})^2}, \tag{87}$$

with μ, ν as given in (83). Again, with no inhibitor this expression simplifies dramatically,

$$\tilde{f}_{11} = \frac{8\alpha K^{\text{intra}} R_{\text{tot}}}{2R_{\text{tot}} + K^{\text{intra}} + 4R_{\text{tot}} K^{\text{intra}}(1+\alpha R_{\text{tot}})}. \tag{88}$$

We shall use these key results (85)–(88) below.

6.6.1 Determining the Dissociation Constants

We now outline how to use this analysis to determine the dissociation constants of the full system. Again we use dimensional quantities in the discussion below, referring back to (1)–(20) where the dimensional and dimensionless equations are first set out. The idea is to consider the early stages of the usual titration experiment with ligand added slowly to fixed amounts of receptor, R^*_{tot} and inhibitor, I^*_{tot}. As ligand is added, the concentration $C^* = R^*_{\text{tot}} \tilde{f}_1$ of complexes containing $n \geq 2$ receptor molecules is measured at a few selected values of total ligand concentration L^*_{tot}, that are small relative to R^*_{tot} (say $L^*_{\text{tot}} < 0.1 R^*_{\text{tot}}$). Measure the initial gradient of this concentration profile of C^* versus L^*_{tot},

$$G = \left.\frac{dC^*}{dL^*_{\text{tot}}}\right|_{L^*_{\text{tot}}=0} \equiv R_{\text{tot}} \left.\frac{d\tilde{f}_1}{dL_{\text{tot}}}\right|_{L_{\text{tot}}=0} = R_{\text{tot}} \tilde{f}_{11}, \tag{89}$$

where the quantity $\tilde{f}_{11}$ is as defined by (84) (given by one of the expressions (85)–(88)). Just a few data points in each experiment should suffice to get a reasonable value for G. Cases (i) and (ii) differ, so we consider them separately here.

6.6.2 Case (i), Short Bivalent Ligand

Step 1: No Inhibitor Consider first two experiments with (known) receptor concentrations R^*_{tot} and mR^*_{tot} ($m \neq 1$), but with no inhibitor present, $I^*_{\text{tot}} = 0$. For the two experiments, we have two measured gradients G_1 and G_m which, by (86), satisfy

$$G_1 = \frac{8\alpha R^2_{\text{tot}}}{1 + 4R_{\text{tot}}(1+\alpha R_{\text{tot}})}, \qquad G_m = \frac{8\alpha m^2 R^2_{\text{tot}}}{1 + 4mR_{\text{tot}}(1+\alpha m R_{\text{tot}})}.$$

Since G_1, G_m, and m are known, these represent two equations for R_{tot} and α, with solution

$$4R_{\text{tot}} = \frac{(m-1)G_m(2-G_1)}{m(G_m(2-G_1) - mG_1(2-G_m))} - 1, \qquad \alpha = \frac{G_1(1+4R_{\text{tot}})}{4R^2_{\text{tot}}(2-G_1)}, \tag{90}$$

from which we can extract $K^{*\text{inter}}_d = R^*_{\text{tot}}/R_{\text{tot}}$.

Step 2: With Inhibitor With α and $K^{*\text{inter}}_d$ fixed we now carry out a third experiment with a nonzero quantity of inhibitor present, $I^*_{\text{tot}} \neq 0$, and total receptor concentration R^*_{tot}. Make the same plot of receptor-complex concentration versus total ligand concentration, and extract its initial gradient, G_I say,

$$G_I = \left.\frac{d(R^*_{\text{tot}}\tilde{f}_1)}{dL^*_{\text{tot}}}\right|_{L^*_{\text{tot}}=0} \equiv R_{\text{tot}} \left.\frac{d\tilde{f}_1}{dL_{\text{tot}}}\right|_{L_{\text{tot}}=0} = R_{\text{tot}} \tilde{f}_{11}, \tag{91}$$

where $\tilde{f}_{11}$ is given in (85) above. This gives a nonlinear equation

$$G_I = \frac{2\alpha R_{\rm tot}(\mu^2 - 4K^{\rm mono}R_{\rm tot} + \nu\mu)}{R_{\rm tot}(2(1+\nu) + \alpha(\mu^2 - 4K^{\rm mono}R_{\rm tot}) + \mu(2+\alpha\nu))}, \tag{92}$$

with ν, μ as defined in (83), where the only unknown is now $K^{\rm mono}$ (we have G_I, α, $R_{\rm tot} = R^*_{\rm tot}/K^{*\rm inter}_d$, and $I_{\rm tot} = I^*_{\rm tot}/K^{*\rm inter}_d$). Hence, we can solve (92) numerically for $K^{\rm mono}$, and finally extract $K^{*\rm mono} = K^{*\rm inter}_d K^{\rm mono}$.

6.6.3 Case (ii), Long Bivalent Ligand

Step 1: No Inhibitor In case (ii) the additional unknown $K^{\rm intra}$ appears in the no-inhibitor expression (88), so here it seems we must carry out a third no-inhibitor experiment, with receptor concentration $qR^*_{\rm tot}$ ($q \neq 1$, m) if we are to determine the three constants $R_{\rm tot}$, α, $K^{\rm intra}$ (and hence, the dimensional quantities $K^{*\rm inter}_d$, $K^{*\rm intra}_d$). So, suppose we carry out three experiments with receptor concentrations $R^*_{\rm tot}$, $mR^*_{\rm tot}$, $qR^*_{\rm tot}$, and as described above we estimate the initial gradients of the titration curve in each experiment, G_1, G_m, G_q. By (88),

$$G_1 = \frac{8\alpha K^{\rm intra}R_1^2}{2R_1 + K^{\rm intra} + 4R_1K^{\rm intra}(1+\alpha R_1)}, \tag{93}$$

$$G_m = \frac{8\alpha m^2 K^{\rm intra}R_1^2}{2mR_1 + K^{\rm intra} + 4mR_1K^{\rm intra}(1+\alpha mR_1)}, \tag{94}$$

$$G_q = \frac{8\alpha q^2 K^{\rm intra}R_1^2}{2qR_1 + K^{\rm intra} + 4qR_1K^{\rm intra}(1+\alpha qR_1)}, \tag{95}$$

representing three equations for R_1, α, and $K^{\rm intra}$ (all other quantities are known). However, (93)–(95) are not independent. Eliminating $R_{\rm tot}$ and then α between (93) and (94):

$$\begin{aligned} R_{\rm tot} &= \frac{K^{\rm intra}(G_m - m^2G_1)}{2m(1+2K^{\rm intra})(mG_1 - G_m)}, \\ \alpha &= \frac{G_1(2R_{\rm tot} + K^{\rm intra} + 4R_{\rm tot}K^{\rm intra})}{4K^{\rm intra}R_{\rm tot}^2(2-G_1)} \end{aligned} \tag{96}$$

and substituting in (95), we find (after simplification) that either $K^{\rm intra} = 0$ (which we know is not the case) or that

$$G_q = \frac{2G_1G_m(m-1)q^2}{G_m(-2+G_1(m-1)(q-1))(m-q) - 2G_1m^2(q-1)},$$

a consistency condition on the gradient in the third experiment. So, carrying out further experiments without inhibitor does not help; we can only find two equations for three unknowns. We therefore neglect (95), and consider the case with inhibitor to make further progress.

Step 2: With Inhibitor With a total of four dimensionless unknowns $R_{\rm tot}$, α, $K^{\rm intra}$, and $K^{\rm mono}$ to determine, and only two independent equations from Step 1 above, we need to carry out a further two experiments with inhibitor. Since the details are messy, and do not lead to a closed-form expression for the required parameters, we

give only the outline. Carry out two experiments as in Step 1, but with also known quantities of inhibitor: experiment 1, with receptor concentration $R^*_{\rm tot}$ and inhibitor concentration $I^*_{{\rm tot},1} = \delta_1 R^*_{\rm tot}$; and experiment 2, with receptor concentration $mR^*_{\rm tot}$ and inhibitor concentration $I^*_{{\rm tot},m} = \delta_m R^*_{\rm tot}$. Extract the initial gradients, $G_{I,1}$, $G_{I,m}$ of the titration curves as before, and use (89), together with (87), to write down the two equations that the parameters satisfy. In terms of the dimensionless quantities that appear in (87) then, we must consider $R_{\rm tot} = R^*_{\rm tot}/K^{*\rm inter}_d$ as a quantity to be solved for; and $I_{{\rm tot},1} = I^*_{{\rm tot},1}/K^{*\rm inter}_d = \delta_1 R_{\rm tot}$, while $I_{{\rm tot},m} = \delta_m R_{\rm tot}$ (with δ_1, δ_m known). Substituting for these values of $I_{\rm tot}$ in the appropriate gradient equations, with $R_{\rm tot}$ and α given by (96), and with m, G_1, G_m, $G_{I,1}$, $G_{I,m}$, δ_1, δ_m given, leads to two very complicated algebraic equations for $K^{\rm intra}$ and $K^{\rm mono}$, which must be solved numerically. Once this is done, substitute back in (96) to obtain values for $R_{\rm tot}$ and α. There are obviously questions of uniqueness here: It is quite possible that there will be more than one solution set that leads to physically-sensible values for $K^{\rm intra}$, $K^{\rm mono}$, $R_{\rm tot}$, and α; as a minimum we insist that all values be real and positive. Hence, finally, we obtain all the dissociation constants: $K^{*\rm inter}_d = R^*_{\rm tot}/R_{\rm tot}$, $K^{*\rm intra}_d = K^{*\rm inter}_d K^{\rm intra}$, $K^{*\rm mono}_d = K^{*\rm inter}_d K^{\rm mono}$.

7 Discussion and Conclusions

We have taken an established system of equations (Mack et al. 2011) that govern equilibria between symmetric bivalent receptors, bivalent ligands, and univalent ligands (inhibitor), a system that forms a model for the immune response. In certain simplified cases and asymptotic limits, we derived analytical results that allow dissociation constants and cooperativity parameters in the system to be estimated by conceptually-simple experiments, in which the degree of receptor-ligand cross-linking is measured. Such measurements may be made by, for example, flow cytometry techniques (Hlavacek et al. 1999; Posner et al. 2002; Sklar et al. 2002). In addition to providing a practical means to calculate the dissociation constants, the asymptotic limits provide clear insight into the behavior of this system that is not apparent from numerical computations alone. The results can also provide guidance as to conditions under which the immune response, as measured by the degree of aggregation, is maximized. For ease of reference, the notation we use and our main results are summarized in Tables 1 and 2 at the end of this paper. It is hoped that biochemists and biologists characterizing the binding of antibodies to antigens can use our simple approximations as a quantitative tool to guide the design and screening of recombinant antibodies, which can be used to treat human disorders.

Our analysis is restricted to the case of a system involving symmetric bivalent receptors (antibodies or immunoglobulins), bivalent ligands (antigens; pathogens or allergens), and monovalent ligands. This is, of course, not the most general biochemical model of the immune response. A more comprehensive analysis would account for (in the first instance) multivalent ligands, that allow more complicated crosslinking than is considered here (Hlavacek et al. 1999). Nonetheless, given that we are able to obtain explicit analytical results, we believe that our model represents an important step in enabling quantitative predictions about such receptor-ligand systems

Table 1 Definitions of parameters used in the mathematical model

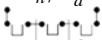
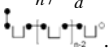
Parameter	Definition
n	No. of bivalent receptors found in a given molecular complex (as in Fig. 1).
Z_1^*	Concentration of free (unbound) bivalent receptor; depicted as ⊔.
Z_1	Normalized concentration of free (unbound) bivalent receptor; $Z_1 = Z_1^*/K_d^{\text{inter}}$.
L^*	Concentration of free (unbound) bivalent ligand; depicted as •—•.
L	Normalized concentration of free (unbound) bivalent ligand; $L = L^*/K_d^{\text{inter}}$.
I^*	Concentration of free (unbound) monovalent ligand (inhibitor); depicted as ∘.
I	Normalized concentration of free (unbound) monovalent ligand; $I = I^*/K_d^{\text{inter}}$.
$K_d^{*\text{inter}}$	Intermolecular dissociation constant.
$K_d^{*\text{mono}}$	Monovalent dissociation constant.
K^{mono}	Normalized monovalent dissociation constant $K^{\text{mono}} = K_d^{*\text{mono}}/K_d^{*\text{inter}}$.
$K_{d,n}^{*\text{intra}}$	Intramolecular dissociation constant for molecules with n bivalent receptors.
K_1^{intra}	Normalized intramolecular dissociation constant for molecules with only one bivalent receptor: $K_1^{\text{intra}} = K_{d,1}^{*\text{intra}}/K_d^{*\text{inter}}$. $K_1^{\text{intra}} = \infty$ for short bivalent ligands (case (i)); $K_1^{\text{intra}} = K_n^{\text{intra}}$ for long bivalent ligands (case (ii)).
K_n^{intra}	Normalized intramolecular dissociation constant for molecules with n bivalent receptors: $K_n^{\text{intra}} = K_{d,n}^{*\text{intra}}/K_d^{*\text{intra}}$.
R_{tot}^*	Total concentration of bivalent receptor; includes both free and bound receptors.
R_{tot}	Normalized total concentration of bivalent receptor: $R_{\text{tot}} = -R_{\text{tot}}^*/K_d^{*\text{inter}}$.
L_{tot}^*	Total concentration of bivalent ligand; includes both free and bound ligands.
L_{tot}	Normalized total concentration of bivalent ligand: $L_{\text{tot}} = L_{\text{tot}}^*/K_d^{*\text{inter}}$.
I_{tot}^*	Total concentration of monovalent ligand (inhibitor); both free and bound.
I_{tot}	Normalized total concentration of monovalent ligand: $I_{\text{tot}} = I_{\text{tot}}^*/K_d^{*\text{inter}}$.
A_n^*	Concentration of the linear complexes depicted as  .
A_n	Normalized total concentration of complexes, $A_n = A_n^*/K_d^{*\text{inter}}$.
B_n^*	Concentration of the linear complexes depicted as  .
B_n	Normalized total concentration of complexes, $B_n = B_n^*/K_d^{*\text{inter}}$.
C_n^*	Concentration of the cyclic complexes depicted as  .
C_n	Normalized total concentration of cyclic complexes, $C_n = C_n^*/K_d^{*\text{inter}}$.
E_n^*	Concentration of the linear complexes depicted as  .
E_n	Normalized total concentration of complexes, $E_n = E_n^*/K_d^{*\text{inter}}$.
F_n^*	Concentration of the linear complexes depicted as  .
F_n	Normalized total concentration of complexes, $F_n = F_n^*/K_d^{*\text{inter}}$.
$\tilde{f}_1$	Fraction of total receptors R_{tot} in complexes with ≥2 bivalent receptors: $\tilde{f}_1 = (1/R_{\text{tot}})\sum_{n=1}^{\infty} = (Z_n + A_n + B_n + C_n + D_n + E_n + F_n)$.
f_1	Fraction of total receptors R_{tot} in complexes with one bivalent receptor: $f_1 = 1 - \tilde{f}_1 = (Z_1 + A_1 + B_1 + C_1 + D_1 + E_1 + F_1)/R_{\text{tot}}$.
$\tilde{f}_{1,\max}$	Maximum value of $\tilde{f}_1$ as *total concentration of bivalent ligand* L_{tot}^* varies. Corresponds to the maximum sample oligomerization as L_{tot}^* varies.
C^*	Concentration of receptors in complexes with ≥2 bivalent receptors: $C^* = -R_{\text{tot}}^*\tilde{f}_1$.
$C_{\max}^*$	Maximum value of C^* as *total concentration of bivalent ligand* L_{tot}^* varies. Corresponds to the maximum sample oligomerization as L_{tot}^* varies.
G	Gradient (slope) of the receptor concentration curve at small total concentrations of bivalent ligand (from (89)): $G = \frac{dC^*}{dL_{\text{tot}}^*}\|_{L_{\text{tot}}^*=0}$.

Table 2 Summary of main analytical results and conditions of validity

Sect.	R_{tot} vs L_{tot} regime	I_{tot} regime	α regime	K_1^{intra} regime	K_n^{intra} regime	Parameter of interest	Equations
4.1	Any	0	1	Any K^{intra}	Any K^{intra}	$\tilde{f}_{1,\max}$	(40)
4.2	Any	0	1	∞	Any K^{intra}	$\tilde{f}_{1,\max}$	(47), (48)
6.1.1	$L_{\mathrm{tot}} < R_{\mathrm{tot}}$	Any	Any	∞	$K^{\mathrm{intra}} \ll 1$	$\tilde{f}_1$	(51)
6.1.2	$L_{\mathrm{tot}} > R_{\mathrm{tot}}$	Any	Any	∞	$K^{\mathrm{intra}} \ll 1$	$\tilde{f}_1$	(53)
6.1.3	$L_{\mathrm{tot}} \approx R_{\mathrm{tot}}$	Any	Any	∞	$K^{\mathrm{intra}} \ll 1$	$\tilde{f}_1$	(55)
6.1.4	$L_{\mathrm{tot}} \gg R_{\mathrm{tot}}$	Any	Any	∞	$K^{\mathrm{intra}} \ll 1$	f_1	(56)
6.3.1	$L_{\mathrm{tot}} < R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	∞	Any K^{intra}	$\tilde{f}_1$	(65)
6.4.1	$L_{\mathrm{tot}} < R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	Any K^{intra}	Any K^{intra}	$\tilde{f}_1$	(65)
6.3.2	$L_{\mathrm{tot}} > R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	∞	Any K^{intra}	$\tilde{f}_1$	(68)
6.4.2	$L_{\mathrm{tot}} > R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	Any K^{intra}	Any K^{intra}	$\tilde{f}_1$	(68)
6.3.3	$L_{\mathrm{tot}} \approx R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	∞	Any K^{intra}	$\tilde{f}_1$	(72)
6.4.3	$L_{\mathrm{tot}} \approx R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	Any K^{intra}	Any K^{intra}	$\tilde{f}_1$	(72)
6.3.4	$L_{\mathrm{tot}} \gg R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	∞	Any K^{intra}	$\tilde{f}_1$	(74)
6.4.4	$L_{\mathrm{tot}} \gg R_{\mathrm{tot}}$	Any	$\alpha \gg 1$	Any K^{intra}	Any K^{intra}	$\tilde{f}_1$	(74)
6.6	Any	Any	Any	∞	Any K^{intra}	$\tilde{f}_{11} \approx \tilde{f}_1/L_{\mathrm{tot}}$	(85)
6.6	Any	Any	Any	Any K^{intra}	Any K^{intra}	$\tilde{f}_{11} \approx \tilde{f}_1/L_{\mathrm{tot}}$	(87)

to be made. Our predictions are largely restricted to certain asymptotic limits and the issue of deciding whether, in an experiment, a given asymptotic limit is valid, is not obvious. The many explicit relations such as (75) that we derived in our analysis can be useful in this regard as a consistency check. The values obtained for dissociation constants in practice will obviously be limited by the precision with which the experimental curve $C^*(L_{\mathrm{tot}}^*)$ can be determined; errors in the dissociation constant values obtained should be in line with these experimental errors.

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