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Exact Analysis of Ligand-Induced Dimerization of Monomeric Receptors

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This paper analyzes the equilibria involved in the dimerization of monomeric receptors with homo-bifunctional ligands. We provide analytical expressions that can be used to estimate the concentration of each species present in a mixture of homo-bifunctional ligand and monomeric proteins, given initial conditions defining the total concentration of bivalent ligand $[L_2]_0$, the total concentration of protein $[P]_0$, one dissociation constant K_d, and a parameter to account for cooperativity α. We demonstrate that the fraction of protein present in a complex of two proteins and one bivalent ligand ($P \cdot L_2 \cdot P$) is maximized at $[L_2]_0 = K_d/2 + [P]_0/2$.

This paper analyzes the equilibria involved in the dimerization of monomeric receptors (e.g., proteins in solution or bound to a membrane) with homo-bifunctional ligands (Figure 1B). Our objective was to develop reliable equations that would allow us to predict the concentrations of protein and ligand at which the fraction of protein present in a complex of two proteins and one bivalent ligand ($P \cdot L_2 \cdot P$) is maximized. We provide analytical expressions that can be used to estimate the concentration of each species (L_2, P, $P \cdot L_2$, $P \cdot L_2 \cdot P$) present in a mixture of homo-bifunctional ligand and monomeric proteins, given initial conditions defining the total concentration of bivalent ligand $[L_2]_0$, the total concentration of protein $[P]_0$, one dissociation constant K_d, and a parameter to account for cooperativity α. Our expressions are based only on the assumption (commonly made for dilute solutions) that the thermodynamic activities of the species are equal to their molar concentrations.

Numerous biological pathways and processes involve the ligand-induced dimerization of monomeric proteins—e.g., the proliferation of B lymphocytes, triggering of degranulation by cells involved in the immune response, and the production of erythrocytes.[1–3] Our analysis provides researchers guidelines for designing and conducting assays that use or measure the ligand-induced dimerization of proteins.

The ligand-induced dimerization of soluble proteins and the ligand-induced dimerization of monomeric receptors on the membrane of a cell are strategies important in determining the response of a cell to external stimuli. Exploiting oligovalency is a strategy also widely used in the (semi)rational design of ligands for proteins.[4–6] The term "chemical inducers of dimerization" has been applied to both homo- and hetero-bifunctional ligands that dimerize soluble proteins, and this class of molecules includes both small molecules and peptides.[5] For example, Schreiber and Crabtree reported that FK1012, a synthetic dimer of FK506, binds two molecules of FKBP-12.[7] Belshaw et al. reported the synthesis of a dimer of cyclosporin and found that this compound is capable of triggering apoptosis in certain engineered T-cells.[8]

The binding of soluble homo-bifunctional ligands (L_2) to receptors bound in a membrane is treated by the theory described in this paper (Figure 1C). Human growth hormone, erythropoietin, and granulocyte colony-stimulating factor are cytokines that bind two receptors simultaneously and transmit a signal across the cell membrane.[3] Antibodies are bivalent receptors, and the theory developed herein applies to bivalent immunoglubulins binding to monovalent antigens (P_2 and L, respectively in Figure 1D).

Perelson and DeLisi derived an analytical expression to calculate the fraction of dimerized proteins in a mixture of homo-bifunctional ligands and monomeric proteins.[9] Their model—based on the simplifying assumption that the concentration of total bivalent ligand is equal to the concentration of free or unbound bivalent ligand—is limited to circumstances where this assumption is valid (that is, where the total concentration of the bivalent ligand $[L_2]_0$ is much greater than the total concentration of monomeric protein $[P]_0$). For many systems, this assumption is inappropriate. In particular, for systems where the objective is to maximize $[P \cdot L_2 \cdot P]$ (signaling assays, therapeutic treatments, separations, etc.), it fails entirely. Whitty and Borysenko reviewed the Perelson treatment in the context of surface-bound receptors and provided several guidelines for the identification and optimization of lead

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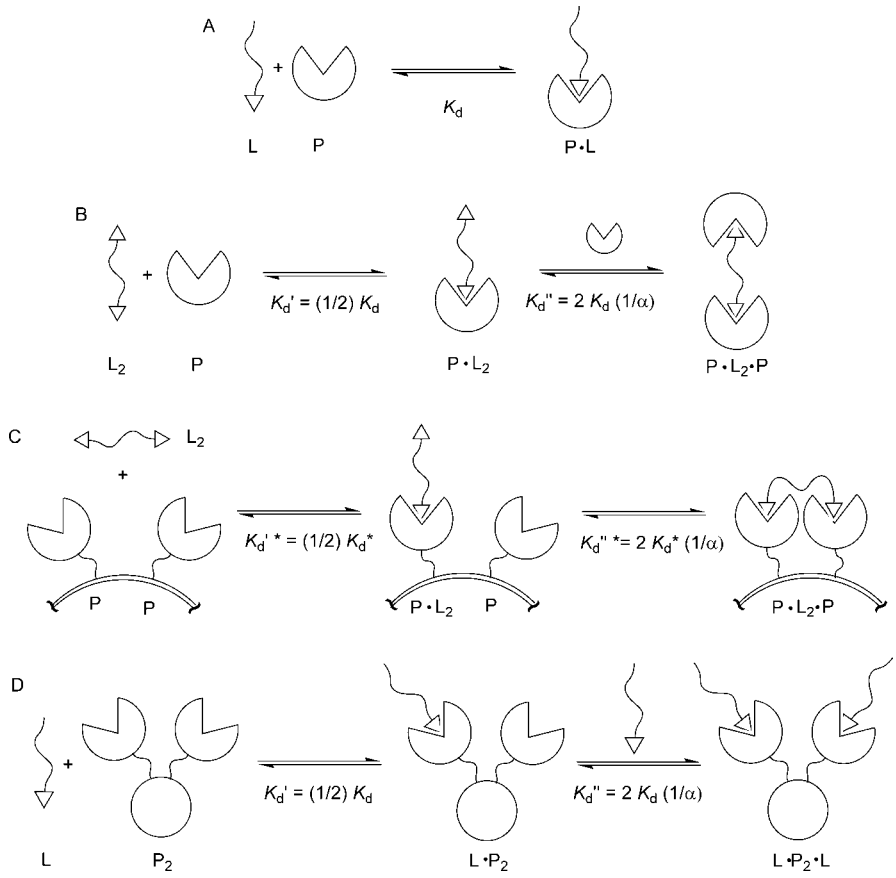

Figure 1. Thermodynamic schemes describing the binding of monovalent and bivalent ligands to monovalent and bivalent receptors. (A) The equilibrium between a monovalent ligand (L), a monomeric protein (P), and a protein−ligand complex (P·L) is characterized by a dissociation constant (K_d). (B) The equilibrium between a bivalent ligand (L_2) and P can be conceptualized as two equilibria: the equilibrium between L_2, P, and P·L_2 is characterized by a dissociation constant $K_d' = (1/2)\ K_d$, and the equilibrium between L_2, P·L_2, and a complex composed of two proteins and one bivalent ligand (P·L_2·P) is characterized by a dissociation constant $K_d'' = 2K_d(1/\alpha)$, where K_d in each equilibrium are assumed to be the same and α is a parameter to account for cooperativity. Further details of K_d, K_d', and K_d'' are provided in the text. (C) The binding of L_2 to proteins bound in a surface is also described in this theory; although, the monovalent dissociation constant for this equilibrium (K_d^*) will likely have different values than K_d for soluble ligand and soluble protein. (D) The equilibrium between a bivalent receptor (P_2) and monovalent ligand (L) is exactly analogous, with the terms P and L interchanged.

molecules.[10] Their conclusions are subject to the limitations of the Perelson model. Erickson and co-workers described a mathematical model to analyze the dimerization of the Fab portion of anti-DNP immunoglubins with bivalent DNP ligands and used a numerical solver to fit the binding polynomial to the their data.[11] These authors did not provide an analytical solution to the binding polynomial. The groups of Wagner and Hu studied separately the dimerization of dihydrofolate reductase with bivalent ligands presenting two molecules of methotrexate. Each group presented a mathematical model suitable for analyzing the data.[12,13] These models require an initial guess for certain concentrations followed by an iterative process to determine the parameters of interest. The dependence of the system on the various concentrations and binding constants was not explored. Braun and Wandless developed a general method to describe the concentrations of proteins and ligands in a system at equilibrium using differential equations.[14] The resolution of these equations requires a knowledge of numerical calculus.

Our objectives were as follows: (i) develop an equation that accurately describes the equilibria described in Figure 1B, and especially to understand the response of $[P{\cdot}L_2{\cdot}P]/\ [P]_0$ (that is, the fraction of the protein present as dimer) to concentrations and binding constants; (ii) provide a series of calculated curves to illustrate the dependence of the system on these concentrations and binding constants; and (iii) provide all the relevant expressions in a format that can be easily adopted and used by other researchers (an Excel worksheet is provided in the Supporting Information) without any limitation imposed by knowledge of numerical calculus.

RESULTS

Equilibria between Monomeric Proteins and a Bivalent Ligand. Figure 1A represents the equilibrium between a monovalent ligand (L), a monomeric protein (P), and a complex of protein and ligand (P·L). This equilibrium is characterized by the dissociation constant K_d (eq 1).

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$$K_d = \frac{[L][P]}{[P\cdot L]} \quad (1)$$

P and P·L typically have units of concentration (i.e., molar) in solution or units of density (i.e., moles area^{-1}) when bound to a surface. The value for the dissociation constant in solution can be drastically different from the dissociation constant when either the receptor or ligand is tethered to a surface. This difference requires that K_d be determined for the particular equilibrium under study.

Figure 1B represents the two possible equilibria between a bivalent ligand (L_2) and monomeric proteins (P). The equilibrium between P, L_2, and a complex of one P and one L_2 ($P\cdot L_2$) is characterized by the dissociation constant K_d'—the product of a statistical factor of $^1/_2$ and the monovalent dissociation constant K_d (eq 2). The statistical factor of $^1/_2$ reflects the fact that there are two ways to form $P\cdot L_2$ from P and L_2 but only one way to form P and L_2 from $P\cdot L_2$.[15]

$$K_d' = \frac{K_d}{2} = \frac{[L_2][P]}{[P\cdot L_2]} \quad (2)$$

The complex $P\cdot L_2\cdot P$ contains two monomeric proteins for each bivalent ligand. The equilibrium between P, $P\cdot L_2$, and $P\cdot L_2\cdot P$ is characterized by the dissociation constant K_d''—the product of a statistical factor of 2, the monovalent dissociation constant K_d and a term to account for cooperativity $1/\alpha$ (eq 3).

$$K_d'' = \frac{2K_d}{\alpha} = \frac{[P][P\cdot L_2]}{[P\cdot L_2\cdot P]} \quad (3)$$

The factor of 2 is a statistical factor that accounts for the existence of two ways to form $P\cdot L_2$ from $P\cdot L_2\cdot P$ but only one way to form $P\cdot L_2\cdot P$ from $P\cdot L_2$. We assume the monovalent dissociation constant for the dissociation of $P\cdot L_2\cdot P$ into $P\cdot L_2$ and P to have the same value as the dissociation constant K_d for the dissociation of P·L into P and L (eq 1) and subsume deviations from these values into a cooperativity factor α.

The parameter α accounts for interactions among the components of the system: if $\alpha < 1$ the system is negatively cooperative and P binds less strongly to PL_2 than P binds to L_2. Unfavorable steric interactions between the proteins in $P\cdot L_2\cdot P$ can result in negatively cooperative ($\alpha < 1$) binding. When $\alpha = 1$, the system is noncooperative and P binds to $P\cdot L_2$ with the same affinity as P binds to L_2. Noncooperative binding could arise if the distance between the ends of L_2 is sufficiently large that the binding of one P to one end of L_2 does not affect the binding another P at the other end of L_2. In other words, the formation of $P\cdot L_2\cdot P$ is not affected by the presence of the linker comprising L_2. If $\alpha > 1$, the system is positively cooperative and the binding of P to $P\cdot L_2$ is more favorable than the binding of P to L_2. Positively cooperative binding could result from the formation of favorable "contacts" between the two proteins in $P\cdot L_2\cdot P$. Nonspecific interactions among proteins on a surface or in solution can cause deviations from thermodynamic ideality (thermodynamic activities ≠ 1); such deviations are not accounted for by changes in α.[16]

Derivation and Solutions of the Binding Polynomials. In the formulation of the problem, we describe the concentration of unbound or free protein [P] in terms of the total concentration of protein $[P]_0$, the total concentration of bivalent ligand $[L_2]_0$, α, and K_d. Equations 2 and 3 characterize the equilibria of Figure 1B, and eqs 4 and 5 are the equations accounting for the mass balance of the bivalent ligand and the protein, respectively.

$$[L_2]_0 = [L_2] + [P\cdot L_2] + [P\cdot L_2\cdot P] \quad (4)$$

$$[P]_0 = [P] + [P\cdot L_2] + 2[P\cdot L_2\cdot P] \quad (5)$$

Equations 2−5 can be arranged as a cubic equation (eq 6) which, written out in full, is equation 7.

$$[P]^3 + a[P]^2 + b[P] + c = 0 \quad (6)$$

$$[P]^3 + \left(\frac{2K_d}{\alpha} + 2[L_2]_0 - [P]_0\right)[P]^2 + \frac{K_d}{\alpha}(K_d + 2[L_2]_0 - 2[P]_0)[P] - \frac{K_d^2}{\alpha}[P]_0 = 0 \quad (7)$$

Equation 7 can be solved analytically to yield three roots. The only physically real solution of equation 7 is equation 8, where Q and R are defined in eqs 9 and 10.[17,18]

$$[P] = -\frac{a}{3} + \sqrt[3]{R + \sqrt{Q^3 + R^2}} + \sqrt[3]{R - \sqrt{Q^3 + R^2}} \quad (8)$$

$$Q = \frac{3b - a^2}{9} \quad (9)$$

$$R = \frac{9ab - 27c - 2a^3}{54} \quad (10)$$

Equations 11 and 12 are the solutions of eq 7 for $[P\cdot L_2]$ and $[P\cdot L_2\cdot P]$, respectively. The Supporting Information provides further details on deriving the equations and an Excel worksheet for using them.

$$[P\cdot L_2] = \frac{2[L_2][P]}{K_d} = \frac{2K_d[L_2]_0[P]}{K_d^2 + 2K_d[P] + \alpha[P]^2} \quad (11)$$

$$[P\cdot L_2\cdot P] = \alpha\frac{[L_2][P]^2}{K_d^2} = \frac{\alpha[L_2]_0[P]^2}{K_d^2 + 2K_d[P] + \alpha[P]^2} \quad (12)$$

The fractions of the total amount of protein in the form P, $P\cdot L_2$, and $P\cdot L_2\cdot P$ (θ_P^P, $\theta_P^{PL_2}$, and $\theta_P^{PL_2P}$, respectively) are given by eqs 13−15 where the superscript variable (P, PL_2, or PL_2P) represents the numerator of the ratio and the subscript P reminds us that the denominator is the total concentration of protein.

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$$\theta_P^{P} = \frac{[P]}{[P]_0} \quad (13)$$

$$\theta_P^{PL_2} = \frac{[P \cdot L_2]}{[P]_0} \quad (14)$$

$$\theta_P^{PL_2P} = \frac{2[P \cdot L_2 \cdot P]}{[P]_0} = 1 - \theta_P^{PL_2} - \theta_P^{P} \quad (15)$$

Figure 2 illustrates the behavior of the system represented in Figure 1B for a representative set of specific values and concentrations. As seen in Figure 2, when $[L_2]_0$ is much smaller than K_d and $[P]_0$, the mixture contains primarily free monovalent protein. As $[L_2]_0$ increases, the concentration of dimerized protein $[P \cdot L_2 \cdot P]$ increases to a maximum at $[L_2]_0 = K_d/2 + [P]_0/2$ (vide infra). When $[L_2]_0$ is increased further, the equilibrium is shifted to favor the formation of complexes comprising one protein and one bivalent ligand ($P \cdot L_2$).

DISCUSSION

In the vast majority of in vivo assays involving homobifunctional ligands, the observed response (e.g., apoptosis, expression of protein, etc.) is a function of the quantity of receptor that is dimerized ("quantity" may mean concentration or fraction of total protein, depending on circumstance). We have therefore prepared the following results in terms of $\theta_P^{PL_2P}$, as defined in eq 15.

Dependence on $[P]_0$ and K_d. Figure 3A shows a series of curves of $\theta_P^{PL_2P}$ as a function of changing $[P]_0/K_d$ ($[P]_0/K_d = 0.05$, 0.5, 5, 50, and 500), where each curve corresponds to a calculation with $\alpha = 1$. The calculations depicted in Figure 3A predict that the ratio of $[L_2]_0/K_d$ for which $\theta_P^{PL_2P}$ reaches a maximum value depends on $[P]_0$ and K_d. More importantly, and unsurprisingly, the value of $[L_2]_0/K_d$ where $\theta_P^{PL_2P}$ reaches a maximum in Figure 3A is of the same order of magnitude as $[P]_0/K_d$ (i.e., $[P]_0 \approx [L_2]_0$), which indicates that for large values of $\theta_P^{PL_2P}$ (i.e., $\theta_P^{PL_2P} > 0.1$ and $[P \cdot L_2 \cdot P] > 0.1[P]_0$) the value of $[L_2]_0$ is of the same order of magnitude as $[P \cdot L_2 \cdot P]$ because $[P \cdot L_2 \cdot P] > 0.1[L]_0$. As a consequence, setting $[L_2]_0 = [L_2]$ (that is, assuming $[P \cdot L_2] + [P \cdot L_2 \cdot P] < [L_2]$ as did Perelson and DeLisi) is not a valid assumption.

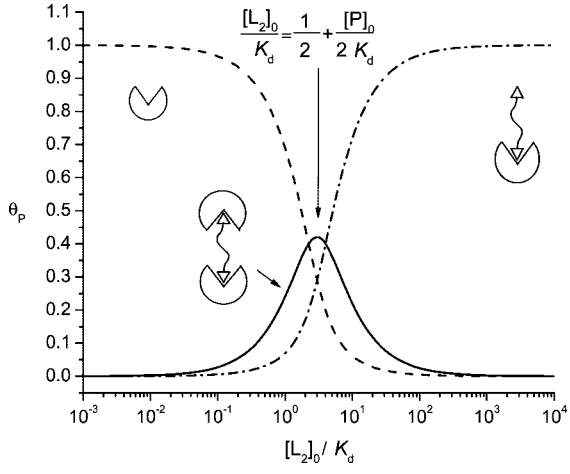

Figure 2. Plot of the fraction of P present as P, $P \cdot L_2$, and $P \cdot L_2$, θ_P (θ_P represents $\theta_P^{P} = [P]/[P]_0$, $\theta_P^{PL_2} = [P \cdot L_2]/[P]_0$, and $\theta_P^{PL_2P} = 2[P \cdot L_2 \cdot P]/[P]_0$, where $[P]_0$ is the total concentration of protein) as a function of the ratio of the total concentration of bivalent ligand ($[L_2]_0$) to K_d. For the specific results shown here, calculations were performed using $K_d = 0.02$ μM, $\alpha = 1$, and $[P]_0 = 0.1$ μM, according to eqs 13–15. A bell-shaped curve is obtained for $\theta_P^{PL_2P}$.

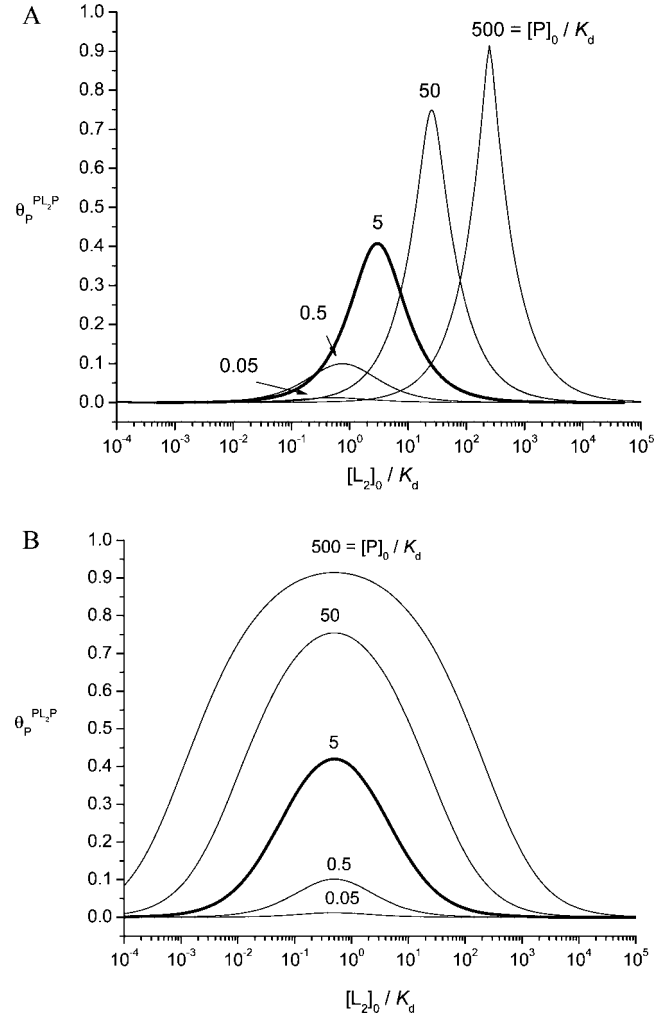

Figure 3. Plots of $\theta_P^{PL_2P}$ as a function of $[L_2]_0/K_d$ with $\alpha = 1$ showing the variations in $\theta_P^{PL_2P}$ with changing ratios of $[P]_0/K_d$. (A) Calculations using the exact solution (eq 15), and (B) calculations based on a model that assumes $[L_2]_0 = [L_2]$ (eq S6).

For comparison, Figure 3B also shows a series of curves calculated assuming that $[L_2]_0 = [L_2]$ (eq S6), as proposed by Perelson and DeLisi (summarized in the Supporting Information). The parameters for the curves in Figure 3B are identical to the parameters used in the calculation of Figure 3A; that is, $\alpha = 1$, and $[P]_0/K_d = 0.05$, 0.5, 5, 50, 500. Under the assumption that $[L_2]_0 = [L_2]$ (Figure 3b), $\theta_P^{PL_2P}$ reaches a maximum at a total concentration of ligand that does not depend on $[P]_0$. The maximum in $\theta_P^{PL_2P}$ is incorrectly predicted to occur when $[L_2]_0 = K_d/2$. These results are both misleading and physically unreasonable for all values of $[P]_0/K_d > 0.5$. For example, in the curve with $[P]_0/K_d = 500$, if we assume a value for K_d of 1 μM, the maximum value in $\theta_P^{PL_2P}$ of 0.9 would require a value for $[L_2]_0 \geq 225$ μM; the total concentration of bivalent ligand $[L_2]_0$ is only 0.5 μM.

Dependence on α. Figure 4 shows curves of $\theta_P^{PL_2P}$ versus $[L_2]_0/K_d$ as a function of changing α with $[P]_0/K_d = 5$. The curves in Figure 4 show that the value of $[L_2]_0/K_d$ at which $\theta_P^{PL_2P}$ is maximum is independent of the value of α. The maximum

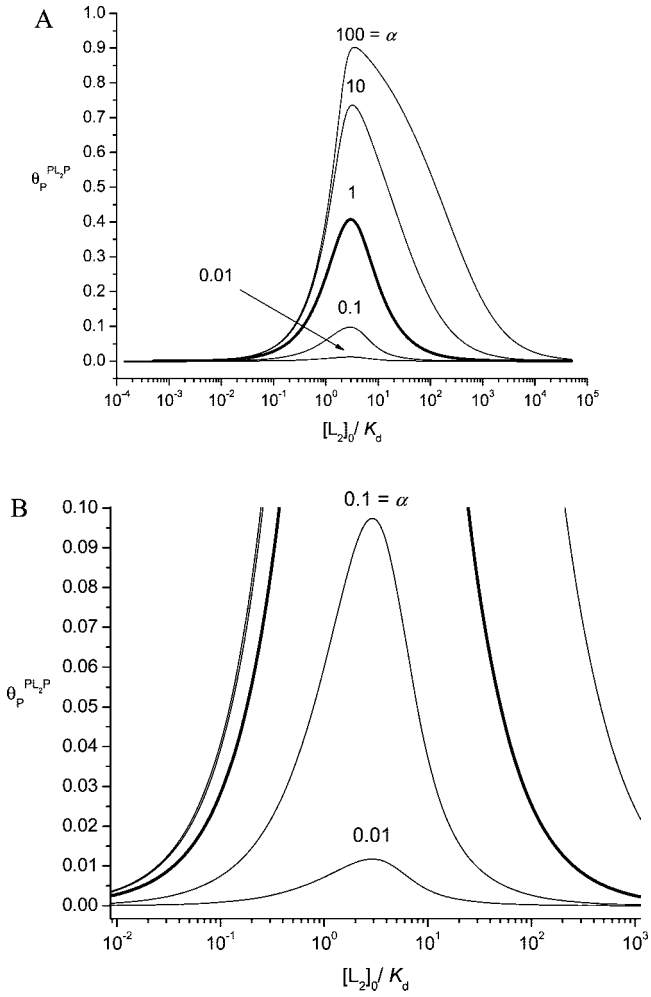

Figure 4. Plots of $\theta_P^{PL_2P}$ as a function of $[L_2]_0/K_d$ showing the variations in $\theta_P^{PL_2P}$ with changing α and fixed $[P]_0/K_d = 5$ (e.g., $[P]_0 = 0.1\ \mu M$ and $K_d = 0.02\ \mu M$). (A) Curves for α ranging from 0.001 to 100 and (B) expanded view of $\theta_P^{PL_2P}$ with α = 0.1 and 0.01.

concentration of P·L_2·P is proportional to α. In other words, when α >1, the system is positively cooperative and more P·L_2·P is formed than when α ≤ 1. A curve of $\theta_P^{PL_2P}$ versus $[L_2]_0/K_d$ is symmetrical about the maximum when α =1. If the system is positively cooperative (α > 1), the slope of the curve is steep and positive when $[L_2]_0 < [L_2]_{0,max}$. The slope is negative and shallow when $[L_2]_0 > [L_2]_{0,max}$. Positive cooperativity in the system shifts the equilibrium from P·L_2 toward P·L_2·P because the binding of P to P·L_2 is more favored than the binding of P to L_2.

Maximum in $\theta_P^{PL_2P}$. The total concentration of bivalent ligand at which $\theta_P^{PL_2P}$ reaches a maximum ($[L_2]_{0,max}$) can be found using eq 16.

$$[L_2]_{0,max} = \frac{K_d}{2} + \frac{[P]_0}{2} \qquad (16)$$

The derivation of eq 16 is in the Supporting Information (eq S42). Equation 16 predicts that the position of the maximum in $\theta_P^{PL_2P}$ is independent of α, a result in agreement with Figure 4. A practical consequence of using eq 16 is that knowledge of the position of the maximum of $\theta_P^{PL_2P}$, together with knowledge of $[P]_0$ (the value of $[P]_0$ is usually a known parameter of in vitro assays), allows one to estimate K_d. Alternatively, one can estimate $[P]_0$ (usually unknown in in vivo assays) using eq 16 and the knowledge of the position of the maximum of $\theta_P^{PL_2P}$ and of K_d.

The maximum value of $\theta_P^{PL_2P}$ ($\theta_P^{PL_2P}{}_{max}$) can be calculated using eq 17 (a result initially described correctly by Perelson and DeLisi[9]). The derivation of eq 17 is in the Supporting Information (eq S41).

$$\theta_P^{PL_2P}{}_{max} = 1 + \frac{2K_d}{\alpha[P]_0} - \sqrt{\left(\frac{2K_d}{\alpha[P]_0}\right)^2 + \frac{4K_d}{\alpha[P]_0}} \qquad (17)$$

According to eq 17, and as shown in Figures 3 and 4, $\theta_P^{PL_2P}{}_{max}$ depends on $[P]_0$, K_d, α, and $[L_2]_0$. With knowledge of $\theta_P^{PL_2P}{}_{max}$, $[P]_0$, and K_d, 17eq 17 can provide an estimate of the cooperativity α. For example, variations in the experimental conditions leading to increasing values of $\theta_P PL_2P_{max}$ at constant $[L_2]_{0,max}$ indicates that the cooperativity is increasing in the system while K_d remains constant.

A practical consequence of using both eqs 16 and 17 is that knowledge of $\theta_P^{PL_2P}{}_{max}$ and $[L_2]_{0,max}$ along with knowledge of $[P]_0$ allows a simple determination of both the dissociation constant K_d and the cooperativity α.

CONCLUSIONS

The model introduced in this paper is exact—it does not include approximations—and relates the concentrations of each species to parameters characteristic of the equilibria, $[P]_0$, $[L]_0$, K_d, and α. Experimental results, therefore, can be related accurately to the model and can provide a clear understanding of the behavior of the equilibria.

This paper makes it possible to estimate the concentrations of $[L_2]_0$ and $[P]_0$ (for specific values of K_d and α) at which $\theta_P^{PL_2P}$ is maximum; it thus makes it possible—in a particular system—to maximize the value of $\theta_P^{PL_2P}$. The results of this paper could find direct application in a variety of fields, including the analysis of assays for cell signaling, the design and optimization of bivalent ligands to be used as drugs, and the precipitation of proteins in the presence of bivalent ligands.

In application of this theory to assays measuring cell signaling, eq 16 provides a convenient way to study the expression of cell membrane receptors simply using the relationship that $[L_2]_{0,max}$ is directly proportional to $[P]_0$. For instance, the relative levels of expression of homo-dimeric receptors in two cell lines could be assayed by measuring the concentration of bivalent ligand (a cytokine in this case) required to elicit a maximum response; the larger the value of $[L_2]_{0,max}$ the larger the value of $[P]_0$.

To maximize the conversion of proteins (P) to dimeric complexes (P·L_2·P), one should design ligands with low values of K_d and, if possible, with positive cooperativity (α >1). A simple protocol for screening bivalent ligands can be developed based on the homodimerization model presented in this paper. For instance, one could screen bivalent ligands with different linkers (e.g., linkers of different length) by measuring $\theta_P^{PL_2P}{}_{max}$. Assuming that K_d is equivalent for all ligands, the largest value of $\theta_P^{PL_2P}{}_{max}$ observed in the experimental curves would indicate the bivalent ligand that binds with the most favorable value of α.

The model of homodimerization in solution that we present in this paper is universal; it does not include approximations or

numerical calculations that may limit the accuracy of the results obtained with other presently available models. It is also applicable to interactions on surfaces, although on surfaces, the equations summarized here may not adequately describe all interactions (especially lateral steric interactions involving more than two proteins). We believe that our model will be useful in a spectrum of applications, particularly in the design of assays for studying homodimeric receptors.

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SUPPORTING INFORMATION AVAILABLE

Includes Figures S1 and S2 (showing the dependence of $\theta_P^{PL_2P}$ on $[P]_0$ and K_d), the detailed derivation of the analytical expressions used in this paper (eqs 1−17) (pdf file), and an Excel file (xls file) in which eqs 1−17 were implemented for easy quantification. This material is available free of charge via the Internet at http://pubs.acs.org.

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