An exact mathematical expression for describing competitive binding of two different ligands to a protein molecule

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Abstract The dissociation constant for the binding of a spectroscopically invisible or non-radioactive ligand to its protein receptor can be determined in a competition experiment by using a structural analog that contains a reporter group. Many plotting and numerical analysis methods have been developed to calculate the binding constant of unlabeled ligand from the displacement experiments. However, a common problem with these plotting methods is that the equation transformations inevitably result in non-standard error distribution, and thus simple linear regression can not be used to extract correct values for the parameters. In the case of the numerical analysis methods, one would be faced with the possible existence of multiple solutions. In this paper, the exact mathematical expression for describing competitive binding of two different ligands to a protein molecule is presented in terms of the total concentrations of species in the system. Thus, using a commercially available non-linear regression program, all unknown parameters for describing this system can be determined by fitting the experimental data to the algebraically explicit equation without any data transformations. The distribution curves of all the species in the system can also be constructed with this equation. It is particularly useful for the cases in which the concentrations of all the species in the system are comparable to each other.

Key words: Protein–ligand interaction; Competitive displacement; Ligand binding; Determination of binding constant

1. Introduction

Of a number of physical techniques known for determining the binding constants of ligand–protein complexes, spectroscopic titration and equilibrium dialysis with radioactively labeled ligand are the most commonly used [1–3]. However, many ligands of interest are neither chromophores nor available in labeled form. In this case, the binding constants of these ligands can be determined from measurement of the competitive displacement of a bound chromophoric or radiolabeled ligand. To obtain the binding constant for the chromophoric ligand alone, experimental data can be fitted to an algebraically explicit equation [4–6]. However, it is not easy to extend the analytical, algebraically explicit description of binding equilibria to mixtures of competing ligands. For a system consisting of a protein and two competing ligands, when the total concentrations of all the components are of the same order of magnitude, combining all partial equilibria and mass conservation, one obtains a cubic algebraic equation [7,8]. Roots of polynomials degree $n > 2$ are usually extracted by methods of numerical analysis [9], and polynomial equations up to the fourth degree can even be solved analytically [10,11]. In the case of both the numerical and the analytical methods, however, one would be faced with the possible existence of multiple real roots.

In the present paper, the properties of the roots of the general cubic equation have been analyzed in detail. For a system consisting of a protein and two competing ligands, a unique proper root has been identified unambiguously. Thus, this algebraically explicit equation can be put into a commercially available non-linear regression computer program, and all unknown parameters for describing this system can be determined by fitting the experimental data to this equation without any data transformations. The distribution curves of all the species in the systems can also be constructed with this equation.

2. Theory

Let us consider a protein binding experiment that includes a mixture of two ligands, a chromophoric ligand A and an invisible ligand B according to the following equations

$$P + A \overset{K_A}{\rightleftharpoons} PA, \quad P + B \overset{K_B}{\rightleftharpoons} PB$$

$$K_A = \frac{[P][A]}{[PA]}, \quad K_B = \frac{[P][B]}{[PB]} \tag{1}$$

Conservation of mass requires that

$$[A]_0 = [A] + [PA] \tag{2}$$

$$[B]_0 = [B] + [PB] \tag{3}$$

$$[P]_0 = [P] + [PA] + [PB] \tag{4}$$

where K_A and K_B are dissociation constants for the binding of A and B, and [P], [A] and [B] are the concentrations of free protein and free ligands, respectively. In the titration experiment, A is added to a mixture of protein and ligand B. The total concentrations of protein and ligand B are $[P]_0$ and $[B]_0$, respectively. The total concentration of ligand A is varied, and the spectroscopic signal is measured for each ternary mixture.

From eqs. 1–3, we have

$$[PB] = \frac{[P][B]_0}{K_B + [P]} \tag{5}$$

$$[PA] = \frac{[P][A]_0}{K_A + [P]} \tag{6}$$

Substitution of eqs. 5 and 6 into eq. 4 and rearrangement yields

$$[P]^3 + a[P]^2 + b[P] + c = 0 \tag{7}$$

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where

$$a = K_A + K_B + [A]_0 + [B]_0 - [P]_0$$
$$b = K_B([A]_0 - [P]_0) + K_A([B]_0 - [P]_0) + K_A K_B$$
$$c = -K_A K_B [P]_0$$

By the substitution $[P] = u - (a/3)$, the eq. 7 becomes

$$u^3 - qu - r = 0 \quad (8)$$

where

$$q = \frac{a^2}{3} - b, \qquad r = -\frac{2}{27}a^3 + \frac{1}{3}ab - c$$

The discriminant of eq. 8 is given by

$$\Delta = \frac{r^2}{4} - \frac{q^3}{27} \quad (9)$$

Since $\Delta < 0$, the three real roots of eq. 8 are given by [11]

$$u_1 = \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\frac{\theta}{3} \quad (10)$$

$$u_2 = \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\frac{2\pi - \theta}{3} \quad (11)$$

$$u_3 = \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\frac{2\pi + \theta}{3} \quad (12)$$

where

$$\theta = arc\cos\frac{-2a^3 + 9ab - 27c}{2\sqrt{(a^2 - 3b)^3}}$$

According to the definition of u and the physical conditions of the problem proposed, it can be verified that u_1 expresses the unique physically meaningful root of eq. 8, and u_2 and u_3 have no reference to the problem proposed (see Appendix). Thus, the proper root of eq. 7 can then be written as

$$[P] = -\frac{a}{3} + \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\frac{\theta}{3} \quad (13)$$

and the expressions for [PA] and [PB] are given by

$$[PA] = \frac{[A]_0\{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}}{3K_A + \{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}} \quad (14)$$

$$[PB] = \frac{[B]_0\{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}}{3K_B + \{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}} \quad (15)$$

In a competitive displacement titration experiment, let

$$F_0 = \varepsilon_A[A]_0$$
$$F_0' = \varepsilon_B[B]' + \varepsilon_P[P]' + \varepsilon_{PB}[PB]'$$
$$F = \varepsilon_B[B] + \varepsilon_P[P] + \varepsilon_{PB}[PB] + \varepsilon_A[A] + \varepsilon_{PA}[PA]$$

where F_0, F_0' and F are the spectroscopic signal of ligand A alone, protein–ligand B mixture and the triple mixture containing the protein, ligand A and ligand B at the same total concentrations, respectively. If the binding of ligand B to the protein gives no change in spectroscopic signal at the given wavelength, we have $\varepsilon_{PB} = \varepsilon_P + \varepsilon_B$, and the change in spectroscopic signal due to the interaction between ligand A and protein P can consequently be described by

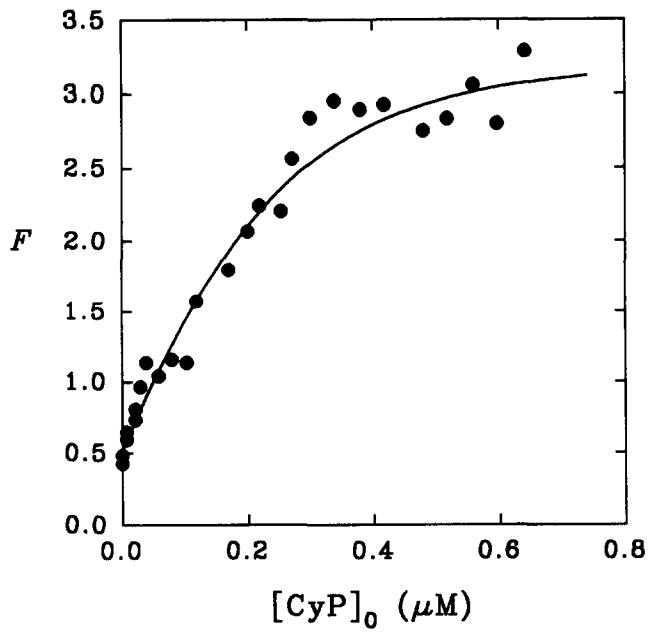

Fig. 1. Fluorescence intensities observed in the competitive binding of cyclosporin A (CsA) and [D-Lys(Dns)]^8-CsA to cyclophilin. The concentrations of [D-Lys(Dns)]^8-CsA and CsA are 0.2 μM and 0.5 μM, respectively. The dissociation constant for [D-Lys(Dns)]^8-CsA was kept constant (5.3 nM). The curve represents the best fit to eq. 17.

$$F - F_0 - F_0' = \Delta F = C[PA] = \frac{C[A]_0\{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}}{3K_A + \{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}} \quad (16)$$

In absorption spectroscopy, the constant, $C = \varepsilon_{PA} - \varepsilon_P - \varepsilon_A$, is the difference in the molar extinction coefficient between the PA complex and other species. Thus, when the volume change during the titration experiments is negligible, the total concentrations of both enzyme and ligand B are constant, and K_B can then be determined by fitting experimental data to eq. 16.

If protein P has no absorbance or fluorescence at the given wavelength, the competitive titration experiment can be performed by adding a different amount of protein into a solution with fixed concentrations of ligand A and B. Similarly, let

$$F_0 = \varepsilon_A[A]_0 + \varepsilon_B[B]_0$$
$$F = \varepsilon_B[B] + \varepsilon_P[P] + \varepsilon_{PB}[PB] + \varepsilon_A[A] + \varepsilon_{PA}[PA]$$
$$= \varepsilon_B[B]_0 + \varepsilon_A[A]_0 + (\varepsilon_{PA} - \varepsilon_A)[PA]$$

and

$$F - F_0 = (\varepsilon_{PA} - \varepsilon_A)[PA]$$

When [P] approaches infinity, $[PA] = [A]_0$, we then have $F_{max} - F_0 = (\varepsilon_{PA} - \varepsilon_A)[A]_0$ and

$$F = F_0 + (\varepsilon_{PA} - \varepsilon_A)[PA] =$$
$$F_0 + (F_{max} - F_0)\frac{\{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}}{3K_A + \{2\sqrt{(a^2 - 3b)}\cos(\theta/3) - a\}} \quad (17)$$

As an example of the use of the new method, data were taken from Fig. 2 of Kuzmic et al.'s paper [12] and re-analyzed using the present method. The experimental data (Fig. 1, filled circles)

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were fitted to eq. 17 using a modified computer program [13] or a commercially available non-linear regression program, SigmaPlot 5.0. The optimized values of K_B, F_0 and F_{max} were 43.4 ± 10.3 nM, 0.514 ± 0.047, and 2.694 ± 0.084, respectively. It can be seen from the result that the value of the dissociation constant for cyclosporin A, K_B, obtained by the present method is quite close to that obtained by Kuzmic et al.

3. Discussion

In a competitive displacement titration experiment, if the spectroscopic signal for the interaction is strong enough to detect the low concentration of the protein–ligand complex in solution, and the binding of both ligands to protein is relatively weak, it would be possible to carry out the titration experiments under conditions where $[A]_0$, $[B]_0 \gg [P]_0$. In these situations, the concentration of each protein–ligand complex is negligibly small with respect to one of its molecular components, and the concentration of free ligands can be treated as equal to their total concentrations. Thus the dissociation constant of the 'invisible' ligand B can then be easily determined by either a graphic method used in enzyme kinetics [14], or a non-linear least square regression method. However, such situations rarely prevail. Most often, the spectroscopic signals for protein–ligand complexes are detectable only at relatively high protein concentrations ($[P]_0 > K_A, K_B$). Under this condition, a significant fraction of the total ligand is bound to protein, and thus $[A]_0$ and $[B]_0$ can not be treated as equal to the free ligand concentration. In the case of a direct titration experiment, this causes little problem because the corresponding equation is only quadratic [4,5], and the physically meaningful root can be easily identified. In the case of a competitive displacement experiment, however, the corresponding equation becomes cubic and thus one would be faced with the possible existence of multiple roots [8,12]. A similar problem was also encountered in the determination of a dissociation constant for the receptor–non-radioactive ligand complex from displacement curves [7]. Although many plotting methods have been developed to calculate the binding constant of unlabeled ligand from the displacement experiments [7,15,16], a common problem with these plotting methods is that the equation transformations inevitably result in a non-standard error distribution, and thus simple linear regression can not be used to extract correct values for the parameters [17,18]. Recently, Kuzmic et al. have presented an alternative method to circumvent this problem [12]. However, the use of their method requires a special computer program which may not be available in most laboratories. The best solution for this problem seems to be to perform regression analysis on the original non-linear form of the equation [18]. Thus, an analytical, algebraically explicit equation is necessary for most commercially available non-linear regression programs.

In this paper, we discussed a typical multiple thermodynamic system encountered in biochemical studies. The results obtained show that for a special system, although there are multiple roots, the physically meaningful root may be unique, and can be identified unambiguously from analysis of properties of the system. Thus, with this exact analytical expression and a usual least square regression computer program, all unknown parameters for describing the equilibrium system can be accurately determined from the experimental data.

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Appendix

In order to identify the physically meaningful root of eq. 7, let us discuss some properties of the three roots of eq. 8 first. If the angle θ is between 0 and $\pi/2$ or if its cosine is positive, then $(2\pi - \theta)/3$ and $(2\pi + \theta)/3$ are both greater than $\pi/2$, and their cosines are negative: in other words, one root of the corresponding equation is positive, and two are negative; but if θ is between $\pi/2$ and π or if its cosine is negative, then $\theta/3$ and $(2\pi - \theta)/3$ are less than $\pi/2$, and $(2\pi + \theta)/3$ is greater than $\pi/2$, or, in other words, two of the three real roots are positive, and one of them is negative. Thus, it can be seen from the discussion given above that u_1 is always a positive root and u_3 a negative root of eq. 8, whatever the value of θ may be.

According to the definition of u and the physical conditions of the problem proposed, we have

$$u = (a/3) + [P] = \{3[P] + K_A + K_B + [A]_0 + [B]_0 - [P]_0\}/3$$
$$= \{2[P] + K_A + K_B + ([A]_0 - [PA]) + ([B]_0 - [PB])\}/3 > 0$$

On the other hand, since [P] is the concentration of free protein, one can obtain

$$[P] = u - (a/3) > 0 \quad \text{or} \quad u > (a/3)$$

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Therefore, a physically meaningful root of eq. 8, u, must satisfy the following condition:

$$u > \max\{a/3, 0\} \quad \textbf{(A1)}$$

That is, $u > (a/3)$ if $a > 0$, and $u > 0$ if $a < 0$. As mentioned earlier, u_3 is always a negative root and hence can be excluded first. For u_2, since $\pi/3 < (2\pi - \theta)/3 < 2\pi/3$ and $-0.5 < \cos\{(2\pi - \theta)/3\} < 0.5$, when $a > 0$ we have $u_2 - (a/3) < \{\sqrt{(a^2 - 3b)} - a\}/3 < 0$. On the other hand, when $a < 0$, $-2a^3 + 9ab - 27c > 0$ and hence $\cos(\theta) > 0$. It can be seen from the discussion given above that in this case we have $u_2 < 0$ because $(2\pi - \theta)/3$ is greater than $\pi/2$. Thus, according to inequality A1, u_2 should also be excluded and u_1 expresses the unique proper root of eq. 8. u_2 and u_3 have no reference to the problem proposed.

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