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Fluorescence Displacement Method for the Determination of Receptor–Ligand Binding Constants

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The equilibrium constant for the binding of a spectroscopically invisible ligand to its protein receptor can be determined in a competition experiment, by using a structural analog that contains a reporter group (fluorophor). A novel mathematical treatment of the multiple equilibria allows the analysis to be performed under tight-binding conditions. The equilibrium equation for mixtures of two mutually competitive tight-binding ligands can be expressed in a *recursive* form, a form in which the dependent variable appears on both sides and the solution is found iteratively. The algorithm is also applicable to the special case of weak binding, where the concentration of the bound ligand can be neglected in the mass balance. The fluorescence displacement method is demonstrated on the determination cyclophilin binding to cyclosporin A (CsA), in competition with its fluorescent derivative, [D-Lys(Dns)]^8-CsA. © 1992 Academic Press, Inc.

Studies of protein–ligand interactions by fluorescence spectroscopy were reviewed by Ward (1). In this paper we describe a method to determine the binding constant for a nonfluorescent ligand in competition with its fluorescent analog. To obtain the binding constant for the fluorescent ligand alone, experimental data can be fitted to an algebraically explicit equation (2). A crude estimate for K_d can be made from the linear transformation devised by Stinson and Holbrook (3,4). However, it is not easy to extend the analytical, algebraically explicit description of binding equilibria to mixtures of competing ligands. Combining all partial equilibria and mass balances, one obtains a *cubic* algebraic equation. This situation was recently encountered by Corin *et al.* (5) in their studies of cytochrome c binding to a fluorescent derivative of cytochrome c peroxidase, in competition with nonfluorescent mutants of the same enzyme. Roots of polynomials degree $n > 2$ are usually extracted by methods of numerical analysis (6), and polynomial equations up to the fourth degree can even be solved analytically (7). In the case of both the numerical and the analytical methods one is faced with the possible existence of multiple real roots. Moreover, the performance of numerical methods such as the Newton–Raphson algorithm depends on a suitable choice of initial estimates.

In the present paper we describe a convenient alternative to the above methods, a rapidly converging iterative numerical formalism which avoids the multiple root problem. From any given set of concentrations (of the receptor and both ligands) and from values of the two corresponding binding constants, the overall fluorescence intensity is calculated iteratively by using a simple *recursive* formula. Within a given range of receptor concentrations, the entire theoretical binding curve can be calculated in this way. Then for a set of experimental receptor concentrations and fluorescence intensities, a best-fit binding curve is found by the usual least-squares minimization. In this process, the binding constant for the invisible ligand is obtained as one of the optimized parameters.

THEORY

Let us consider a receptor binding experiment that includes a mixture of two ligands, the reporting ligand L_1 and the invisible ligand L_2, which are kept at constant concentrations $[L_1]_0$ and $[L_2]_0$, respectively. The total analytical concentration of the receptor $[R]_0$ is varied, and the fluorescence intensity is measured for each ternary mixture. The fluorescence intensity at zero receptor concentration is designated F_0, and F_s is the fluorescence observed at complete saturation (i.e., at infinite $[R]_0$). At any finite receptor concentration $[R]_0$, the fluorescence intensity is determined by the molar fraction of L_1 in the bound state according to Eq. [1].

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Substitution for $[L_1R]$ from the definition of the dissociation constant, $K_{d1} = [R]\,[L_1]/[L_1R]$, yields Eq. [2]:

$$F = F_0 + (F_s - F_0)\frac{[L_1R]}{[L_1R] + [L_1]} \quad [1]$$

$$F = F_0 + (F_s - F_0)\frac{[R]}{[R] + K_{d1}}. \quad [2]$$

The required free receptor concentration $[R]$ can be obtained from the mass balance equations [3] through [5]. Combining these equations with the definitions of both equilibrium constants and fully expanding the intermediate expression [6], one would obtain a cubic polynomial in $[R]$. It could be shown by using the method of determinants (7) that the polynomial in $[R]$ always has three real roots, so that two of them would have to be eliminated. We instead consider the Eq. [6] as a recursive computational formula. Note that the quantity to be calculated, $[R]$, appears on both sides of Eq. [6]:

$$[L_1]_0 = [L_1] + [L_1R] \quad [3]$$

$$[L_2]_0 = [L_2] + [L_2R] \quad [4]$$

$$[R]_0 = [R] + [L_1R] + [L_2R] \quad [5]$$

$$[R] = \frac{[R]_0}{1 + ([L_1]_0/K_{d1} + [R]) + ([L_2]_0/K_{d2} + [R])}. \quad [6]$$

$[R]$ can be expressed in terms of the fluorescence intensity F as in Eq. [7], which immediately follows from [2]. Substituting for $[R]$ from [7] into [6] and rearranging, we obtain the final recursive Eq. [8] for the intensity of fluorescence. Note that the quantity to be calculated, F, appears on both sides. In the first cycle of the recursion ($i = 1$) we make an arbitrary estimate $F^{(1)}$. An improved estimate of fluorescence intensity $F^{(2)}$ is then calculated from Eq. [8], and in the next cycle the value of $F^{(2)}$ is used to compute $F^{(3)}$ in the same fashion. In each cycle of the recursion, the ith estimate $F^{(i)}$ is compared with the $(i + 1)$th estimate $F^{(i+1)}$. If the two values differ only insignificantly, for example by less than 0.01%, the recursion is terminated and the last computed value of F is considered as the desired result. In this fashion, theoretical fluorescence intensities are calculated point by point at experimental receptor concentrations—a procedure required in the least-squares fit of experimental data:

$$[R] = K_{d1}\frac{F - F_0}{F_s - F}. \quad [7]$$

Convergence properties of formula [8] are similar to an analogous recursive enzymatic rate equation for mixtures of two competitive tight-binding inhibitors (8):

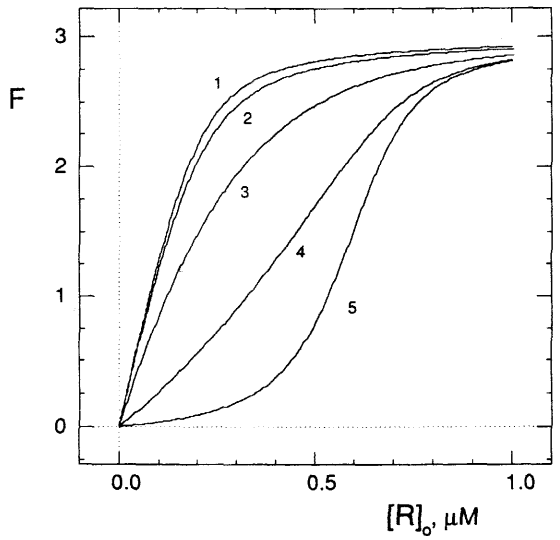


FIG. 1. Simulation of fluorescence intensity in dependence on the binding constant for the spectroscopically invisible ligand L_2. Parameters: $[L_1]_0$ 200 nM; K_{d1} 20 nM; $[L_2]_0$ 500 nM, K_{d2} 10, 1, 0.1, 0.01, and 0.001 μM (curves 1, 2, 3, 4, and 5, respectively); F_0 0.0; F_s 3.0.

$$F^{(i+1)} = F_0 + (F_s - F_0)\left\{[R]_0\Big/[R]_0 + \frac{F_s - F^{(i)}}{F_s - F_0}[L_1]_0 + \frac{1}{(F^{(i)} - F_0)/(F_s - F^{(i)}) + K_{d2}/K_{d1}}[L_2]_0 + K_{d1}\right\}. \quad [8]$$

As in the previous case (8), initial estimates spanning six orders of magnitude were found acceptable (data not shown). Optimum convergence is assured by setting the initial estimate $F^{(1)}$ to an average of the limiting values F_0 and F_s.

Figure 1 illustrates the shape of simulated fluorescence intensity curves for a mixture of two hypothetical tight-binding ligands. The fluorescent ligand (dissociation constant 20 nM) is present at 200 nM. The invisible ligand is present at 500 nM, and its dissociation constant varies between 10 μM and 1 nM for different theoretical curves displayed.

EXPERIMENTAL

Materials. Tetrahydrofuran was distilled from sodium benzophenone; lithium chloride was dried by heating at 300°C *in vacuo* overnight. Recombinant human cyclophilin was a generous gift from Dr. Thomas F. Holzman (Abbot Laboratories). Cyclosporin A was obtained from Sandoz AG (Switzerland). The synthesis of [D-Lys]^8-CsA[2] was described elsewhere (9). The fluorescent cyclosporin derivative [D-Lys(Dns)]^8-CsA was pre-

[2] Abbreviations used: CsA, cyclosporin A; THF, tetrahydrofuran.

pared from [D-Lys]^8-CsA by using a standard dansylation procedure (dansylchloride 1.0 eq, diethylamide 1.3 eq in dry dimethylformamide) and purified by thin-layer chromatography.

Fluorescence measurements. Fluorescence (excitation 350 nm, emission 500 nm) was measured by using a Greg PC fluorimeter (ISS Inc., Champaign, IL) equipped with a Y-42 cutoff filter (Hoya Optics Inc., Freemont, CA). Data acquisition software was written by Dr. Catherine A. Royer (University of Wisconsin). A stock solution of [D-Lys(Dns)]^8-CsA was prepared in dry 0.47 M lithium chloride in tetrahydrofuran (20 mg LiCl/ml THF). A solution of cyclophilin (0.001 to 2.0 μM) in the buffer (10 mM Tris, pH 7.0) was placed in the fluorometric cuvette, and [D-Lys(Dns)]^8-CsA was added as a solution in LiCl/THF (final concentration 200 nM). Fluorescence was measured after 50 s at room temperature. Alternatively, cyclosporin A was also added as a solution in 0.47 M LiCl/THF to give a final concentration 500 nM.

Determination of the kinetic inhibition constant for [D-Lys(Dns)]^8-CsA. The tight-binding inhibition constant for [D-Lys(Dns)]^8-CsA was determined by monitoring the peptidylprolyl *cis–trans* isomerase activity of cyclophilin in a chymotrypsin-coupled assay (10). Enzymatic progress curves were fitted to an implicit integrated rate equation evaluated by the Newton–Raphson method (11); model optimization was performed by using the Marquardt algorithm (12). The time dependence of the apparent inhibition constant was analyzed as reported elsewhere (13). All calculations were performed on a Macintosh II computer by using the software package KineTic version 1.3 (BioKin Ltd., Madison, WI).

RESULTS

The binding constant for the fluorescent cyclosporin derivative [D-Lys(Dns)]^8-CsA was determined by nonlinear least-squares fit of fluorescence intensities (Fig. 2, open circles) to Eq. [9] (2). The Marquardt algorithm was used to optimize F_0, F_s, and K_{d1}. The error of estimation for each parameter was determined from the final curvature matrix (14). The value thus obtained for K_{d1} was 5.2 ± 3.8 nM:

$$F = F_0 + (F_s - F_0) \times \frac{[R]_0 - [L_1]_0 - K_{d1} + \sqrt{([R]_0 - [L_1]_0 - K_{d1})^2 + 4[R]_0K_{d1}}}{[R]_0 - [L_1]_0 + K_{d1} + \sqrt{([R]_0 - [L_1]_0 - K_{d1})^2 + 4[R]_0K_{d1}}}. \quad [9]$$

The inhibition of cyclosporin's peptidylprolyl *cis–trans* isomerase activity by [D-Lys(Dns)]^8-CsA was monitored by using the chymotrypsin-coupled assay

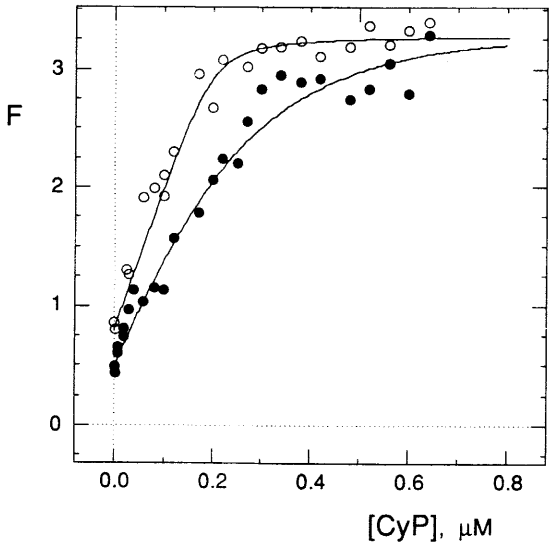


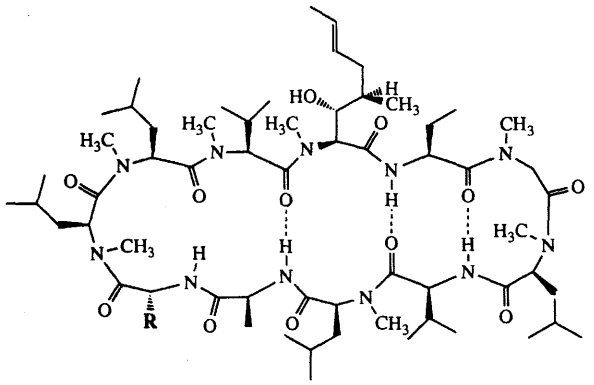
FIG. 2. Fluorescence intensities observed in the competitive binding of cyclosporin A (CsA) and [D-Lys(Dns)]^8-CsA to cyclophilin. Open circles: [D-Lys(Dns)]^8-CsA only (200 nM); the curve represents the best fit to Eq. [9]. Filled circles: [D-Lys(DNS)]^8-CsA (200 nM) and CsA (500 nM); the curve represents the best fit to Eq. [8].

(10). As in the case of cyclosporin A itself (12), the apparent inhibitory potency of the fluorescent analog depends on time of incubation with the enzyme, and on the solvent in which the inhibitor is dissolved prior to the assay. When this CsA analog was delivered to the aqueous buffer as a solution in 0.47 M lithium chloride in tetrahydrofuran, the competitive inhibition constant observed immediately after mixing was 13.0 ± 3.2 nM. Over a period of 60 min, the apparent inhibitory potency of [D-Lys(Dns)]^8-CsA increased and the final value of the inhibition constant was 5.0 ± 2.0 nM.

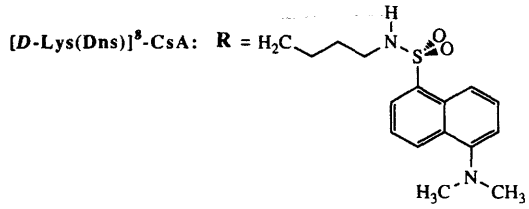
The dissociation constant for the complex between cyclosporin A and recombinant human cyclophilin was determined by nonlinear least-squares fit of fluorescence intensities measured for a mixture of CsA (0.5 μM) and [D-Lys(Dns)]^8-CsA (0.2 μM). The results (Fig. 2, filled circles) were fitted to Eq. [8]. Parameters F_0, F_s, and K_{d2} were optimized by using the Marquardt algorithm (13) while the dissociation constant K_{d1} was kept constant (5.3 nM) throughout the calculation. The optimized value of K_{d2} and the associated error of estimation (14) were 32 ± 18 nM.

DISCUSSION

Cyclosporin A (Scheme 1) is an important immunosuppressive drug frequently used to treat the rejection of transplanted human organs. It is also an inhibitor of cyclophilin, a *cis–trans* peptidylprolyl isomerase, but the relevance of cyclophilin binding to immunosuppression has not been completely elucidated (15). The



CsA: R = CH_3



[D-Lys(Dns)]^8-CsA: R = H_2C

SCHEME 1

search continues for protein receptors of cyclosporin other than cyclophilin.

Cyclosporin–cyclophilin binding can be monitored kinetically, by using the PPIase inhibition assay originally designed by Fischer *et al.* (16) and later modified by us (10). However, it is possible that cyclosporin binds to proteins that do not have PPIase activity or any other enzymatic activity, and thus the methods to monitor the binding must be based on physico-chemical phenomena such as fluorescence. To illustrate the applicability of the newly proposed recursive equation for determining tight-binding equilibrium constants in those cases, the cyclosporin-cyclophilin system is used here as an example. The advantage is that the results of the physical binding study can be compared with the kinetic results (12).

The cyclosporin A analog [D-Lys(Dns)]^8-CsA (Scheme 1) was used as the fluorescent probe, whose binding constant was determined in a separate experiment. The binding constant (5.3 nM) compares favorably with the competitive tight-binding inhibition constant (5.0 nM); physical binding to cyclophilin correlates with the inhibition of its peptidyl prolyl *cis–trans* isomerase activity. In the experiments reported herein, both cyclosporin A and [D-Lys(Dns)]^8-CsA were dissolved in 0.47 M lithium chloride in tetrahydrofuran, prior to being brought into contact with the protein receptor. Under these conditions, CsA exists exclusively as a single conformer characterized by a competitive inhibition constant 20 nM (12). This value is in good agreement with the dissociation constant for the cyclosporin–cyclophilin complex obtained by the newly proposed fluorescence displacement method (K_{d2}, 32 ± 18 nM).

In interpreting the experimental data shown above, two observations deserve special attention. First, there is a considerable noise in the data due to the time dependence of cyclosporin–cyclophilin binding (12). The fluorescence intensity is changing over time, and the initial value used to determine the binding constant is obtained by extrapolation. The resulting inaccuracy in fluorescence intensities is the most probable reason for a considerable uncertainty in the estimate of K_{d2}, and also for the deviations between the theory and experiment in Fig. 2. Second, the initial fluorescence (at zero receptor concentration) of the dansylated cyclosporin derivative is somewhat altered by the excess of CsA itself. It is possible that these very hydrophobic molecules form aggregates in an aqueous buffer and that fluorescence properties of the heterodimer CsA + [D-Lys(Dns)]^8-CsA differ from the monomer.

In conclusion, the recursive numerical formalism can be conveniently used to determine binding constants for nonradioactive, nonfluorescent ligands. The method is applicable under tight-binding conditions, where mutual depletion of the receptor and the ligands is significant, and under weak-binding conditions, where the concentration of each receptor–ligand complex is negligibly small with respect to one of its molecular components. The method can be implemented in any computing environment by encoding the recursive formula [8], with a suitable convergence criterion. It thus represents a simple counterpart to more general, but also more complex numerical methods for the analysis of biomolecular binding data (17).

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