Quantitative Analyses of Bifunctional Molecules†

Patrick D. Braun‡ and Thomas J. Wandless*,§

Department of Chemistry and Department of Molecular Pharmacology Stanford University, Stanford, California 94305

Received October 10, 2003; Revised Manuscript Received March 5, 2004

ABSTRACT: Small molecules can be discovered or engineered to bind tightly to biologically relevant proteins, and these molecules have proven to be powerful tools for both basic research and therapeutic applications. In many cases, detailed biophysical analyses of the intermolecular binding events are essential for improving the activity of the small molecules. These interactions can often be characterized as straightforward bimolecular binding events, and a variety of experimental and analytical techniques have been developed and refined to facilitate these analyses. Several investigators have recently synthesized heterodimeric molecules that are designed to bind simultaneously with two different proteins to form ternary complexes. These heterodimeric molecules often display compelling biological activity; however, they are difficult to characterize. The bimolecular interaction between one protein and the heterodimeric ligand (*primary* dissociation constant) can be determined by a number of methods. However, the interaction between that protein−ligand complex and the second protein (*secondary* dissociation constant) is more difficult to measure due to the noncovalent nature of the original protein−ligand complex. Consequently, these heterodimeric compounds are often characterized in terms of their activity, which is an experimentally dependent metric. We have developed a general quantitative mathematical model that can be used to measure both the primary (protein + ligand) and secondary (protein−ligand + protein) dissociation constants for heterodimeric small molecules. These values are largely independent of the experimental technique used and furthermore provide a direct measure of the thermodynamic stability of the ternary complexes that are formed. Fluorescence polarization and this model were used to characterize the heterodimeric molecule, SLFpYEEI, which binds to both FKBP12 and the Fyn SH2 domain, demonstrating that the model is useful for both predictive as well as ex post facto analytical applications.

Small molecules can perturb biological systems by binding to protein targets, and these molecules are widely used as therapeutic agents as well as probes of biological processes (*1*). Recently, molecules that possess two protein-binding moieties have been of particular interest. One class of these compounds, the chemical inducers of dimerization, includes FK1012, AP1889, and others that have been used to examine cellular processes through the recruitment of a chimeric protein to a cellular target of interest (*2*−*6*). These compounds are designed to bind simultaneously to two proteins, presumably with little or no effect on the intrinsic binding affinities of the ligands.

A second class of dimeric molecules is designed to possess altered affinity for their cognate binding targets (*7*−*12*). We have demonstrated a strategy that uses protein−protein interactions to modulate the activity of synthetic, bifunctional molecules, where the presence or absence of a nontarget protein affects binding to the target protein of interest (*7*, *10*). For example, the bifunctional molecule SLFpYEEI (Figure 1) is comprised of a ligand for the FKBP[1] protein (SLF) covalently linked to a ligand for the SH2 domain of the Fyn protein (phosphotyrosyl-glutamyl-glutamyl-isoleucine, pYEEI). Binding experiments using several techniques have shown that the IC_{50} of SLFpYEEI for FynSH2 increases 6-fold in the presence of FKBP (*7*).

The binding affinities of both classes of compounds have typically been described in terms of activity (IC_{50}, etc.). While these metrics provide a general indication of the change in binding affinity (increase in IC_{50} = increase in K_d) for any given binding event, the dependence on the particular experimental conditions often makes it difficult to compare compounds. We desired a means of characterizing the activity of bifunctional molecules that is independent of experimental conditions in the hope that a knowledge of the specific binding constants could lead to more efficient designs of and broader uses for bifunctional molecules.

Consider the system shown schematically in Figure 1. The bifunctional molecule SLFpYEEI competes with the fluorescein-labeled ligand FLGpYEEI (FL) to bind to the FynSH2 target protein. We describe the SLFpYEEI·FynSH2 complex by the *primary* dissociation constant (Fyn K_d1) because FynSH2 is the first protein bound to SLFpYEEI. The situation becomes complicated when the FKBP presenter

† This research was supported by the NSF (CHE-9985214) as well as the Sloan and Dreyfus Foundations. P.D.B. was partially supported by an Abbott Laboratories predoctoral fellowship.

* To whom correspondence should be addressed. E-mail: wandless@stanford.edu.

‡ Department of Chemistry.

§ Department of Molecular Pharmacology Stanford University.

[1] Abbreviations: FKBP, FK506- and rapamycin-binding protein 12 kDa; FynSH2, Src homology 2 domain (residues 102−205) of the Fyn kinase; SLF, synthetic ligand for FKBP; pYEEI, phosphotyrosyl-glutamyl-glutamylisoleucine; FP, fluorescence polarization; FLSLF, fluorescein-SLF; FLGpYEEI, fluorescein-glycyl-phosphotyrosyl-glutamyl-glutamylisoleucine.

10.1021/bi035839g CCC: $27.50
Published on Web 04/14/2004

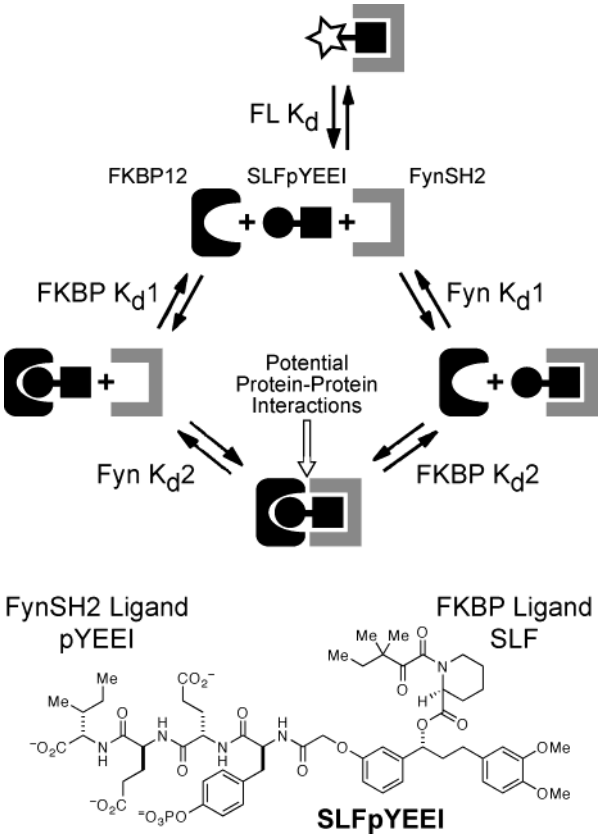

FIGURE 1: Schematic representation of a fluorescence polarization competition binding experiment in the presence of presenter protein. The bifunctional molecule (SLFpYEEI) can bind to the presenter protein (FKBP) and the target protein (FynSH2) to form a dimeric or trimeric complexes. Dimeric complex formation is described by the primary dissociation constants FKBP K_d1 and Fyn K_d1. Trimeric complex formation is described by the secondary dissociation constants FKBP K_d2 and Fyn K_d2. Free fluorescent tracer is not shown for clarity, but is treated explicitly by the model.

protein is included in the system. At equilibrium, the solution may contain the monomeric species FynSH2, FKBP, SLFpYEEI, and FLGpYEEI; the dimeric complexes FLGpYEEI·FynSH2, SLFpYEEI·FynSH2, and FKBP·SLFpYEEI; and the trimeric complex FKBP·SLFpYEEI·FynSH2. The binding of the FKBP·SLFpYEEI complex to FynSH2 to form the trimeric complex FKBP·SLFpYEEI·FynSH2 is differentiated from SLFpYEEI·FynSH2 formation by labeling the former as the *secondary* dissociation constant (Fyn K_d2) because FynSH2 in this case is the second protein to bind to the bifunctional molecule. The same terminology is used for the FKBP complexes. The binding of SLFpYEEI to form the dimeric species FKBP·SLFpYEEI is described by the *primary* dissociation constant, FKBP K_d1, and the binding of the SLFpYEEI·FynSH2 complex to FKBP is described by the *secondary* dissociation constant, FKBP K_d2.

This distinction is important because differences between the primary and secondary dissociation constants provide a direct measure of the stability of the trimeric complex. If $K_d2 > K_d1$ for a given ligand−protein interaction, then the trimeric complex is less stable than the dimeric complex, and unfavorable protein−protein interactions may be responsible for the reduced stability. Conversely, if $K_d2 < K_d1$, the formation of trimeric complex is favored, possibly due to energetically stabilizing protein−protein interactions. It is difficult to design experiments that directly measure a secondary dissociation constant owing to the noncovalent nature of the bifunctional molecule−protein complex. For example, an experiment to determine the value of Fyn K_d2 (FKBP·SLFpYEEI + FynSH2) will likely include a contribution from Fyn K_d1 because the FKBP·SLFpYEEI complex can dissociate and free SLFpYEEI can directly compete for FynSH2.

Many methods are available to measure the dissociation constant of a simple ligand−protein binding event (*13*); however, fewer methods exist for determining the dissociation constant of a competitor in a competition binding experiment. Comparison of IC_{50} values rather than dissociation constants is standard practice. One of the earliest attempts to extract dissociation constants from competition binding experiments focused on the effects of antagonistic drugs in isolated tissue samples (*14*). These studies yielded the expression shown in eq 1, where labeled ligand L competes with competitor ligand C to bind to protein P. P_t represents total protein, K_L is the dissociation constant for P·L, and K_C is the dissociation constant for P·C.

$$[P{\cdot}L] = \frac{[L][P_t]}{[L] + K_L\left(\frac{[C]}{K_C} + 1\right)} \quad (1)$$

The value of K_L can usually be determined using a standard saturation binding experiment. Total protein concentration is a known experimental variable, and [P·L] is obtained from the measured signal. Closer examination of eq 1 reveals that the concentration of P·L also depends on the concentrations of free labeled ligand and free competitive ligand rather than the total ligand concentrations, and neither of these values are constant over a range of competitor concentrations. For example, at low $[C_t]$, [L] will be very similar to initial [L]. However, as $[C_t]$ increases and more P·C is formed, [L] increases as well. To circumvent this problem, experiments using eq 1 must be designed so that $[L_t] \gg [P_t]$, which allows the use of the approximations that $[L] \approx [L_t]$ and $[C] \approx [C_t]$.

Another method to derive a dissociation constant from a competition binding experiment was proposed by Cheng and Prusoff (*15*). Their method transforms an IC_{50} value determined by a competitive enzymatic inhibition experiment into a K_d for the competitor. This protocol was later modified by Munson and Rodbard to give eq 2 (*16*, *17*). The argument for the modification is that the original expression assumed that there would be little difference between the total and free concentrations of competitor and labeled ligand, as was also the case with eq 1. Under the experimental conditions treated by the Cheng-Prusoff method (concentration of enzyme is much lower than the concentration of either competitor or labeled ligand), the assumption that the concentrations of either free competitor or free labeled ligand are equivalent to the respective total concentrations is usually reasonable. However, this assumption can lead to substantial errors when the Cheng-Prusoff method is applied to nonenzymatic competition binding experiments.

$$K_i = \frac{IC_{50}}{1 + y_o + \frac{[L_t](y_o + 2)}{2K_L(y_o + 1)}} - \frac{K_L y_o}{y_o + 2} \quad (2)$$

The modified Cheng-Prusoff method (eq 2) relies upon the concentration of competitor required to reduce the signal by 50% (IC_{50}), the total concentration of labeled ligand ($[L_t]$), the dissociation constant of labeled ligand for the target protein (K_L), and the initial ratio of bound labeled ligand to free labeled ligand (y_o). This expression provides the dissociation constant (K_i) of the competitor ligand for the protein target. The quantity y_o implicitly includes the total protein concentration because this variable, along with K_L, will dictate the initial ratio of bound to free labeled ligand (*17*). Furthermore, this method explicitly considers the total concentration of labeled ligand (rather than free concentration). However, the modified Cheng-Prusoff method depends substantially on the value of the IC_{50}. The IC_{50} values are often judged by inspection, although nonlinear fit procedures have been used to generate IC_{50} values (*18*). Additionally, modifications to this method to allow for the treatment of bifunctional molecules capable of binding to two or more proteins are not straightforward.

An ideal method to derive dissociation constants from competition binding experiments would explicitly consider all species and possible equilibria to generate theoretical binding curves. Those curves could then be tested using a least-squares method to determine the experimental parameters that most accurately describe the experiment. Several computer programs have been developed that aim to achieve that goal (*19*, *20*). They appear to be useful for most ligand binding conditions and can be expanded to consider multiple ligands and protein receptors. However, they make the assumption that each ligand−protein complex undergoes no further reactions once formed. More recently, Wang has described an exact expression of competitive binding (*21*), although this solution cannot be applied to our dimeric molecules.

Bifunctional molecules that are the focus of our studies will typically be characterized by more than one dissociation constant (i.e., both primary and secondary binding constants). We found that solving the explicit equilibrium binding expressions for an interaction of interest was operationally inflexible and computationally intensive. As an alternative, we considered describing bifunctional molecule dissociation constants using ratios of the rate constants for the kinetic assembly and disassembly of all possible complexes. Equilibrium dissociation constants are ratios of rate constants, so all dimeric and trimeric complexes can be described using rate equations. Using Mathematica to aid in the computations, we have developed a method to describe complex binding equilibria for multiple molecules as a collection of partial differential rate equations.

This method possesses several attractive characteristics, beginning with the fact that all free and bound species are treated explicitly. There are no assumptions made that concentration of free protein is equivalent to the concentration of total protein or that an IC_{50} value is equivalent to a K_d value. Furthermore, binary complexes are allowed to be involved in additional binding reactions and can subsequently form ternary or even higher-order complexes. The method is easy to expand, and the inclusion of multiple proteins or ligands is allowed. Two different parameters can be varied to simultaneously generate multiple theoretical isotherms that are then compared to the experimental data. Evaluation of these fits using Pearson's coefficient of determination (R^2) provides a quantitative tool to determine the experimental parameters that best fit the observed data. The studies reported herein demonstrate the utility of this procedure through the analysis of the bifunctional molecule, SLFpYEEI, using fluorescence polarization competition binding experiments to quantitate the effects of the FKBP presenter protein on the affinity of SLFpYEEI for the FynSH2 protein.

MATERIALS AND METHODS

Curve-Fitting Equations. Dissociation constants are related to kinetic rate constants (eq 3), so the formation of the dimeric and trimeric complexes can be described by combinations of rate constants. The formation of FKBP·SLFpYEEI can be described by the association of FKBP and SLFpYEEI (k_a1FKBP), the dissociation of FKBP·SLFpYEEI complex (k_d1FKBP; FKBP $K_d1 = k_d1FKBP/k_a1FKBP$), the association of FKBP·SLFpYEEI complex and FynSH2 (k_a2Fyn), and the dissociation of FKBP·SLFpYEEI·FynSH2 (k_d2Fyn; Fyn $K_d2 = k_d2Fyn/k_a2Fyn$), where the descriptors 1 or 2 indicate a primary or secondary binding event (eq 4). This expression is then expanded so that each monomeric species is expressed in terms of initial concentrations and complex concentrations, as shown for FKBP·SLFpYEEI (eq 5). The same analysis is performed for all possible species in an equilibrium binding reaction (see Supporting Information).

$$\frac{[\text{SLFpYEEI}][\text{FKBP}]}{[\text{FKBP·SLFpYEEI}]} = \text{FKBP } K_d1 = \frac{k_d1\text{FKBP}}{k_a1\text{FKBP}} \quad (3)$$

$$\frac{d}{dt}[\text{FKBP·SLFpYEEI}] = [\text{FKBP}][\text{SLFpYEEI}]k_a1\text{FKBP} - [\text{FKBP·SLFpYEEI}]k_d1\text{FKBP} - [\text{FKBP·SLFpYEEI}][\text{Fyn}]k_a2\text{Fyn} + [\text{FKBP·SLFpYEEI·Fyn}]k_d2\text{Fyn} \quad (4)$$

$$\frac{d}{dt}[\text{FKBP·SLFpYEEI}] = ([\text{FKBP total}] - [\text{FKBP·SLFpYEEI}] - [\text{FKBP·SLFpYEEI·Fyn}])([\text{SLFpYEEI total}] - [\text{FKBP·SLFpYEEI}] - [\text{SLFpYEEI·Fyn}] - [\text{FKBP·SLFpYEEI·Fyn}])k_a1\text{FKBP} - [\text{FKBP·SLFpYEEI}]k_d1\text{FKBP} - [\text{FKBP·SLFpYEEI}]([\text{Fyn total}] - [\text{SLFpYEEI·Fyn}] - [\text{FKBP·SLFpYEEI·Fyn}] - [\text{FLGpYEEI·Fyn}])k_a2\text{Fyn} + [\text{FKBP·SLFpYEEI·Fyn}]k_d2\text{Fyn} \quad (5)$$

The equations describing the formation of the complexes were treated as partial differential equations and solved simultaneously as a function of time using software such as Mathematica (*22*). The concentration of SLFpYEEI was treated as a variable, emulating experimental conditions. As the calculation was run, the concentrations of each potential noncovalent complex increased from zero and settled to constant amounts once equilibrium was reached. The equilibrium concentrations of the complexes were then exported to a plotting program to generate a theoretical competition curve that is comparable to a typical thermodynamic

competition binding experiment. The theoretical data were tested against the experimental data using Pearson's coefficient of determination (R^2). A more complete description of the generation of theoretical binding isotherms and their application to experimental data can be found in Supporting Information.

Saturation Binding Experiments. The dissociation constants of the fluorescein-labeled ligands for their target proteins were determined using saturation binding experiments. The degree to which the labeled ligand emits polarized light upon excitation is the experimental indicator of protein−ligand binding, so the concentration of the fluorescent ligand was held constant and polarization was measured as the concentration of target protein was increased. As with all saturation binding experiments, it is important that the concentration of the constant species is below (ideally at least 10-fold below) the dissociation constant (*23*).

A representative experiment to measure the affinity of fluorescein-labeled phosphopeptide (FLGpYEEI) for the FynSH2 protein is described. A stock solution of FLGpYEEI at 2 nM was prepared in fluorescence polarization (FP) buffer (50 mM KH_2PO_4, 150 mM NaCl, pH 7.8, 0.002% Tween-20, 0.1 mg/mL bovine γ-globulin). A stock solution of FynSH2 (100.4 μM in FP buffer) was prepared and used to make further 3-fold dilutions of the protein. Equal volumes (50 μL) of the FLGpYEEI and the FynSH2 stock solutions were added to a black, flat-bottomed, polystyrene 96-well plate (Corning, Cat. No. 3915, Corning, NY). Four independent binding reactions were prepared at each protein concentration, and the plate was covered with aluminum foil and incubated at room temperature. After 45 min, the plate was placed in the spectrophotometer (LJL Analyst) and analyzed. For all FP experiments, analysis was repeated at 60 and 75 min to verify that equilibrium had been acheived.

The resulting data file was opened with Excel, the four polarization values at each protein concentration were averaged, and the standard deviations were determined. The data were processed to determine the fraction of bound tracer at each protein concentration and fitted for the K_d value of FLGpYEEI, as described in Supporting Information. The data were also fitted using eq 6 with known values entered for F_0 = 0% bound FLGpYEEI, F_{100} = 100% bound FLGpYEEI, $[F]_T$ = total fluorescent tracer concentration, and $[P]_T$ = total protein. The sole fitted variable, K_d, is the dissociation constant of FLGpYEEI for FynSH2 in units identical to the entered protein concentrations.

$$F_0 - (F_0 - F_{100}) \times \frac{(K_d + [F]_T + [P]_T) - \sqrt{(K_d + [F]_T + [P]_T)^2 - (4[F]_T[P]_T)}}{2[F]_T} \quad (6)$$

Competition Binding Experiments. A representative competition binding experiment between the FLGpYEEI·FynSH2 complex and various concentrations of SLFpYEEI is described. The FLGpYEEI tracer was prepared as a 4× stock solution (4 nM) in FP buffer. The FynSH2 protein was diluted into FP buffer to prepare excess (22 μM) and 2× protein (204 nM) stock solutions. The excess protein stock was used to determine the polarization signal of 100% bound FLGpYEEI. The SLFpYEEI competitor was dissolved in FP buffer to give a maximum competitor stock solution (500 μM) that was used to make further 3-fold dilutions. The stock solution of FLGpYEEI and the various SLFpYEEI dilutions were added in 25-μL aliquots to a black, flat-bottomed, polystyrene 96-well plate. The FynSH2 solutions were added to each well as 50-μL aliquots, and the plate was covered with aluminum foil and incubated at room temperature. After 45 min, the plate was placed in the spectrophotometer and analyzed.

The resulting data file was opened with Excel, the four polarization values at each competitor concentration were averaged, and the standard deviations were determined. The data were processed to determine the fraction of bound tracer at each competitor concentration, and the data were fit to determine the K_d value of SLFpYEEI as described in Supporting Information.

Competition Binding Experiments with Presenter Protein. A representative competition binding experiment between the FLGpYEEI·FynSH2 complex and various concentrations of SLFpYEEI in the presence of the FKBP presenter protein is described. The FLGpYEEI tracer was prepared as a 4× stock solution (4 nM) in FP buffer. The FynSH2 protein was diluted into FP buffer to prepare excess (22 μM) and 2× protein (404 nM) stock solutions. The excess protein stock was used to determine the polarization signal of 100% bound FLGpYEEI. FKBP was diluted into FP buffer to prepare a 4× stock solution of presenter protein at 20 μM. A prior saturation binding experiment was performed between FLGpYEEI and FKBP to verify the lack of any interaction between the two molecules. The SLFpYEEI competitor was dissolved in FP buffer to give a maximum competitor stock solution (500 μM) that was used to make further 3-fold dilutions.

The stock solution of FLGpYEEI and the various SLFpYEEI dilutions were added in 25-μL aliquots to a black, flat-bottomed, polystyrene 96-well plate. The FKBP solution was added in 25-μL aliquots, and the plate was covered with aluminum foil and incubated at room temperature for 15 min. The FynSH2 solutions were then added in 25-μL aliquots, and the plate was covered with aluminum foil and incubated at room temperature. After 45 min, the plate was placed in the spectrophotometer and analyzed. The resulting data file was opened with Excel, the four polarization values at each competitor concentration were averaged, and the standard deviations were determined. The data were processed to determine the fraction of bound tracer at each competitor concentration and fitted for the SLFpYEEI dissociation constant in the presence of the FKBP presenter protein as described in Supporting Information.

Curve-Fitting Procedure. Theoretical binding isotherms were generated using equations that describe the formation of each possible protein complex. All known starting concentrations and dissociation constants were entered, and data were generated for multiple concentrations of SLFpYEEI and multiple values of the dissociation constant to be fitted. It is difficult to determine the best curve fit by inspection so Pearson's coefficient of determination (R^2) was used to compare the experimental data to the calculated data (eq 7). The dissociation constant provided by the theoretical curve with the maximum R^2 value was taken as the final

value. A detailed description of this procedure is provided in Supporting Information

$$R^2 = 1 - \frac{\bar{\imath}(y_i - \hat{y}_i)^2}{\bar{\imath}(y_i - \bar{y}_i)^2} \quad (7)$$

where y_i = observed datum; $\hat{y}_i$ = theoretical datum; $\bar{y}_i$ = mean observed data.

RESULTS AND DISCUSSION

Validation of Curve-Fitting Procedure. Fluorescence polarization (FP) was chosen to analyze the behavior of SLFpYEEI for several reasons (*24*). First, FP measures direct interactions in the solution phase, thus ensuring experimental homogeniety of all species. Second, FP is quite sensitive, which allows dissociation constants as low as 1 nM to be measured using relatively small quantities of reagents. Third, experiments can be performed in 96-well plate format, which allows for high throughput and simultaneous replicates. Fourth, the amount of bound fluorescein-labeled ligand can be measured directly without requiring an experimental step to physically separate the bound and unbound labeled ligand that is required by radiolabeled competition binding experiments.

As is true for any curve-fitting procedure, there are several combinations of parameters that may provide a good fit with the data even though the results may have limited physical meaning. How can we verify that our model is correctly describing the experimental results? We compared the ability of our curve-fitting procedure to determine the dissociation constant from a saturation binding experiment (Figure 2) with that of a standard binding equation (eq 6). If our model is correct, it should be possible to use the proposed curve-fitting procedure to describe the binding of FLGpYEEI to FynSH2 (Figure 2).

Theoretical curves were generated for the experimental data and analyzed as described above. The experimental data were also analyzed using eq 6. The results obtained using both methods were identical (K_d = 93 nM), and the generated isotherms had the same fit for the experimental data (R^2 = 0.9987) (Figure 2A). The experiment was repeated, and the results were averaged for K_d = 92 ± 3 nM. The analogous process was performed for FKBP and the fluorescein-labeled ligand FLSLF provided an average K_d = 3.3 ± 0.3 nM (Figure 2B).

Primary Dissociation Constants of SLFpYEEI. Once the dissociation constants between fluorescein-labeled ligands and their cognate proteins are measured, competition binding experiments can be used to determine the K_d1 values for the competitor ligand, SLFpYEEI. A competition experiment is typically performed by adding various concentrations of competitor to a fixed concentration of the fluorescent ligand−protein complex. For a traditional competition experiment, conditions are typically chosen to saturate the protein with labeled ligand. This design feature is unnecessary with FP experiments because the experimental output does not indicate how much fluorescein ligand is bound to protein, but rather provides a measure of the fraction of fluorescein ligand bound to protein.

Competition binding experiments were performed using constant concentrations of FLGpYEEI and FynSH2 and varying amounts of the bifunctional molecule, SLFpYEEI. These studies provided an average dissociation constant, Fyn K_d1 = 1.16 ± 0.12 μM (Figure 3A). The same experiment was performed using constant concentrations of FLSLF and FKBP to provide an average FKBP K_d1 = 12 ± 5 nM (Figure 3B). Both of these measured dissociation constants are similar to the K_d values of the corresponding fluorescein ligands as well as to binding affinities reported for other related compounds (*25*−*28*), suggesting that our protocol can be used to measure dissociation constants from competition binding experiments.

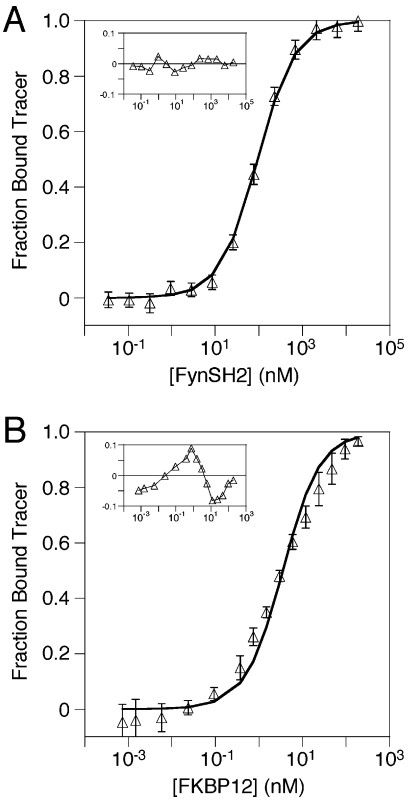

FIGURE 2: Determination of the labeled ligand dissociation constants. (A) The fluorescence polarization of 1 nM fluorescent ligand FLGpYEEI with increasing FynSH2 protein (△) was fit using the described method (bold line) for K_d = 93 nM (R^2 = 0.9987). Error bars represent the standard deviation of four repetitions, and a plot of the residuals is shown in the inset. Duplicate experiments were averaged to yield a final K_d = 92 ± 3 nM. (B) The fluorescence polarization of 0.2 nM fluorescent ligand FLSLF with increasing FKBP protein (Δ) was fit using the described method (bold line) for K_d = 3.4 nM (R^2 = 0.9905). Error bars represent the standard deviation of four repetitions. Duplicate experiments were averaged to yield a final K_d = 3.3 ± 0.3 nM. In both cases, identical results were obtained with fits using eq 6.

Secondary Dissociation Constants of SLFpYEEI. The competition binding experiment that was used to measure the affinity of SLFpYEEI for the FynSH2 protein was repeated in the presence of 5 μM FKBP presenter protein (Figure 1). Since we have measured the primary dissociation constants for this interaction, the only variables that can be used to describe perturbations of FKBP on the affinity of SLFpYEEI for the FynSH2 protein are the secondary dissociation constants. There are two possible paths to form trimeric complex (Figure 1), and the total free energy of each path should be equal (eq 8). Transformation of the free energies yields an expression in which the primary dissociation constants are shown to be proportional to the secondary

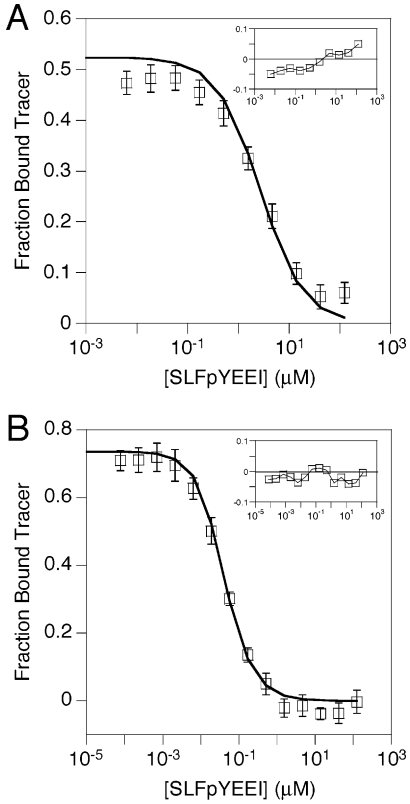

FIGURE 3: Determination of the primary dissociation constants of SLFpYEEI. (A) Increasing concentrations of the bifunctional molecule SLFpYEEI in the presence of 1 nM FLGpYEEI and 102 nM FynSH2 were examined by fluorescence polarization (□). Error bars represent the standard deviation of four repetitions. Duplicate experiments were fit using the described method and averaged to yield Fyn $K_d1 = 1.16 \pm 0.12$ μM. (B) Increasing concentrations of the bifunctional molecule SLFpYEEI in the presence of 1 nM FLSLF and 10.1 nM FKBP were examined by fluorescence polarization (□). Error bars represent the standard deviation of four repetitions, and a plot of the residuals is shown in the inset. Triplicate experiments were fit using the described method and averaged to yield FKBP $K_d1 = 12 \pm 5$ nM.

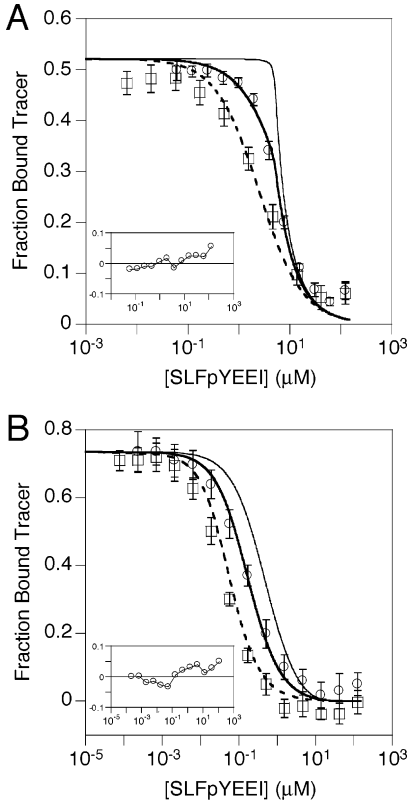

FIGURE 4: Determination of the secondary dissociation constants of SLFpYEEI. (A) Increasing concentrations of the bifunctional molecule SLFpYEEI in the presence of 1 nM FLGpYEEI, 101 nM FynSH2, and 5 μM FKBP presenter protein were examined by fluorescence polarization (○). Error bars represent the standard deviation of four repetitions. At least five experiments were fit using the described method and averaged to yield Fyn $K_d2 = 3.9K_d1$. (B) Increasing concentrations of the bifunctional molecule in the presence of 1 nM FLSLF, 10 nM FKBP, and 10 μM FynSH2 presenter protein were examined by fluorescence polarization (○). Error bars represent the standard deviation of four repetitions, and a plot of the residuals is shown in the inset. At least five experiments were fit using the described method and averaged to yield FKBP $K_d2 = 4.8K_d1$. Data from Figure 3 (open squares) are repeated in panels A and B for comparison.

dissociation constants. Therefore, the fractional change between FKBP K_d1 and FKBP K_d2 should be proportional to the fractional change between Fyn K_d1 and Fyn K_d2 (eq 9).

$$\Delta G_{\mathrm{FKBP1}} + \Delta G_{\mathrm{Fyn2}} = \Delta G_{\mathrm{Fyn1}} + \Delta G_{\mathrm{FKBP2}} \qquad (8)$$

$$\ln(K_d1\mathrm{FKBP}) + \ln(K_d2\mathrm{Fyn}) = \ln(K_d1\mathrm{Fyn}) + \ln(K_d2\mathrm{FKBP})$$

$$\ln(K_d1\mathrm{FKBP}{*}K_d2\mathrm{Fyn}) = \ln(\mathrm{K_d1Fyn}{*}K_d2\mathrm{FKBP})$$

$$\frac{K_d1\mathrm{FKBP}}{K_d1\mathrm{Fyn}} = \frac{K_d2\mathrm{FKBP}}{K_d2\mathrm{Fyn}} \qquad (9)$$

If the secondary dissociation constants are equal to the primary dissociation constants ($K_d2 = K_d1$), then the binding of SLFpYEEI to FKBP does not affect its binding to FynSH2, and the calculated theoretical isotherm (Figure 4A, dashed line) matches the experiment without presenter protein (Figure 4A, squares). Alternatively, if SLFpYEEI is unable to bind to both proteins simultaneously and the FKBP·SLFpYEEI·FynSH2 trimeric complex cannot form, then the secondary dissociation constants can be considered to be infinitely large ($K_d2 \approx \infty$). The resulting theoretical isotherm is shown in Figure 4A (thin line).

The fact that neither theoretical curve matches the experimental data obtained in the presence of 5 μM FKBP (Figure 4A, circles) argues that trimeric complex does form, but its energetics are such that trimeric complex is destabilized relative to either dimeric complex. Therefore, the actual K_d2 values must be between the K_d1 values and infinity ($K_d1 < K_d2 < \infty$). Theoretical isotherms were generated for multiple values of K_d2 and were compared to the experimental data using Pearson's coefficient of determination. This procedure was repeated for several competition experiments in the presence of FKBP to provide $K_d2 = 3.9 \times K_d1$.

One would expect to obtain the same secondary dissociation constants when the competition experiment is performed using FKBP as the target protein and FynSH2 as the presenter protein. As before, the theoretical isotherms generated by $K_d2 = K_d1$ (Figure 4B, dashed line) and $K_d2 \approx \infty$ (Figure 4B, thin line) do not match the experiment when 10 μM FynSH2 is included as the presenter protein (Figure 4B, circles). To quantitate the effects of the FynSH2 protein on the secondary dissociation constant, multiple competition

experiments were fit as described above, and the results were averaged providing a K_d2 for this experiment that is 4.8-fold higher than the primary dissociation constant. The fits from the two experiments were used to calculate the K_d2 values. From the experiment using FKBP as the presenter protein, the results were K_d2 FynSH2 = 4 ± 1 μM and K_d2 FKBP = 50 ± 20 nM. The results from the complementary experiment using FynSH2 as presenter protein were K_d2 FynSH2 = 6 ± 1 μM and K_d2 FKBP = 60 ± 30 nM.

CONCLUSION

Important biological processes such as signal transduction pathways are comprised of networks of molecules whose interactions are mediated by noncovalent interactions. The lack of covalent bonds between the binding partners allows these interactions to be both concentration-dependent and reversible, characteristics that increase the regulatory power and complexity of these biological networks. Bifunctional molecules (i.e., homo- or heterodimeric compounds) can act as powerful manipulators of cellular processes, but it is often unclear how noncovalent protein−protein interactions that may arise in a ternary complex impact the activity of these molecules. Understanding these effects can be important because the relative value of these small molecules is a function of their affinity and specificity for their protein targets. The recruitment of a second protein, and the noncovalent nature of the interactions, makes for an intricate thermodynamic system. While programs exist that can be used to describe such systems once all values are known, these programs are unable to fit experimental data to determine the experimental parameters that describe trimeric complex formation, which we label the secondary dissociation constants.

We have developed a method to describe complicated bifunctional molecule equilibria using partial differential equations. The equations are solved simultaneously to generate theoretical isotherms that are then compared to the experimental data. Evaluation using Pearson's coefficient of determination reveals the parameters that best fit the experimental data. In an important test of validity, our method successfully reproduces fits of a saturation binding experiment using known binding partners (Figure 2). This method can also be used to measure dissociation constants from a competition binding experiment (Figure 3), and the K_d1 values that were obtained from our protocol are consistent with precedents for related compounds (*24*−*27*). Our current results do differ slightly from earlier experiments wherein isothermal titration calorimetry was used to characterize SLFpYEEI binding (*7*). However, operational considerations prevented us from performing our competitions binding assays reported in this manuscript using the same buffer that was used for the calorimetry experiments. These differences in buffer composition are likely responsible for the minor differences that are observed.

Particular strengths of our method are that it can be expanded to describe any system for which rate equations can be generated, no assumptions are made concerning the free concentration of any species, and any variable can be treated as unknown and fitted. These factors make this protocol particularly well-suited for determining the secondary dissociation constants created by bifunctional molecules in the presence of both target and presenter proteins. The utility of this method was demonstrated by analyzing the interactions of SLFpYEEI with the proteins FKBP and FynSH2, and these studies yielded similar values for secondary dissociation constants regardless of how the system was probed.

ACKNOWLEDGMENT

We wish to thank Joseph Barco and Emily Hanan for reagents, helpful discussions, and preliminary experiments as well as Profs. James Ferrell and Eric Anslyn for advice and encouragement during the formative stages of this research.

SUPPORTING INFORMATION AVAILABLE

Description of a protocol that makes use of pairs of rate equations to represent binding constants between two or more species. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Hung, D. T., Jamison, T. F., and Schreiber, S. L. (1996) Understanding and controlling the cell cycle with natural products. *Chem. Biol. 3*, 623−639.
2. Spencer, D. M., Wandless, T. J., Schreiber, S. L., and Crabtree, G. R. (1993) Controlling signal transduction using synthetic ligands. *Science 262*, 1019−1024.
3. MacCorkle, R. A., Freeman, K. W., and Spencer, D. M. (1998) Synthetic activation of caspases: Artificial death switches. *Proc. Natl. Acad. Sci. U.S.A. 95*, 3655−3660.
4. Pollock. R., and Rivera, V. M. (1999) Regulation of gene expression with synthetic dimerizers. *Methods Enzymol. 306*, 263−281.
5. Althoff, E. A., and Cornish, V. W. (2002) A bacterial small-molecule three-hybrid system. *Angew. Chem. Int. Ed. 41*, 2327−2330.
6. Koide, K., Finkelstein, J. M., Ball. Z., and Verdine, G. L. (2001) A synthetic library of cell-permeable molecules. *J. Am. Chem. Soc. 123*, 398−408.
7. Briesewitz, R., Ray, G. T., Wandless, T. J., and Crabtree, G. R. (1999) Affinity modulation of small-molecule ligands by borrowing endogenous protein surfaces. *Proc. Natl. Acad. Sci. U.S.A. 96*, 1953−1958.
8. Rosen, M. K., Amos, C. D., and Wandless, T. J. (2000) Mechanistic studies of affinity modulation. *J. Am. Chem. Soc. 122*, 11979−11982.
9. Kar, A. K., Braun, P. D., and Wandless, T. J. (2000) Synthesis and evaluation of daunorubicin-paclitaxel dimers. *Bioorg. Med. Chem. Lett. 10*, 261−264.
10. Braun, P. D., Barglow, K. T., Lin, Y.-M., Akompong, T., Briesewitz, R., Ray, G. T., Haldar, K., and Wandless, T. J. (2003) A bifunctional molecule that displays context-dependent cellular activity. *J. Am. Chem. Soc. 125*, 7575−7580.
11. Kopytek, S. J., Standaert, R. F., Dyer, J. C. D., and Hu, J. C. (2000) Chemically induced dimerization of dihydrofolate reductase by a homobifunctional dimer of methotrexate. *Chem. Biol. 7*, 313−321.
12. Carlson, J. C. T., Kanter, A., Thuduppathy, G. R., Cody, V., Pineda, P. E., McIvor, R. S., and Wagner, C. R. (2003) Designing protein dimerizers: the importance of ligand conformational equilibria. *J. Am. Chem. Soc. 125*, 1501−1507.
13. Fersht, A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, Chapter 6, Freeman, New York.
14. Gaddum, J. H. (1937) Quantitative effects of antagonistic drugs. *J. Physiol. (Lond.) 89*, 7P−9P.
15. Cheng, Y.-C., and Prusoff, W. H. (1973) Relationship between inhibition constant (K1) and concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol. 22*, 3099−3108.
16. Munson, P. J., and Rodbard, D. (1988) An exact correction to the Cheng-Prusoff correction. *J. Recept. Res. 8*, 533−546.

17. Munson, P. J., and Rodbard, D. (1989) *J. Recept. Res. 9*, 511−511.
18. Clackson, T., Yang, W., Rozamus, L. W., Hatada, M., Amara, J. F., Rollins, C. T., Stevenson, L. F., Magari, S. R., Wood, S. A., Courage, N. L., Lu, X., Cerasoli, F., Jr., Gilman, M., and Holt, D. A. (1998) Redesigning an FKBP-ligand interface to generate chemical dimerizers with novel specificity. *Proc. Natl. Acad. Sci. U.S.A. 95*, 10437−10442.
19. Munson, P. J., and Rodbard, D. (1980) Ligand − A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem. 107*, 220−239.
20. Yuryev, D. K. (1991) Affinogen − A program for affinity spectrum reconstruction. *J. Immunol. Methods 139*, 297−297.
21. Wang, Z. X. (1995) An exact mathematical expression for describing competitive-binding of 2 different ligands to a protein molecule. *FEBS Lett. 360*, 111−114.
22. Wolfram, S. (1999) *The Mathematica Book*, 4th ed., Cambridge University Press, Cambridge, England.
23. Kenakin, T. (1997) *Pharmacologic Analysis of Drug-Receptor Interaction*, Chapter 8, Lippincott-Raven, Philadelphia, PA.
24. Dandliker, W. B., Hsu, M.-L., Levin, J., and Rao, B. R. (1981) Equilibrium and kinetic inhibition assays based upon fluorescence polarization. *Methods Enzymol. 74*, 3−27.
25. Cousins-Wasti, R. C., Ingraham, R. H., Morelock, M. M., and Grygon, C. A. (1996) Determination of affinities for Lck SH2 binding peptides using a sensitive fluorescence assay: comparison between the pYEEIP and pYQPQP consensus sequences reveals context-dependent binding specificity. *Biochemistry 35*, 16746−16752.
26. Charifson, P. S., Shewchuk, L. M., Rocque, W., Hummel, C. W., Jordan, S. R., Mohr, C., Pacofsky, G. J., Peel, M. R., Rodriguez, M., Sternbach, D. D., and Consler, T. G. (1997) Peptide ligands of pp60(c-src) SH2 domains: a thermodynamic and structural study. *Biochemistry 36*, 6283−6293.
27. Bradshaw, J. M., Mitaxov, V., and Waksman, G. (1999) Investigation of phosphotyrosine recognition by the SH2 domain of the Src kinase. *J. Mol. Biol. 293*, 971−985.
28. Keenan, T., Yaeger, D. R., Courage, N. L., Rollins, C. T., Pavone, M. E., Rivera, V. M., Yang, W., Guo, T., Amara, T. F., Clackson, T., Gilman, M., and Holt, D. A. (1998) Synthesis and activity of bivalent FKBP12 ligands for the regulated dimerization of proteins. *Bioorg. Med. Chem. 6*, 1309−1335.

BI035839G