Isothermal titration calorimetry to determine association constants for high-affinity ligands

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An important goal in drug development is to engineer inhibitors and ligands that have high binding affinities for their target molecules. In optimizing these interactions, the precise determination of the binding affinity becomes progressively difficult once it approaches and surpasses the nanomolar level. Isothermal titration calorimetry (ITC) can be used to determine the complete binding thermodynamics of a ligand down to the picomolar range by using an experimental mode called displacement titration. In a displacement titration, the association constant of a high-affinity ligand that cannot be measured directly is artificially lowered to a measurable level by premixing the protein with a weaker competitive ligand. To perform this protocol, two titrations must be carried out: a direct titration of the weak ligand to the target macromolecule and a displacement titration of the high-affinity ligand to the weak ligand—target macromolecule complex. This protocol takes approximately 5 h.

INTRODUCTION

One aim of drug development is to optimize the binding affinities of inhibitors and ligands for their biological targets. Binding affinities are usually determined by using various techniques: spectroscopy, dialysis, ultracentrifugation, calorimetry, surface plasmon resonance, etc. A common problem with these techniques is that accurately determining the binding affinity of molecules that have very high (that is, nanomolar level or higher) affinity becomes difficult. Different methods have been devised to deal with this problem. For example, changing the pH or the temperature in the experiment may cause a decrease in the binding affinity to measurable levels. By performing binding experiments at several pH values or several temperatures, the binding affinity can be extrapolated back to the original conditions.

In the approach presented here, we perform a titration of the target protein with a weak ligand or inhibitor followed by a displacement titration with the stronger analyte ligand[1,2]. The heat released or absorbed upon binding is measured using an isothermal titration calorimeter. Unlike other techniques, ITC allows determination of the binding affinity and the binding enthalpy simultaneously, providing a complete thermodynamic characterization of the ligand binding in one experiment.

The advantages of the displacement titration method are: (i) experimental conditions are not changed and, therefore, the binding affinity is determined at the intended experimental conditions; (ii) the kinetic or the thermodynamic stability of the macromolecule is not compromised by changing the experimental conditions (for example, aggregation or partial unfolding due to temperature or pH being too low or too high); (iii) linkage equations for extrapolating the binding affinity to other temperatures or pH values are not needed.

The limitations of the displacement titration method are: (i) the binding affinities of the weak ligand and the strong ligand must differ by a factor of 10 or more, and (ii) the binding enthalpies of the weak ligand and the strong ligand should be as different as possible, otherwise the measured heat signal will be small (if the binding enthalpies were equal, the heat of binding would not be measurable because the thermal effect associated with the dissociation of the weak ligand would completely compensate for the thermal effect associated with the binding of the strong ligand).

The displacement titration method has been used successfully to determine the association constants for inhibitors binding to HIV-1 protease[3–6], peptides binding to Src kinase SH2 domain[7], peptide analogs binding to PTP1B[8], carbohydrate binding to glucoamylase[1] and nucleotide inhibitors binding to RNase A[2]. In the procedure presented here, we show the application of the displacement titration approach by measuring the binding constant of a high-affinity HIV-1 protease inhibitor using acetyl-pepstatin as the weak inhibitor (**Fig. 1**).

In drug development, the initial compounds (inhibitors or ligands) usually have binding affinities in the micromolar range and need to be optimized to nanomolar and, sometimes, subnanomolar levels. For that reason, weaker ligands are usually available, as they can be obtained from previous iterations of the optimization process. In this protocol, ITC is used to determine the binding thermodynamics of the clinical inhibitor indinavir to its target, the HIV-1 protease. Acetyl-pepstatin, a generic aspartyl protease inhibitor, is used as the weak ligand. The binding affinity of indinavir is too great to be measured by direct titrations.

This protocol requires performing two titrations: a standard titration with the weak ligand binding to the macromolecule and a displacement titration with the strong ligand binding to the macromolecule pre-bound to the weak ligand. Both titrations are performed following the same steps, and they differ only in that weak ligand is added to the macromolecule solution in the calorimetric cell in the displacement titration.

MATERIALS

REAGENTS

- HIV-1 protease (purified according to refs. 6,9)
- Indinavir (Crixivan, Merck), or any other chemically pure clinical or experimental inhibitor
- Acetyl-pepstatin (Bachem AG, cat. no. N1250; see REAGENT SETUP)
- Sodium acetate 10 mM, pH 5.0
- Sodium hydroxide 9 mM
- Dimethylsulfoxide (DMSO)

EQUIPMENT

- Isothermal titration calorimeter (for example, VP-ITC, MicroCal LLC; Nano ITC III, Calorimetry Sciences Corporation) or equivalent
- Origin 7.0 or equivalent data analysis software with the ability of performing nonlinear least-squares data fitting using user-specified functions
- Vacuum pump
- 2.5 ml long-needle syringe (for example, Hamilton 1002LLSN)
- 12 × 75 mm (or 15 × 50 mm) and 6 × 50 mm glass tubes for sample preparation

REAGENT SETUP

Things to be considered before beginning. In a displacement titration, the weak competitive ligand must be present in the calorimetric cell at a concentration sufficient to reduce the affinity of the high-affinity ligand to measurable levels ($K_a \leq 10^9\ M^{-1}$). The apparent binding affinity of the high-affinity ligand, K_L^{app}, is reduced by a factor, RF, dependent on the binding affinity, $K_{a,X,}$ and concentration of the weak ligand:

$$K_L^{app} = \frac{K_{a,L}}{RF} = \frac{K_{a,L}}{1 + K_{a,X}[X]} \quad (1)$$

where [X] is the concentration of the free weak ligand X, which is not known. For practical purposes, the total concentration of weak ligand required to achieve a predetermined reduction factor is approximately:

$$[X]_T = \frac{RF - 1}{K_{a,X}} + [M]_T \quad (2)$$

where $[X]_T$ and $[M]_T$ are the total weak ligand and total macromolecule concentration in the calorimetric cell.

To obtain a complete binding isotherm within the specified number of injections, the ligand solution in the syringe should be more concentrated than the macromolecule in the cell, so that at the end of the experiment, the molar ratio of ligand to macromolecule inside the cell is 2–3 to ensure near saturation (for 1:1 stoichiometry). Considering the volume of the sample cell (1.4 ml), the typical injection volume (10 µl) and the typical number of injections (~25–30), it is advisable to use a concentration of ligand in the syringe 10–20 times higher than the solution in the cell. As a general rule, the macromolecule concentration should be set to 10–50 µM and the concentration of ligand 15-fold higher in the syringe.

In addition, in order to maximize the instrument signal and obtain optimal results, the binding affinity of the weak ligand must differ by a factor of 10 or more from the affinity of the strong ligand, and the binding enthalpy of the weak ligand must be as different (at least 2–3 kcal/mol difference) from the binding enthalpy of the high-affinity ligand as possible (if feasible, their enthalpies must be of opposite sign).

Therefore, before beginning the experiment: choose an appropriate weak competing ligand and choose appropriate concentrations of the target molecule, the weak ligand and the analyte strong ligand. (In the case of HIV-1 protease, the solution in the calorimetric cell contains 20 µM HIV-1 protease and 200 µM acetyl-pepstatin (weak ligand). The solution in the syringe contains 300 µM indinavir (high-affinity ligand).)

Preparing solutions. Experimental conditions should be selected by taking into account the stability and the solubility of the reactants and the biological considerations of the system under study. In the case of HIV-1 protease, the majority of equilibrium experiments have been conducted at mildly acidic pH and low ionic strength (pH 5 and 10–20 mM ionic concentration). These conditions guarantee high conformational stability and low catalytic activity (preventing autocatalysis).

Prepare reactant solutions for the cell and the syringe under identical conditions and with the same buffer composition. For convenience, the injected reactant located in the syringe and the reactant located in the cell are referred to as 'ligand' and 'macromolecule,' respectively. The terms 'ligand' and 'macromolecule' are used only in reference to their location and do not reflect any other attribute. Usually the less-soluble reactant should be placed in the cell. Sometimes reverse titrations (reversing the role of macromolecule and ligand) are conducted to check the stoichiometry or the suitability of the binding model.

The composition, concentration, pH and ionic strength of the buffer all affect the thermodynamic parameters, and the quality of the experiment depends on maintaining a perfect match of the buffer in the cell and syringe samples. For the reactant solutions, buffer type, buffer concentration, pH, ionic strength and co-solvents must be the same. In an ITC assay, consistency between the exact composition of the buffer in the cell and the syringe is of crucial importance to prevent dominance of nonspecific heat effects.

One effective way to achieve this goal is to dialyze the macromolecule against the desired buffer and then use the filtered dialysis buffer to prepare the ligand solution. Certain additives such as DMSO, which increase the solubility of hydrophobic ligands, have an enormous effect on the ITC signal, and therefore extreme care should be taken to keep the concentration of DMSO in the cell and syringe as close as possible to each other.

Determine the concentrations of the weak inhibitor or ligand and the analyte inhibitor or ligand stock solutions by analytical determination of quantitative nitrogen content or any other appropriate technique. Determine the concentration of the target protein solution by measuring its absorbance in a spectrophotometer (the HIV-1 protease has an extinction coefficient of 25,500 $M^{-1}cm^{-1}$ at 280 nm). Reliable estimates of the binding enthalpy, stoichiometry and association constant (although the latter is slightly less dependent) depend on the accurate determination of the concentrations of the reactants.

Solutions used in the HIV-1 protease experiment *Acetyl-pepstatin stock solution*. Dissolve 5 mg of the lyophilized acetyl-pepstatin powder in 9 mM NaOH (this compound is very insoluble in pure water) to a concentration of 8–9 mM. Prepare indinavir stock solution at ≥15 mM in 100% DMSO from lyophilized powder. Indinavir is not water soluble at the required concentration.

Cell solution. For the direct titration with acetyl-pepstatin, dilute the protease to a final concentration of 20 µM in 10 mM sodium acetate, pH 5.0, DMSO 2% (vol/vol), in a total volume of 2.2 ml. For the displacement titration with indinavir, dilute the protease to a final concentration of 20 µM in 10 mM sodium acetate, pH 5.0, DMSO 2% (vol/vol), adding acetyl-pepstatin to the solution to a final concentration of 200 µM, in a total volume of 2.2 ml.

Syringe solution. For the direct titration with acetyl-pepstatin, dilute acetyl-pepstatin to a final concentration of 300 µM in 10 mM sodium acetate, pH 5.0, in a total volume of 0.5 ml, adding DMSO to reach a final concentration of 2% (vol/vol). For the displacement titration with indinavir, dilute indinavir to a final concentration of 300 µM in 10 mM sodium acetate, pH 5.0, in a total volume of 0.5 ml. If necessary, add DMSO to reach a final concentration of 2% (vol/vol).

PROCEDURE

1| Degas all solutions (reactants and buffer solutions used for rinsing the cell) for 10–20 min (stirring and temperature control are optional) using the vacuum pump or any similar device in order to avoid formation of bubbles in the sample cell during the experiment.

2| Lower the calorimeter thermostat setting slightly below the running temperature (with a difference of 0.5–2 °C) to prevent long equilibration delays.

3| Fill the reference cell with degassed distilled water or buffer solution using the long-needle syringe.

4| Rinse the sample cell several times with buffer solution, then remove all liquid.

5| Fill the sample cell with the macromolecule solution, avoiding the appearance of bubbles in the cell.

6| Place the ligand solution in a small glass tube.

7| Fill the calorimeter syringe with ligand solution according to the instructions in the instrument manual. A plastic syringe connected with plastic tubing is used to fill the calorimeter syringe by slowly withdrawing the solution from the small glass tube. A purge-refill cycle may be performed to ensure the absence of air bubbles inside the syringe.

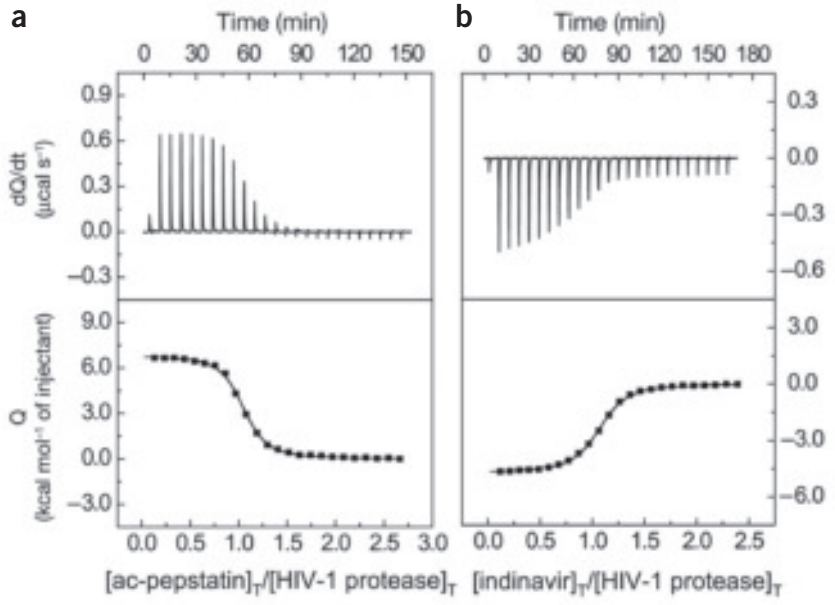

Figure 1 | ITC determination of the binding thermodynamics of indinavir to the HIV-1 protease. Titration of acetyl-pepstatin (300 μM) into a protease solution (20 μM; **a**) and titration of indinavir (290 μM) into a solution of protease (20 μM) in the presence of acetyl-pepstatin (200 μM; **b**). The experiments were performed in 10 mM sodium acetate buffer, pH 5.0, and DMSO 2% (vol/vol) at 25 °C. The cell volume is 1.4 ml and the injection volume is 10 μl. The presence of acetyl-pepstatin in the calorimetric cell reduces the apparent affinity of indinavir by a factor of ~740, which within the measurable range. Analysis of the two sets of data yields the association constant, binding enthalpy and binding entropy of indinavir.

8| Rinse excess ligand solution on the surface of the needle off with water and then carefully dry the surface of the needle with paper.

9| Choose instrument settings appropriate to your experiment, for example: total number of injections (~25–30), measurement temperature (select desired temperature between 5 and 40 °C), reference power (10 μcal s^{-1}), initial injection delay (~200 s), syringe ligand concentration (in mM), cell macromolecule concentration (in mM), stirring speed (~500 r.p.m.), feedback mode gain (high feedback), injection volume (~10 μl), duration of each injection (~20 s), spacing between injections to (~400 s), filter period (~2 s).

▲ **CRITICAL STEP** The reference power, which is in fact the actual signal recorded in the instrument, can be set initially to a higher or lower value if large exothermic or endothermic signals are expected.

▲ **CRITICAL STEP** A stirring speed of ~500 r.p.m. ensures rapid mixing, but it can be modified depending on the sample (e.g., high viscosity, tendency to aggregate or to generate foam, etc.).

▲ **CRITICAL STEP** High feedback gain will provide high sensitivity and small response time, and will allow performing a titration in less than 2 h.

▲ **CRITICAL STEP** The injection volume can be set to lower values (but never lower than 3 μl) if the heat of reaction is expected to be large; in that case it is also possible to modify the concentrations used.

▲ **CRITICAL STEP** Set the volume of the first injection to 3 μl or less (minimizing the amount of reactants wasted). Owing to diffusion of the solutions during the insertion of the syringe or the equilibration stage, the first injection is not useful in the analysis (the heat associated with the first injection is not taken into account in the data analysis).

▲ **CRITICAL STEP** The duration for each injection is usually automatically assigned (2 s μl^{-1}). It should not be lowered in order to avoid large mechanical heat effects.

▲ **CRITICAL STEP** The spacing between injections should be enough to permit the signal to return to baseline before the next injection. However, ligand binding is sometimes accompanied by slow dissociation kinetics. In those cases, the spacing between injections might have to be increased significantly.

10| Initiate the experiment after gently setting the injection syringe in place. The signal equilibration level will be slightly less than the reference power value entered in the parameter setting because of the heat generated due to the stirring.

11| Check the quality of the baseline using a full scale of 1 μcal s^{-1}. Start the injection sequence when the baseline becomes flat, with no significant drift or noise. Usual short-term noise levels are 0.002 μcal s^{-1} (standard deviation) or less. A slight drift in the baseline does not represent a significant problem, given the time length of the peaks (usually less than 5 min).

▲ **CRITICAL STEP** The quality of the baseline is critical for the accurate calculation of the heat effects associated with each injection and, therefore, for the accurate estimation of the thermodynamic binding parameters. **Figure 1** shows the two titrations required for determining the thermodynamic parameters for the binding of indinavir to the HIV-1 protease. The first titration corresponds to the binding of acetyl-pepstatin to the HIV-1 protease and the second one corresponds to the binding of indinavir to the HIV-1 protease in the presence of acetyl-pepstatin, which lowers the binding affinity of indinavir by a factor of ~740. The thermodynamic parameters for the binding of acetyl-pepstatin to the HIV-1 protease are estimated from the first titration: $K_a = 4.1 \times 10^6$ M^{-1}, $\Delta H_a = 6.8$ kcal $mole^{-1}$, $n = 0.98$, $\Delta G_a = -9.0$ kcal/mol, $\Delta S_a = 53$ cal K^{-1} mol^{-1} ($-T\Delta S_a = -15.8$ kcal mol^{-1}). The thermodynamic parameters for the binding of indinavir to the HIV-1 protease are estimated from the displacement titration, and the binding parameters determined for acetyl-pepstatin determined in the first titration. The resulting parameters for indinavir binding to the HIV-1 protease are: $K_a = 2.1 \times 10^9$ M^{-1}, $\Delta H_a = 2.1$ kcal $mole^{-1}$, $n = 0.99$, $\Delta G_a = -12.7$ kcal/mol^{-1}, $\Delta S_a = 50$ cal K^{-1} mol ($-T\Delta S_a = -14.8$ kcal mol^{-1}).

? TROUBLESHOOTING

12| Wash the injection syringe with water (≥50 ml) once the experiment is finished.

▲ **CRITICAL STEP** Regularly, and especially for low-solubility reactants, wash the syringe with methanol or detergent (50 ml) and water (≥300 ml). Then dry the syringe for ≥10 min using the vacuum pump.

13| Wash the plunger tip of the syringe injector with water and dry it.

14| Rinse the sample cell several times with water using the long-needle syringe. For periodic deep cleansing or when using a different macromolecule, or in cases where the sample precipitated or aggregated in the sample cell, the sample cell should be washed with a cleaning solution (compatible with the material the cells are made of) according to the instrument's manual.

▲ **CRITICAL STEP** The sample cell needs to be vigorously cleaned according to manufacturer recommendations, followed by comprehensive rinsing of the cell with water (~1 l).

Data analysis

15| Carefully check the quality of the individual baseline for each peak and, if applicable, manually modify the baseline and the integration interval.

▲ **CRITICAL STEP** Each reaction peak needs to be integrated after tracing the appropriate baseline. The baseline should follow a smooth path, define clearly a peak signal that relaxes and merges with the baseline asymptotically, and go through the noise when the reaction is finished. The appropriate integration interval should cover the entire peak; take care to include all the area corresponding to the relaxation stage.

16| Enter the values of ligand and macromolecule concentration, as well as the sample cell volume to ensure the accuracy of the calculations.

17| Observe the heat effect after saturation; it reflects different nonspecific phenomena and is commonly referred to as 'heat of dilution' even though it contains other factors. A large heat of dilution throughout the titration may render the experiment useless if its magnitude is much larger than the heat of binding, thus masking the binding of the ligand.

▲ **CRITICAL STEP** In addition to the heat effects associated with the binding reaction itself, there are also heat effects associated with the dilution of reactants and heat effects due to friction and turbulence associated with the injection and mixing of the reactants. These effects need to be subtracted from the total heat measured in each injection. The heat due to friction and turbulence upon injection can be estimated by injecting water into water, and should never be larger than 0.5 μcal. A substantially larger heat may be due to large injection volumes, and/or short injection durations (high injection rate); or reflect an instrument malfunction that needs to be addressed to the manufacturer. Two common causes for a large heat of dilution (>10 μcal) are: dilution of the ligand from a concentrated solution in the syringe to a less-concentrated solution in the cell (usually a dilution factor of ~100) and heat due to mismatched (pH or ionic strength) syringe and cell buffer solutions. The first is inevitable, and the experimenter should estimate such effect by performing blank experiments, that is, by injecting ligand solution into buffer solution without macromolecule or by averaging the heat effects measured after saturation has been achieved. In all cases, the heat of dilution is subtracted from the heat associated with each

injection. Sometimes, the heat of dilution is concentration dependent (i.e., it is not constant throughout the titration); in this case the averaging method is not valid, and the actual dilution heats obtained by titrating ligand into buffer need to be subtracted. Failing to properly estimate the dilution heat will result in inaccurate estimates of the thermodynamic binding parameters.

18| Data fitting. The binding affinity and enthalpy for acetyl-pepstatin binding to the HIV-1 protease must be evaluated first using the standard one set of independent sites model (in Origin 7.0 the corresponding fitting function is called *One Set of Sites*). Once this is done, the analysis of the displacement titration should be performed using a binding competition model (the required equations are provided below for those who use custom software; in Origin 7.0 is called *Competitive Binding*). In this fitting function, all the parameters required for analyzing the experiment must be set: total concentration of each reactant, HIV-1 protease, acetyl-pepstatin and indinavir, and the affinity and binding enthalpy of acetyl-pepstatin to the HIV-1 protease. Once this is done, all parameters are estimated by nonlinear least-squares regression.

ANTICIPATED RESULTS

The goal in a displacement titration is to lower the affinity of a high-affinity ligand to a level that can be measured by ITC. This is achieved by adding a weak competitive ligand, X, to the protein solution in the calorimetric cell in order to artificially lower the affinity of the high-affinity ligand, L. The binding equations for this system can be solved by using the following approach.

The total concentration of each reactant inside the calorimetric cell is known and after each consecutive injection i, is given by:

$$\begin{aligned}[M]_{T,i} &= [M]_0\left(1-\frac{v}{V}\right)^i \\ [L]_{T,i} &= [L]_0\left(1-\left(1-\frac{v}{V}\right)^i\right) \\ [X]_{T,i} &= [X]_0\left(1-\frac{v}{V}\right)^i\end{aligned} \tag{3}$$

where $[M]_0$ is the initial concentration of macromolecule in the cell, $[L]_0$ is the concentration of ligand L in the syringe, $[X]_0$ is the concentration of ligand X in the cell, V is the cell volume, v is the injection volume and $(1 - v/V)$ is the factor that accounts for the change in the concentration of reactants due to the dilution that takes place upon each injection. Using the mass action law and the conservation of mass for each species:

$$\begin{aligned}[M]_T &= [M] + [ML] + [MX] = [M] + K_{a,L}[M][L] + K_{a,X}[M][X] \\ [L]_T &= [L] + [ML] = [L] + K_{a,L}[M][L] \\ [X]_T &= [X] + [MX] = [X] + K_{a,X}[M][X]\end{aligned} \tag{4}$$

where $K_{a,L}$ and $K_{a,X}$ are the binding constants for each ligand. Solving this set of equations provides the concentration of any species in the calorimetric cell, in particular, the concentration of both complexes, [ML] and [MX] after each injection i. The heat released or absorbed due to each ligand injection, q_i, is the heat associated with the formation/dissociation of each complex in the injection i:

$$q_i = V\left(\Delta H_{a,L}\left([ML]_i - [ML]_{i-1}\left(1-\frac{v}{V}\right)\right) + \Delta H_{a,X}\left([MX]_i - [MX]_{i-1}\left(1-\frac{v}{V}\right)\right)\right) \tag{5}$$

where $\Delta H_{a,L}$ and $\Delta H_{a,X}$ are the binding or association enthalpy for each ligand. This set of equations constitutes the formalism to analyze binding experiments in which a ligand binds a macromolecule in the presence of a second competitive ligand. Given the initial concentrations of reactants, $[M]_0$, $[L]_0$ and $[X]_0$, the binding affinities, K_{aL} and K_{aX}, and the binding enthalpies, ΔH_{aL} and ΔH_{aX}, it is possible to estimate the heat involved in each injection, which will be the dependent variable used in the analysis, and incorporate that calculation in a fitting function. It is usual to include an additional parameter, n, to consider explicitly the stoichiometry or the percentage of active protein in the solution (substituting $[M]_T$ with $n[M]_T$ in Equation 4 or, alternatively, $[M]_0$ by $n[M]_0$ in Equation 3). The thermodynamic parameters (K_{aL}, ΔH_{aL} and n) will be estimated as adjustable parameters in the fitting procedure. It must be noted that direct titration

experiments in which a protein is titrated with a ligand (in the absence of a competitor ligand) can also be analyzed with the present formalism, because they correspond to the special case in which the total concentration of competitor ligand equals zero ($[X]_T = 0$) and all terms with ligand X in Equations 4 and 5 are not considered.

● TIMING

Each titration may last 2.5 h approximately (sample preparation, 0.5 h; experiment run, 1.5 h; data analysis, 0.5 h; instrument cleaning, 0.5 h). Therefore, the two-titration protocol can be carried out in 5 h.

? TROUBLESHOOTING

For troubleshooting guidance see **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	SOLUTION
Step 11 Poor baseline quality	Several possibilities must be considered. (i) Small irregular noise is probably due to air bubbles in the sample cell. In that case, stop measurement, remove syringe and check cell content. (ii) Strong regular noise (significantly higher than 0.002 μcal s^{-1} (standard deviation)) and/or significant drift indicate that the injection syringe is bent. Stop measurement, and check the needle of the syringe by rolling it on a flat surface. (iii) High baseline level (higher that the reference power set in Step 9) indicates low liquid level or air bubbles in the reference cell. (iv) Low baseline level (1 μcal s^{-1} or more lower than the reference power set in Step 9) indicates that the viscosity of the cell solution is high or the cell is dirty. From a practical point of view, it should always be emphasized that a clean cell and a straight syringe are decisive to avoid spurious results and excellent baselines with high signal-to-noise ratio.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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