Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is the only technique that can directly measure the binding energetics of biological processes, including protein-ligand binding, protein-protein binding, DNA-protein binding, protein-carbohydrate binding, protein-lipid binding, and antigen-antibody binding. ITC has the ability to precisely determine the Gibbs energy, enthalpy, entropy, and heat capacity changes associated with binding.

In this unit several protocols are presented, from the basic ones that are aimed at characterizing binding of moderate affinity (see Basic Protocols 2 and 3) to the advanced ones that are for determining very high or very low affinity (see Alternate Protocols 1 and 2). Also, alternate protocols for special cases (homodimeric proteins and unstable proteins; see Alternate Protocols 3 and 4) and additional information obtainable by ITC (heat capacity and proton transfer coupled to binding; see Alternate Protocols 5 and 6) are presented.

PRINCIPLES OF THE TECHNIQUE

The ITC instrument is a heat-flux calorimeter operating according to the dynamic power compensation principle, i.e., it measures the amount of power (μcal/sec) required to maintain a constant temperature difference (close to zero) between the sample and the reference cell (see Basic Protocol 1). Initially, the feedback system continuously applies a small power to the sample cell, which determines the baseline level. Each injection of the syringe solution (usually termed as ligand) triggers the binding reaction and, depending on the binding affinity and the concentration of reactants (macromolecule, M, and ligand, L) in the cell, a certain amount of macromolecule/ligand (ML) complex is formed. The formation of complex is accompanied by the release (exothermic reaction) or the absorption (endothermic reaction) of heat that causes a difference in temperature between the two cells. Then, the feedback system either lowers or raises the thermal power applied to compensate such temperature unbalance. After each injection, the system reaches equilibrium and the temperature balance is restored. Therefore, the recorded signal shows a typical deflection pattern in the form of a peak (Fig. 17.8.1). Integrating the area under the peak, assuming the baseline as reference, provides the amount of heat associated with the injection. As the reactant in the cell becomes saturated, the heat signal diminishes until only the background heat, due to an unspecific phenomena (e.g., ligand dilution, liquid friction), is observed.

INSTRUMENTATION

Two identical coin-shaped cells, sample and reference, are enclosed in an adiabatic shield (jacket) as illustrated in Figure 17.8.1. The temperature difference between the reference cell and the jacket is continuously monitored to maintain a constant temperature. A feedback control system monitors the difference in temperature between the two cells through a semiconductor Peltier sensor device sandwiched between them. This temperature difference is kept constant and as close to zero as possible at any time. The feedback signal is the measured signal.

One of the reactants is placed in the sample cell and the other one in the injection syringe. During the course of an experiment, the syringe is used to add the titrant reactant to the sample cell in a stepwise fashion and, at the same time, stir the solution in the sample cell to achieve fast mixing. The reference cell serves only as a temperature reference.

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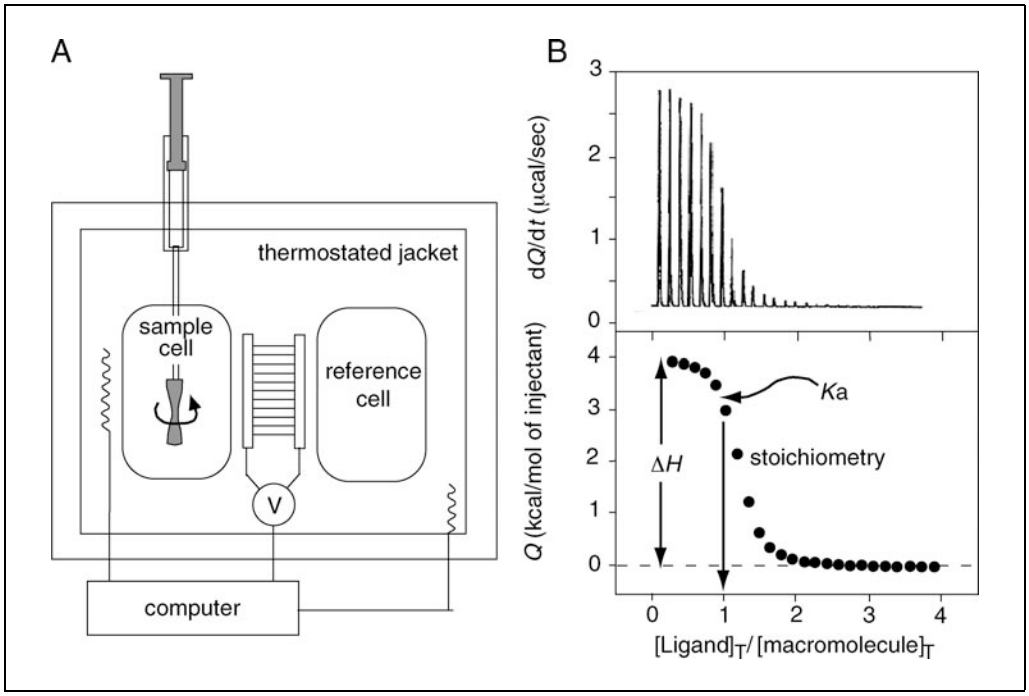

Figure 17.8.1 (**A**) Basic schematic illustration of the ITC instrument, showing the two cells (sample and reference) surrounded by the thermostated jacket, the injection syringe that also works as stirring device, and the computer-controlled thermostatic and feedback systems (using Peltier and resistor devices as sensor and actuator subsystems). (**B**) Example of a typical ITC experiment. The top panel shows the sequence of peaks, each one corresponding to each injection of the solution in the syringe. The monitored signal is the additional thermal power needed to be supplied or removed at anytime to keep a constant temperature in the sample cell and as close as possible to the reference cell temperature. This example corresponds to an endothermic binding. The bottom panel shows the integrated heat plot. The areas under each peak, calculated and normalized per mole of ligand injected in each injection, are plotted against the molar ratio (quotient of the total concentrations of ligand and macromolecule in the sample cell). From this plot, and applying the appropriate model, the thermodynamic parameters of the binding can be obtained: binding affinity, binding enthalpy, and stoichiometry.

BASIC PROTOCOL 1

ISOTHERMAL TITRATION CALORIMETRY

The protocols differ only in the way the experiment is planned and not in the procedure for using the instrument. This protocol consists of a detailed description of setting up and using the ITC instrument. The time required for performing a complete ITC experiment (setting up the instrument, running the experiment, and analyzing the results) is ~2 hr.

This protocol should not be considered a substitute for the instrument manual, where more detailed and specific explanations are provided. This protocol is written based on the VP-ITC MicroCalorimeter (Microcal LLC) and it can serve as a guide for other models.

Materials

Reactant solutions: macromolecule and ligand
Methanol

VP-ITC calorimeter (e.g., Microcal LLC or equivalent)
Vacuum pump
2.5-ml long-needle syringe (e.g., Hamilton)
12 × 75–mm and 6 × 50–mm glass tubes

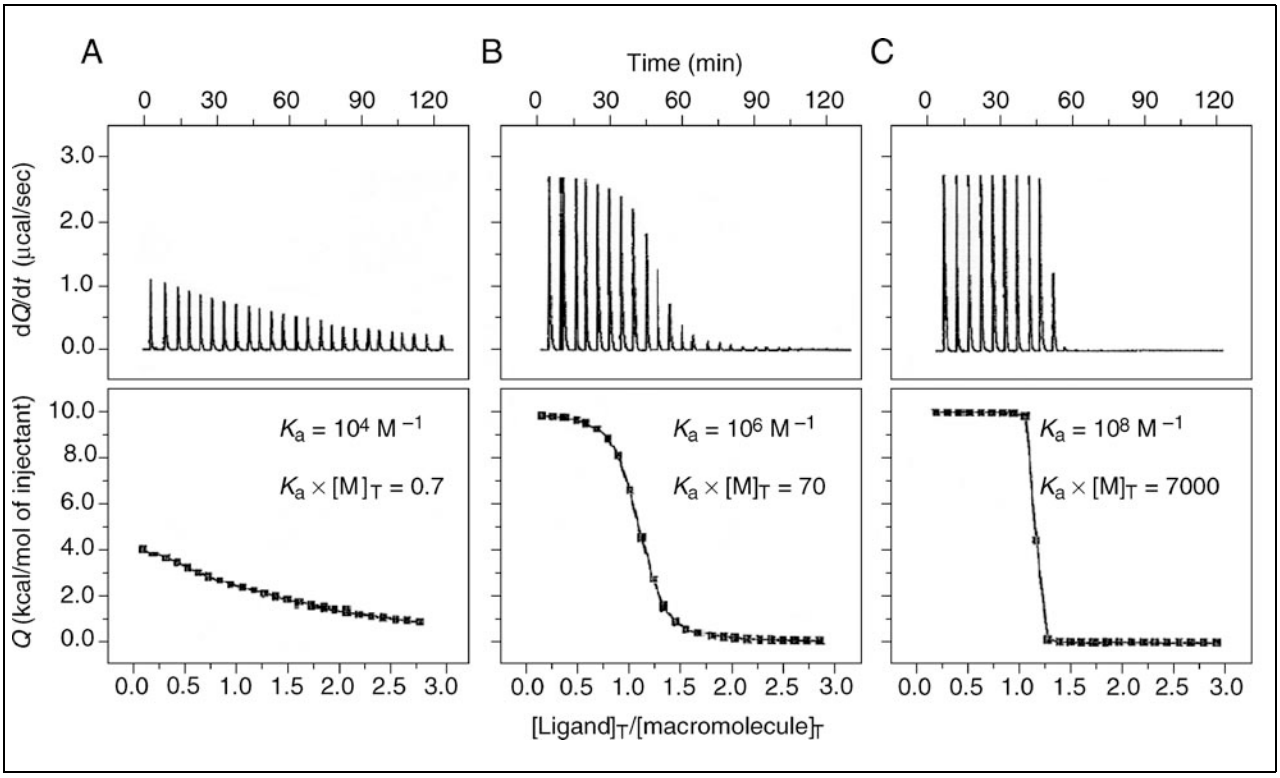

Figure 17.8.2 Illustration of the effect of the association constant value on the shape of a titration curve. The plots represent three titrations simulated using the same parameters (concentrations of reactants and binding enthalpy), but different binding affinities. Low (**A**), moderate (**B**), and high affinity (**C**) binding processes are shown. To obtain accurate estimates of the binding affinity an intermediate case is desirable (**B**, $1 < c = K_a \times [M]_T < 1000$). When the parameter c is large enough, a good estimate of the enthalpy can be determined from the *y*-axis intercept of the curve (if the heat effect associated with dilution is used as a reference). Such an intercept value is given by $(c/c+1) \times \Delta H_a$ (Indyk and Fisher, 1998).

Select concentration of reactants

1. Determine the appropriate concentrations of reactants.

   Typically, the concentrations of reactants are in the micromolar range; if the heat associated with the reaction is significant, however, the concentrations can be lowered.

   An important requirement for an ITC experiment is having appropriate concentrations of interacting molecules such that the heat associated with a given binding reaction is within the calorimetric determination range. Sensitivity of the instrument varies depending on brand and model. The Microcal VP-ITC instrument used in all the experiments described in this unit has a limiting sensitivity of 0.1 μcal. Therefore, to accurately determine the change of heat involved in the interaction between molecules, each injection should have a minimum of 1 μcal of associated heat.

   The affinity of the interaction should also be considered in choosing the required concentrations of the interaction components. A dimensionless constant, c, describes the practical window of the instrument to accurately determine the binding constants (Wiseman et al., 1989) in Equation 17.8.1:

$$c = nK_a[M]_T$$

Equation 17.8.1

   where, K_a is the binding constant, $[M]_T$ the total macromolecular concentration in the cell, and n is the stoichiometry of interaction. As illustrated in Figure 17.8.2, a large c value corresponds to very tight binding, resulting in an isotherm that is rectangular in shape and is devoid of data points in the transition region of binding, therefore, making the estimation of the binding affinity unreliable, although the binding enthalpy can still be obtained accurately. Conversely, a very low c value corresponds to weak binding and

the resultant isotherm is featureless, making the determination of both the binding affinity and enthalpy unreliable. Keeping other parameters constant (K_a, ΔH, etc), the c value could be reduced or increased by changing the $[M]_T$. ITC data with c values between 1 and 1000 can be used for determination of binding constants.

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 to 3 (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 (~30), it is advisable to use a concentration of ligand in the syringe 10 to 20 times higher than the solution in the cell. This will guarantee that the reaction reaches the neutralization point after 7 to 13 injections.

Prepare samples

2. Choose experimental conditions, taking into account the stability and the solubility of the reactants and the biological considerations of the system under study. Prepare reactant solutions for the cell and the syringe under identical conditions and with the same composition.

   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 unspecific heat effects. 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. One effective way of achieving this goal is to dialyze the macromolecule against the desired buffer and then using the filtered dialysis buffer to prepare the ligand solution. Certain additives such as DMSO, which increases the solubility of hydrophobic ligands, have an enormous effect on the ITC signal, therefore, utmost care should be taken to keep the concentration of DMSO in the cell and syringe nearly the same.

3. Prepare the reactant solutions in the appropriate volumes.

   Although the apparent volume of the sample cell is 1.4 ml, the volume of solution required for proper filling of the cell is 2.2 ml. The volume required for the syringe is 0.5 ml.

4. Degas all solutions (reactants and buffer solution used for rinsing the cell) for 10 to 20 min (stirring and temperature control are optional) to avoid formation of bubbles in the sample cell during the experiment.

5. To prevent long equilibration delays, lower the calorimeter thermostat setting slightly below the running temperature (with a difference of 0.5° to 2°C).

Load solutions

6. Place degassed, distilled water or buffer solution in the reference cell using a 2.5-ml, long-needle syringe.

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

8. Fill the cell with macromolecule solution, taking care to prevent the appearance of bubbles in the cell.

   The most common configuration is one in which a macromolecule is placed in the reaction cell and a low-molecular-weight ligand is placed in the injecting syringe. For convenience, the injected reactant located in the syringe is referred to as "ligand" and the one in the reaction cell is referred to as "macromolecule," throughout this unit. The reader should be aware that other configurations are possible.

9. Fill the calorimeter syringe with ligand solution according to the maufacturer's instructions. Rinse off excess ligand solution on the surface of the needle with water and then carefully blot the surface of the needle with a paper towel.

A special plastic syringe is used to fill the calorimeter injecting syringe. A purge-refill cycle may be performed to ensure the absence of air bubbles inside the syringe.

Set instrument settings

10. Set the total number of injections to 30.
11. Set measurement temperature to °C.
12. Set reference power to 10 μcal/sec.

    The reference power can be modified if a large exothermic or endothermic reaction is expected.

13. Set the initial delay (time between the start and the first injection) to 200 sec.
14. Set the syringe ligand concentration to millimolar (mM).
15. Set the cell macromolecule concentration to millimolar (mM).

    During the experiment and analysis, the concentrations are always expressed as millimolar.

16. Set the stirring speed ~500 rpm.

    This speed is selected to ensure good mixing, but it can be modified depending on the sample (e.g., high viscosity, tendency to generate foam, etc.).

17. Set feedback mode/gain to high (high sensitivity and small response time).
18. Set injection volume to 10 μl.

    A different injection volume can be set, for example, if the heat of reaction is expected to be very large or very small. However, if the ligand concentration can be varied, then it is preferable to adjust its concentration in the injecting syringe rather than the injection volume.

19. Set duration for injection to 20 sec.

    This setting is automatically assigned according to the injection volume.

20. Set spacing between injections to 400 sec (usually).

    The spacing might be increased depending on peak size or the reaction kinetics.

    The user should allow enough time between peaks in order to keep the next injection from starting before the signal from the previous injection returns to baseline. Typically, there should be ~200 sec between the end of an injection and the beginning of the next one.

21. Set filter period (the signal-average time period) to 2 sec.
22. Set the volume of the first injection to ≤3 μl. Do not include the heat associated with the first injection in the data analysis.

    Due to inter-diffusion of the solutions during the insertion of the syringe or the equilibration stage, the first injection is not useful in the analysis, therefore, the volume of the first injection should be set to ≤3 μl to minimize the amount of ligand wasted.

Start measurement and troubleshoot the baseline (noise, drift, level)

23. After gently setting the injection syringe in place, initiate the experiment. If fast equilibration is initially selected, both thermal and mechanical equilibration will proceed simultaneously.

    The equilibration level will be slightly less than the reference power value entered in the parameter setting due to the power generated by stirring.

24. Check the quality of the baseline in a full scale of 1 μcal/sec. When the baseline becomes flat, with no significant drift or noise, start the injection sequence.

If the baseline is of poor quality, several possibilities should be considered. (1) Small irregular noise is probably due to air bubbles in reference or sample cell. In the case of bubbles in the sample cell, stop measurement, remove syringe, and check cell content. In the case of bubbles in the reference cell, replace the content of the cell. (2) Strong regular noise and/or drift indicates that the injection syringe is bent. Stop measurement and check the needle of the syringe by rolling it on a flat surface. (3) A high baseline level indicates low liquid level or air bubbles in the reference cell. (4) A low baseline level indicates that the viscosity of the cell solution is high or the cell is dirty.

25. Collect data.

    Once the sequence of injections is initiated, the titration should be continued until saturation is observed.

Clean the ITC

26. Wash the injection syringe with ≥150 ml water. Frequently, and especially for low solubility reactants, wash the syringe with 50 ml methanol (or water-organic co-solvent) and 300 ml water. Dry syringe for ≥10 min.

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

28. Wash the sample cell with water (ten times or more) 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 manufacturer's instructions.

    CAUTION: *Do not leave any methanol or organic solvent in the injection syringe or the cells.*

SUPPORT PROTOCOL

DATA ANALYSIS FOR ITC EXPERIMENTS

The following section is an outline of the foundations for data analysis, including the basic equations describing the binding equilibrium and its implementation. Data analysis is usually performed by non-linear regression using fitting functions defined in the control and analysis software provided by the manufacturer.

Adjust baseline and integrate peaks

1. Carefully check the quality of the automatic baseline and, if applicable, manually modify the baseline and the integration interval.

    The application automatically creates a baseline and integrates each peak when reading the data file. The baseline should follow a smooth path, clearly define a peak signal that relaxes and asymptotically merges with the baseline, and go through the noise when the reaction is finished. The appropriate integration interval should cover the entire peak, taking care to include all the area corresponding to the relaxation stage.

Enter concentration values

2. Enter the values of the ligand and macromolecule concentrations, as well as the sample cell volume to ensure the accuracy of the calculations.

Estimate heat of dilution

3. Note the heat observed after saturation; it reflects different unspecific phenomena, and it is referred to as "heat of dilution." If the fitting procedure does not account for the heat of dilution through an adjustable parameter, make an estimation of such value, e.g., by averaging the values of the last injections or by performing a blank experiment injecting reactant into a buffer solution (see below). If estimated, subtract the heat of dilution from the heat associated with each injection.

Select fitting function

4. Determine the model for binding to be used.

   Although traditional data analysis methods based on linearization of the binding equations have been commonly used, a non-linear regression is a more appropriate methodology for analyzing ITC data. A suitable model, according to the system under study, needs to be considered when selecting a fitting function to use in the non-linear least square regression analysis. The description for different models can be found in the literature (Cantor and Schimmel, 1980; Freire et al., 1990; Wyman and Gill, 1990; Van Holde et al., 1998; Jelesarov and Bosshard, 1999; Sigurskjold, 2000).

5. Express the binding equations in terms of total concentrations. The total concentration of each reactant inside the calorimetric cell is known and after each consecutive injection, *i*, is given by Equation 17.8.2:

$$[\mathrm{M}]_{\mathrm{T},i} = [\mathrm{M}]_o\left(1-\frac{v}{V}\right)^i$$

$$[\mathrm{L}]_{\mathrm{T},i} = [\mathrm{L}]_0\left(1-\left[1-\frac{v}{V}\right]^i\right)$$

Equation 17.8.2

   where $[\mathrm{M}]_0$ is the initial concentration of macromolecule in the cell, $[\mathrm{L}]_0$ is the concentration of ligand in the syringe, 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.

   Since the concentration of each chemical species (free reactants and complex) is not known, the binding equations must be expressed in terms of total concentrations.

6. Determine the heat released or absorbed due to each ligand injection, q_i, i.e., the heat associated with the formation of complex in injection *i* according to Equation 17.8.3:

$$q_\mathrm{i} = V\Delta H_\mathrm{a}\left([\mathrm{ML}]_i - [\mathrm{ML}]_{i-1}\left[1-\frac{v}{V}\right]\right)$$

Equation 17.8.3

   where ΔH_a is the binding or association enthalpy. Again, the dilution factor indicates that the heat change in each injection corresponds to different reactant concentrations.

7. Using the mass action law and the conservation of mass for each species, the expression for the concentration of complex after each injection becomes Equation 17.8.4:

$$[\mathrm{ML}]_i = \frac{1+[\mathrm{M}]_{\mathrm{T},i}K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i} - \sqrt{(1+[\mathrm{M}]_{\mathrm{T},i}K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i})^2 - 4[\mathrm{M}]_{\mathrm{T},i}K_\mathrm{a}^2[\mathrm{L}]_{\mathrm{T},i}}}{2K_\mathrm{a}}$$

Equation 17.8.4

8. It is customary to include in this equation a factor *n*, the stoichiometry, which can be interpreted in two ways: (1) as the generalization for a macromolecule with *n*

equivalent and independent binding sites or (2) to account for the possibility of having a percentage of the macromolecule in a non-competent binding conformation:

$$[\mathrm{ML}]_i = \frac{1 + n[\mathrm{M}]_{\mathrm{T},i} K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i} - \sqrt{(1 + n[\mathrm{M}]_{\mathrm{T},i} K_\mathrm{a} + K_\mathrm{a}[\mathrm{L}]_{\mathrm{T},i})^2 - 4n[\mathrm{M}]_{\mathrm{T},i} K_\mathrm{a}^2 [\mathrm{L}]_{\mathrm{T},i}}}{2K_\mathrm{a}}$$

Equation 17.8.5

Equations 17.8.4 and 17.8.5 constitute the formalism to analyze binding experiments in which a ligand binds a macromolecule with n equivalent and independent binding sites. Given the initial concentrations of reactants, $[M]_0$ and $[L]_0$, the binding affinity, K_a, the binding enthalpy, ΔH_a, and the stoichiometry, n, 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. The thermodynamic parameters (K_a, ΔH_a, and n) will be estimated as adjustable parameters in the fitting procedure.

All examples presented in this unit can be analyzed with built-in functions embedded in Origin version 5.0 or 7.0.

The previous formalism can be applied to other cases (see Basic Protocol 2) by appropriately modifying Equations 17.8.3 and 17.8.4.

BASIC PROTOCOL 2

MACROMOLECULE/LIGAND INTERACTION WITH MODERATE AFFINITY

Ligands with moderate binding affinities to their target macromolecule constitute the majority of the cases in biological systems. However, ligands with very low or very high affinities are also encountered. Conducting ITC assays for a complete thermodynamic characterization of the binding of such ligands requires modified versions of the basic ITC protocol and will be explained later. When the affinity between the macromolecule and the ligand is moderate ($10^4 < K_\mathrm{a} < 10^8\ \mathrm{M}^{-1}$), it is possible to determine the affinity and the enthalpy of binding simultaneously in a single experiment.

This protocol uses commercially available ribonuclease A (RNase A), a well-studied endonuclease of 13.7 kDa, and its commercially available inhibitor 2′-cytidine-monophosphate (2′CMP) of 323 Da, to familiarize first-time users with the setup, procedure, and data analysis of isothermal titration calorimetry. The binding of 2′CMP to RNase A is an example of an exothermic binding reaction, and the binding constant for this reaction is within the range measurable by conventional ITC (Wiseman et al., 1989; Straume and Freire, 1992). It is highly recommended that every attempt at ITC begin with this simple protocol and that conducting experiments with other samples only take place after mastering the technique and analysis explained in this protocol. This protocol presents an efficient way of keeping the conditions in the ITC cell and syringe as close to each other as possible.

Materials

RNase A, lyophilized powder (Sigma)
15 mM potassium acetate buffer, pH 5.5
2′CMP, lyophilized powder (Sigma)

12 × 75–mm and 6 × 50–mm glass tubes
6-kDa MWCO dialysis membrane
0.22-μm filter

Prepare samples

1. Prepare RNase A stock solution in a 12 × 75–mm glass tube. Dissolve the protein in 15 mM potassium acetate buffer, pH 5.5, to a final concentration of 200 to 300 μM.

   RNase A is commercially available as a lyophilized powder. A final volume of 5 ml of this stock solution should be sufficient to conduct several experiments.

2. Dialyze RNase A stock solution against ~1 liter 15 mM potassium acetate buffer, pH 5.5, overnight at 4°C using a dialysis membrane with a ~6.0-kDa MWCO.

3. After dialysis, filter the RNase A solution through a 0.22-μm filter. Determine the exact concentration of the stock solution by measuring its absorbance at 278 nm using an extinction coefficient of 9800 $M^{-1}cm^{-1}$. Store the dialyzed stock of RNase A up to several weeks at 4°C.

4. Prepare 2′CMP stock solution in a 12 × 75–mm glass tube. Filter 15 mM potassium acetate buffer, pH 5.5, used for dialysis using a filter with a 0.22-μm pore size. Dissolve the lyophilized 2′CMP (or 5′CMP) in the filtered buffer to a final concentration of 5 mM. Determine the exact concentration of the stock solution by measuring its

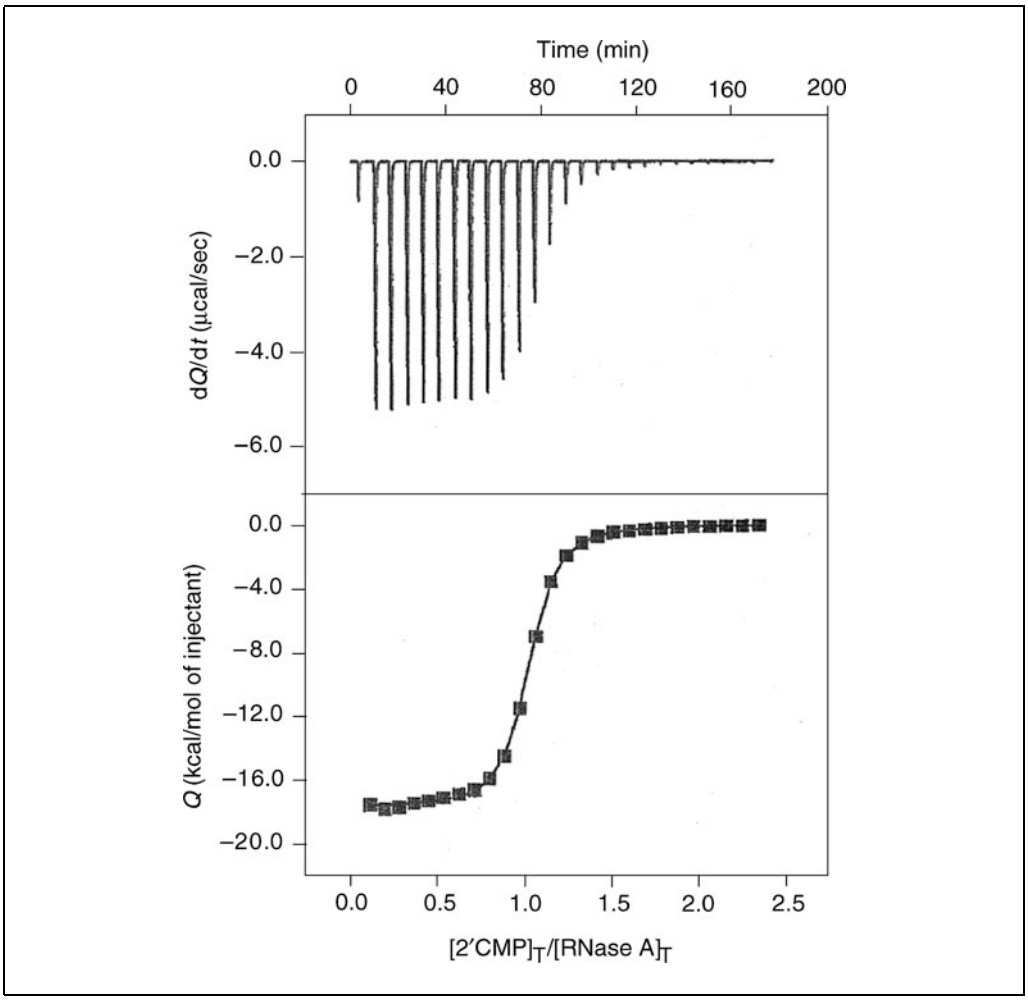

Figure 17.8.3 Titration of RNase A with 2′CMP. The experiment was performed in 15 mM potassium acetate, pH 5.5, at 25°C. The concentration of reactants are 76 μM RNase A and 1.13 mM 2′CMP. The solid line corresponds to the theoretical curve with $n = 1.02$, $K_a = 2.9 \times 10^6$ M^{-1} and $\Delta H = -19.3$ kcal/mol.

absorbance at 260 nm using an extinction coefficient of 7400 $M^{-1}cm^{-1}$ in 100 mM phosphate buffer, pH 7.0.

A final volume of 1 ml should be sufficient to conduct several experiments.

5. Dilute the dialyzed RNase A stock to a final concentration of 50 to 100 μM in 15 mM potassium acetate buffer, pH 5.5.
6. Prepare cell sample (2.2 ml of diluted RNase A) to ensure proper loading of the cell.
7. Dilute the 2′CMP stock to a final concentration of 0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5, depending on the concentration of RNase A.
8. Prepare syringe sample (0.5 ml of diluted 2′CMP) to ensure proper loading of the syringe.
9. Degas 10 ml of the same buffer employed, cell sample solution, and syringe sample solution 20 to 30 min.

   This buffer solution will be used for rinsing the sample cell.

10. After preparation, transfer the syringe solution to a 6 × 50–mm glass tube to fill the syringe.
11. Set the instrument settings, run the experiment, and analyze the data (see Basic Protocol 1 and Support Protocol).

    Figure 17.8.3 shows the result from an experiment in which 2′CMP binds to the enzyme RNase A. The thermodynamic parameters obtained from this experiment are: $K_a = 2.0 \times 10^6\ M^{-1}$, $\Delta H_a = -17.8$ kcal/mol, $n = 0.99$.

BASIC PROTOCOL 3

MACROMOLECULE/MACROMOLECULE INTERACTION WITH MODERATE AFFINITY

The same equations above (see Support Protocol) apply to macromolecule/macromolecule binding. The formalism can be applied to this case by considering the macromolecule in the syringe as the ligand. As in every situation, the less soluble reactant should be placed in the calorimetric cell.

This protocol uses commercially available porcine pancreatic trypsin (PPT), a well-studied serine protease of 23.8 kDa, and its commercially available inhibitor, soybean trypsin inhibitor (STI), of 20.0 kDa. The binding of STI to PPT is an example of an endothermic binding reaction, and the binding constant for this reaction is within the range measurable by conventional ITC (El Harrous and Parody-Morreale, 1997).

Materials

Porcine pancreatic trypsin (PPT), lyophilized powder (Sigma)
25 mM potassium acetate/10 mM calcium chloride, pH 4.5
Soybean trypsin inhibitor (STI), lyophilized powder (Sigma)

10-kDa MWCO dialysis tubing
0.22-μm filter

Prepare samples

1. Dissolve PPT in 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, to a final concentration of ∼400 μM.

   A final volume of 1 ml of this PPT stock solution should be sufficient.

2. Dissolve STI in 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, to a final concentration of ~30 μM.

   A final volume of 5 ml of this STI stock solution should be sufficient.

3. Dialyze PPT and STI against ~1 liter of 25 mM potassium acetate/10 mM calcium chloride buffer, pH 4.5, overnight at 4°C using a dialysis membrane with a ~10-kDa MWCO.

4. After dialysis, filter the solutions through 0.22-μm pore filters.

5. Determine the exact concentration of the PPT and STI stock solutions by measuring their absorbances at 280 nm using extinction coefficients of 35,700 and 18,200 $M^{-1}cm^{-1}$, respectively. Store the dialyzed stocks for several weeks at 4°C.

6. Prepare cell sample. Prepare 2.2 ml of STI at a concentration of ~30 μM.

   Since STI has very low solubility compared to PPT, the former will be placed in the calorimetric cell.

7. Prepare syringe sample. Prepare 0.5 ml of PPT at a concentration of ~400 μM.

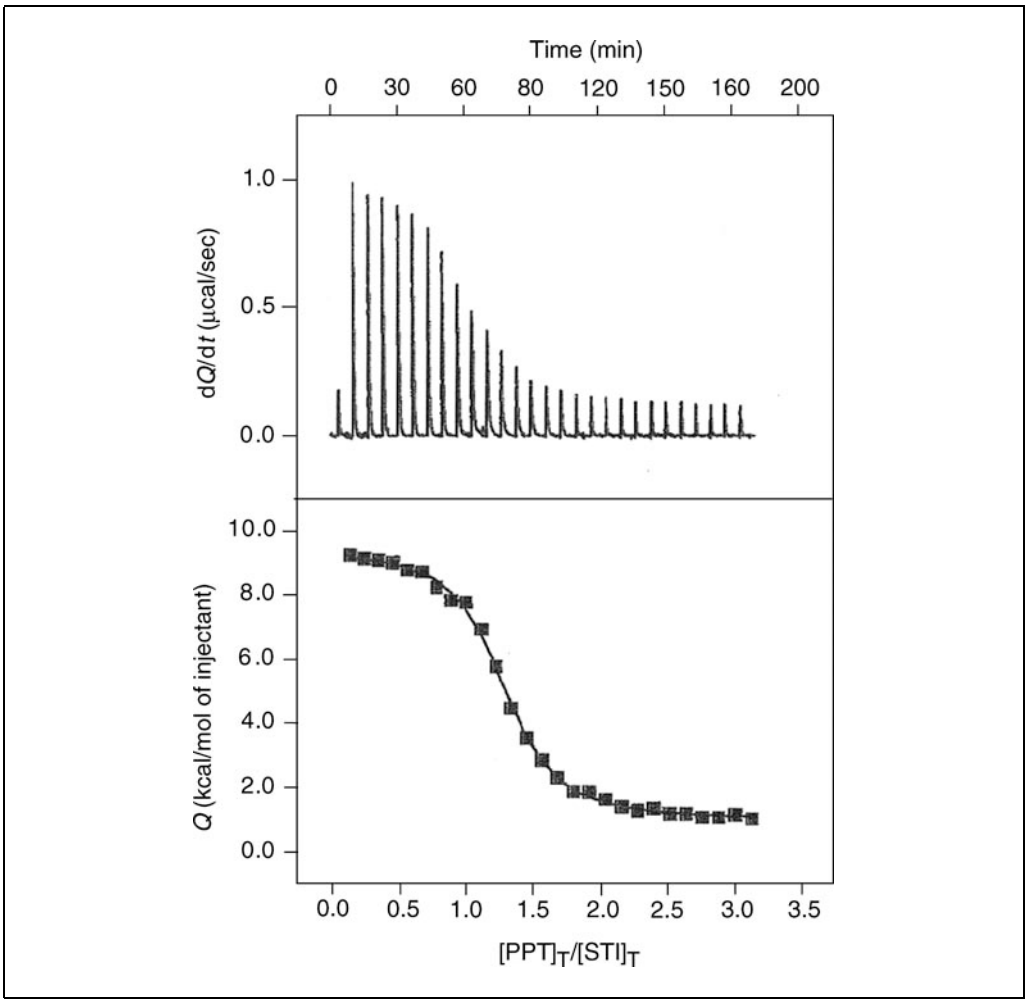

Figure 17.8.4 Titration of STI with PPT. The experiment was performed in 25 mM potassium acetate, pH 4.5/10 mM calcium chloride, at 25°C. The concentrations of reactants are 21 μM STI (in cell) and 312 μM PPT (in syringe). The inhibitor was placed in the calorimetric cell due to its low solubility. The solid line corresponds to theoretical curve with $n = 1.26$, $K_a = 1.5 \times 10^6$ M^{-1} and $\Delta H_a = 8.4$ kcal/mol.

8. Set instrument settings, run the experiment, and analyze the data (see Basic Protocol 1 and Support Protocol).

Figure 17.8.4 shows the result from an experiment in which STI binds to PPT. The thermodynamic parameters obtained from this experiment are: $K_a = 1.5 \times 10^6\ M^{-1}$, $\Delta H_a = 8.4$ kcal/mol, $n = 1.26$.

ALTERNATE PROTOCOL 1

MACROMOLECULE/LIGAND BINDING WITH HIGH AFFINITY

If the affinity between the reactants is too high ($K_a > 10^8\ M^{-1}$), the affinity of binding cannot be reliably measured. To determine the affinity directly, the concentrations of the reactants need to be lowered to levels below the sensitivity limit of the calorimeter. However, in the case of tight binding ligands, the binding enthalpy can be measured very accurately.

Traditionally, several methods have been employed to determine high affinity. All of them rely on changing the experimental conditions (temperature, pH) to reduce the apparent affinity to a measurable value (Doyle et al., 1995; Doyle and Hensley, 1998). The disadvantages are important: (1) appropriate equations (e.g., van't Hoff relationship) have to be used in order to extrapolate (with cumulative errors) to the initially intended experimental conditions; and (2) changing experimental conditions can compromise the stability or the solubility of the reactants.

A method that does not require a change in the experimental conditions is the displacement method (Sigurskjold, 2000). In this method, the ligand solution is placed in the syringe and a solution of the macromolecule pre-bound to a weaker, competitive ligand is placed in the cell. In this way, the apparent affinity of the potent ligand is reduced because it has to displace the weak ligand from the binding site to bind to the macromolecule, therefore, incurring in an energy penalty. Thus, the thermodynamic parameters for the binding of the weak ligand and also the linkage equations for the binding of the potent ligand in the presence of the weak one should be well known.

The same reasoning as in the basic case is made: from the mass conservation equations and the mass action law, it is possible to reach a cubic equation from which the concentration of all species, free and bound, can be obtained and the analysis can be done in an exact manner. The details are explained elsewhere (Sigurskjold, 2000).

However, even if the exact analysis is always the one to be performed, an approximation can be considered when the weak ligand concentration is much higher than the macromolecule concentration, so that the concentration of free weak ligand is assumed to be equal to the concentration of total weak ligand (Zhang and Zhang, 1998). In this approximation, the apparent binding parameters in the displacement binding are related to the individual binding parameters as in Equations 17.8.6 and 17.8.7:

$$K_{a,A}^{app} = \frac{K_{a,A}}{1 + K_{a,B}[B]}$$

Equation 17.8.6

$$\Delta H_{a,A}^{app} = \Delta H_{a,A} - \Delta H_{a,B} \frac{K_{a,B}[B]}{1 + K_{a,B}[B]}$$

Equation 17.8.7

where A and B are the potent and the weak ligands, $K_{a,A}$ and $\Delta H_{a,A}$ are the affinity and enthalpy of binding of the potent ligand, and $K_{a,B}$ and $\Delta H_{a,B}$ are the affinity and enthalpy

of binding of the weak ligand, and the free concentration of ligand B can be approximated by its total concentration.

One of the most important applications of ITC is in the field of structure-based drug design. The goal in any drug design process is to obtain inhibitors with very tight binding affinities to the desired target, while minimizing the binding of the inhibitor to unwanted targets. Another goal is to design inhibitors that are able to adapt to genetic polymorphisms in their target. Genetic polymorphisms can arise as a result of natural population polymorphisms or mutations that cause drug resistance in viral, microbial, and parasitic targets.

Understanding the detailed mechanism and thermodynamic differences in the binding of various inhibitors allows the development of guidelines essential to the design of highly specific drugs that are able to adapt to genetic polymorphisms in their target. The first structure-based drugs were targeted against HIV-1 protease, an aspartic protease essential for the processing of the HIV virus. Measuring the thermodynamic parameters of the binding of HIV-1 protease inhibitors to their target is compounded by the very tight binding of these inhibitors ($K_a > 10^8$ M^{-1}), which makes direct titration impossible. A displacement assay, in which a tight-binding inhibitor competes with a weaker binding inhibitor ($K_a = 10^6 - 10^7$ M^{-1}), allows the measurement of all thermodynamic parameters in a single ITC experiment. This protocol uses HIV-1 protease, a well-studied aspartic protease of 21.6 kDa, and its commercially available inhibitors nelfinavir and acetyl-pepstatin. Unlike acetyl-pepstatin, the affinity of the binding of nelfinavir to HIV-1 protease is beyond the higher limit of measurable range by conventional ITC.

Three titrations should be made: two direct titrations with nelfinavir and acetyl-pepstatin binding to HIV-1 protease, respectively, and one displacement titration of nelfinavir binding to protease prebound to acetyl-pepstatin.

Materials

Acetyl pepstatin (Bachem)
9 mM NaOH
Nelfinavir (Viracept; or any other clinical or experimental inhibitor)
100% DMSO
HIV-1 protease (Todd et al., 1998 and Velazquez-Campoy et al., 2002)
10 mM sodium acetate, pH 5.0/2% (v/v) DMSO

Prepare samples

1. Prepare acetyl-pepstatin stock solution: dissolve the lyophilized acetyl-pepstatin in 9 mM NaOH (this compound is very insoluble in pure water) to a concentration of 8 to 9 mM.

2. Determine the concentration by analytical quantitative nitrogen content determination (Jaenicke, 1974; Pace et al., 1995).

   Acetyl-pepstatin shows no absorbance at 280 nm.

3. Prepare nelfinavir stock solution at >15 mM in 100% DMSO.

   Nelfinavir is not water soluble. The solution in pure DMSO should be very concentrated (15 to 40 mM). When DMSO is diluted in aqueous buffer, the concentration of nelfinavir is closer to 200 to 300 μM.

4. Prepare cell sample for first titration by purifying HIV-1 protease according to Todd et al. (1998) and Velázquez-Campoy et al. (2002) and storing at ∼20°C at a

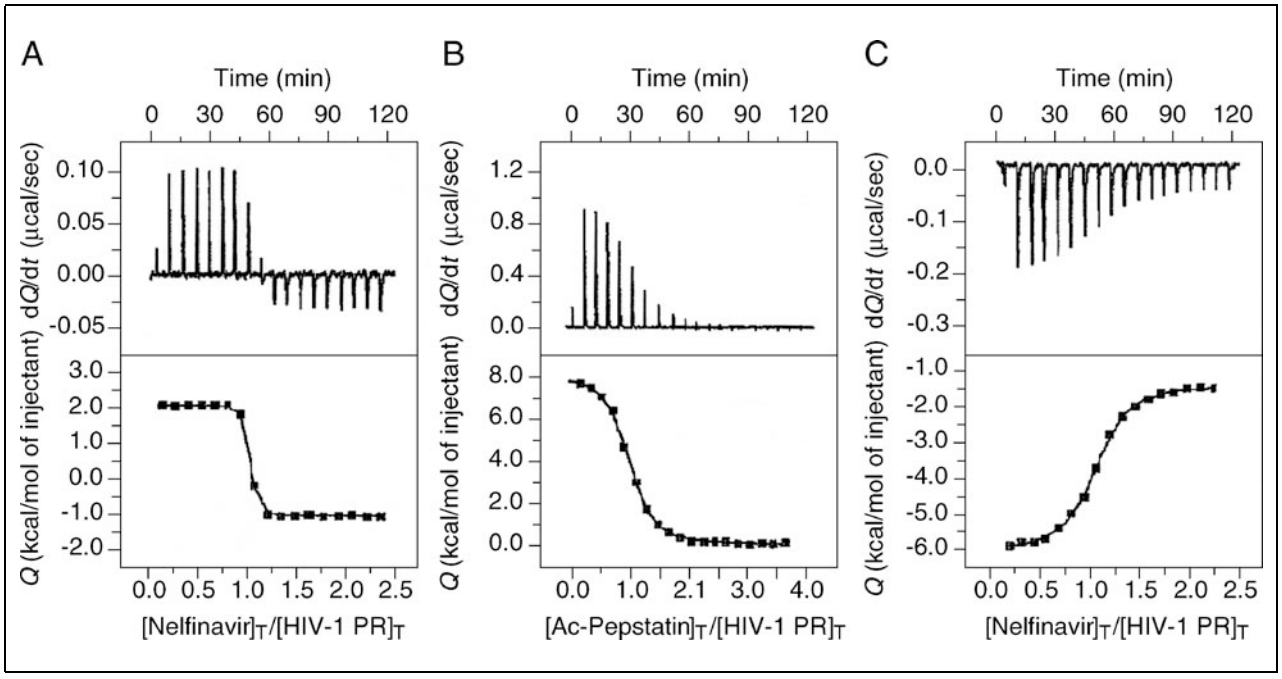

Figure 17.8.5 Set of calorimetric titrations corresponding to the implementation of the displacement protocol for the estimation of very high affinity. The concentrations of reactants are: (**A**) HIV-1 protease 11 μM in cell and nelfinavir 130 μM in syringe; (**B**) HIV-1 protease 19 μM in cell and acetyl-pepstatin 300 μM in syringe; and (**C**) HIV-1 protease 10 μM and acetyl-pepstatin 200 μM in cell, and nelfinavir 130 μM in syringe. The thermodynamic parameters obtained from these experiments are: nelfinavir binding to protease: $\Delta H_a = 3.1$ kcal/mol, $n = 1.02$, and K_a cannot be reliably obtained; acetyl-pepstatin binding to protease: $K_a = 2.3 \times 10^6$ M^{-1}, $\Delta H_a = 8.0$ kcal/mol, $n = 0.98$; nelfinavir binding to protease pre-bound to acetyl-pepstatin $K_a = 2.2 \times 10^9$ M^{-1}, $\Delta H_a = 3.3$ kcal/mol, $n = 0.99$.

concentration of >2.5 mg/ml. Dissolve the protein to a final concentration of 20 μM in 10 mM sodium acetate, pH 5.0/2% DMSO.

5. Determine the exact concentration of the cell sample by measuring its absorbance at 280 nm using an extinction coefficient of 25,500 M^{-1} cm^{-1}.
6. Prepare syringe sample for first titration: 2.2 ml of nelfinavir stock diluted to a concentration of ~300 μM in 10 mM sodium acetate, pH 5.0. If necessary, add DMSO to a final concentration of 2% to ensure that the inhibitor is dissolved in the aqueous buffer.
7. Perform the first titration with HIV-1 protease in the cell and nelfinavir in the syringe.
8. Prepare cell sample for second titration. Repeat step 4.
9. Prepare syringe sample for second titration: 0.5 ml of acetyl-pepstatin diluted to a concentration of ~300 μM in 10 mM sodium acetate, pH 5.0. Add DMSO to a final concentration of 2%.
10. Perform the second titration with HIV-1 protease in the cell and acetyl-pepstatin in the syringe.
11. Prepare cell sample for third titration. Repeat step 4 adding acetyl-pepstatin to the HIV-1 protease in the sample cell solution to a final concentration of 200 μM.
12. Prepare syringe sample for third titration. Repeat step 6.
13. Perform third titration.

Figure 17.8.5 shows the results from an experimental scheme aimed at determining the thermodynamic parameters for the binding of nelfinavir to HIV-1 protease. The thermodynamic

parameters obtained from these experiments are: nelfinavir binding to protease: ΔH_a = 3.1 kcal/mol, n = 1.02; acetyl-pepstatin binding to protease: $K_a = 2.3 \times 10^6\ M^{-1}$, ΔH_a = 8.0 kcal/mol, n = 0.98; nelfinavir binding to protease prebound to acetyl-pepstatin: $K_a = 2.2 \times 10^9\ M^{-1}$, ΔH_a = 3.3 kcal/mol, n = 0.99 (applying the exact equations for the displacement experiment in the data analysis).

MACROMOLECULE/LIGAND BINDING WITH LOW AFFINITY

ALTERNATE PROTOCOL 2

If the affinity between the reactants is too low ($K_a < 10^4\ M^{-1}$), neither the affinity nor the enthalpy of binding can be reliably determined.

One way to determine both is again by using the displacement strategy (Zhang and Zhang, 1998). In this case, the binding parameters for the weak ligand are obtained from the change in the binding parameters of the potent ligand binding to the free macromolecule and its binding to the macromolecule pre-bound to the weak ligand. The same considerations and analysis procedure explained in the case of very high affinity ligands applies here.

Many natural ligands show very low affinity ($K_a < 10^4\ M^{-1}$) for their biological targets. To measure such affinity, the displacement protocol can be implemented again.

In the case of the CMP inhibitors of RNase A, a change in the position of the hydroxyl group from 2′ to 5′ position causes a dramatic decrease in the strength of the interaction. This example illustrates the effect of minor modifications in the ligand on the thermodynamic parameters of binding.

To estimate the binding affinity for 5′-cytidine-monophosphate (5′CMP) binding to RNase A, a similar set of titrations as in the previous section should be done: two direct titrations with 2′CMP and 5′CMP binding to RNase A and one displacement titration of 2′CMP binding to RNase A prebound to 5′CMP.

For materials, see Basic Protocol 2.

Prepare samples

1. Prepare RNase A and inhibitors 2′CMP and 5′CMP stock solutions following instructions in the Basic Protocol 2.
2. Prepare cell sample for first titration: 2.2 ml of RNase A diluted to a concentration of 50 to 100 μM in 15 mM potassium acetate buffer, pH 5.5.
3. Prepare syringe sample for first titration: 0.5 ml of 2′CMP diluted to a concentration of ∼0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5.
4. Prepare cell sample for second titration. Repeat step 2.
5. Prepare syringe sample for second titration: 0.5 ml of 5′CMP diluted to a concentration of ∼0.5 to 1.5 mM in 15 mM potassium acetate buffer, pH 5.5.
6. Prepare cell sample for third titration. Repeat step 2 adding 5′CMP to the solution to a concentration of 0.6 mM.
7. Prepare syringe sample for third titration. Repeat step 3.

Figure 17.8.6 shows the results from an experimental scheme aimed at determining the thermodynamic parameters for the binding of 5′CMP to RNase A. Three titrations are shown: 2′CMP binding to RNase A, 5′CMP binding to RNase A, and 2′CMP binding to RNase A pre-bound to 5′CMP. In the first titration, the binding parameters are obtained as described earlier. It is not possible to obtain a reliable value for either the affinity or the enthalpy of binding from the second titration. On the other hand, applying the exact analysis

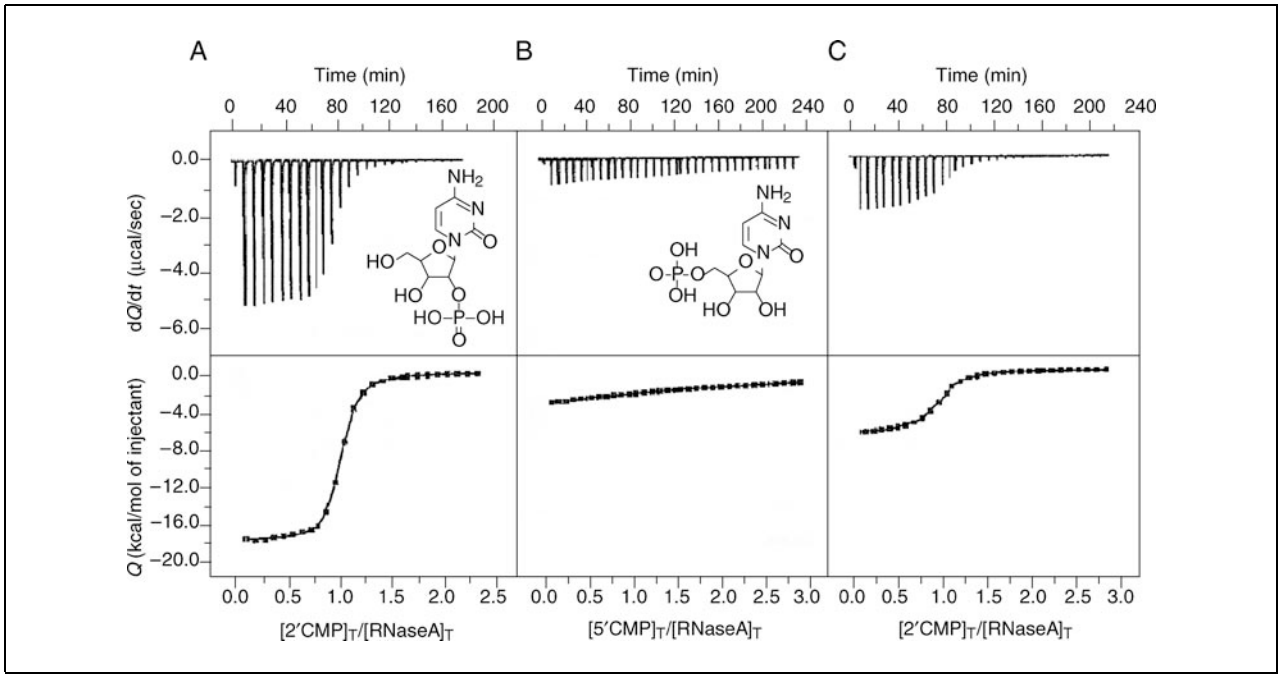

Figure 17.8.6 Set of calorimetric titrations corresponding to the implementation of the displacement protocol for the estimation of very low affinity. The concentrations of reactants are: (**A**) RNase A 76 μM in cell and 2′CMP 1.13 mM in syringe; (**B**) RNase A 76 μM in cell and 5′CMP 1.07 μM in syringe; and (**C**) RNase A 76 μM and 5′CMP 600 μM in cell, and 2′CMP 1.13 mM in syringe. The first titration corresponds to the one shown in Figure 17.8.1. In the second titration neither the binding affinity nor the binding enthalpy can be estimated. In the third titration, the thermodynamic parameters for the binding of 5′CMP obtained applying the exact analysis are: $K_a = 4250\ M^{-1}$, $\Delta H_a = -16.3$ kcal/mol, $n = 0.99$. If using the approximation method, the values obtained for the binding of 5′CMP are: $K_a = 4300\ M^{-1}$, $\Delta H_a = -15.7$ kcal/mol, $n = 0.98$, which are in agreement with the previous ones.

to the third titration yields the following thermodynamic parameters for the binding of 5′CMP: $K_a = 4250\ M^{-1}$, $\Delta H_a = -16.3$ kcal/mol, $n = 0.99$. Using the approximation method, the values obtained for the binding of 5′CMP are: $K_a = 4300\ M^{-1}$, $\Delta H_a = -15.7$ kcal/mol, $n = 0.98$, which are in reasonable agreement with the exact analysis.

ALTERNATE PROTOCOL 3

BINDING OF HOMODIMERIC PROTEINS

When the objective is to determine the thermodynamic parameters for the formation of homodimers, Basic Protocols 1 and 2 are not useful since they require the physical separation between reactants. However, it is still possible to modify the standard procedure towards that goal. In this case, the only reactant is placed in the syringe and the cell is filled with the appropriate buffer solution. The experiment consists of performing a series of injections of the reactant solution into the calorimetric cell. Since the concentration of reactant in the syringe is constant throughout the experiment, the fraction of monomers and dimers within the syringe remains constant. However, the dilution of the reactant in the cell upon injection promotes the dissociation of dimers into monomers. In these circumstances, the area under the peak associated with each injection is simply the heat released or absorbed during the dissociation reaction. Details and examples can be found in the literature (Burrows et al., 1994; Lovatt et al., 1996).

ALTERNATE PROTOCOL 4

BINDING OF UNSTABLE PROTEINS

Sometimes the unique nature of some macromolecules impedes the implementation of Basic Protocol 1 for ITC binding studies. Certain proteins tend to denature or aggregate under the vigorous stirring conditions of the calorimetric cell, which makes conducting standard ITC assays impossible.

However, it is possible to modify the protocol to determine the enthalpy of binding of ligands to such proteins. A solution of the ligand is placed in the calorimetric cell and a solution of the macromolecule is placed in the syringe. Concentrations of reactants should be chosen so that, when injecting, all of the macromolecule binds to the ligand. Therefore, all the heat associated with the injection can be assigned to the binding of all injected macromolecules to the ligand. To obtain the enthalpy of binding, it is necessary to estimate how much heat is coming from binding and how much from unspecific processes (e.g., injection, friction, dilution). Blank experiments, in which buffer solution alone (without ligand) is placed in the cell, are needed to evaluate the non-binding heat. The binding enthalpy is obtained by subtracting such contribution from the observed heat (see Equation 17.8.8):

$$\Delta H_a = \frac{Q_T - Q_{Blank}}{v[S]_0}$$

Equation 17.8.8

where Q_T is the heat associated with the injection of macromolecule into the ligand solution, Q_{Blank} is the heat associated with the injection of macromolecule into the buffer solution, v is the injection volume, and $[S]_0$ is the concentration of reactant in the syringe.

Similar experiments can also be used in Basic Protocol 1 to estimate the heat produced by ligand dilution and to determine if the ligand self-associates or aggregates at high concentration in the syringe. Details and examples can be found in the literature (Nezami et al., 2002, 2003).

MEASURING THE HEAT CAPACITY CHANGE ASSOCIATED WITH BINDING

ALTERNATE PROTOCOL 5

A single ITC assay can provide information regarding the binding constant, the Gibbs free energy of binding, the binding enthalpy, the binding entropy, and the stoichiometry of the binding reaction. In addition to this information, the change in heat capacity upon binding and the change in ionization state upon binding can be obtained by repeating the experiment with varying temperature or buffer conditions.

The heat capacity change, at constant pressure, is simply the temperature derivative of the enthalpy change (see Equation 17.8.9):

$$\Delta C_P = \left(\frac{\partial \Delta H}{\partial T} \right)_P$$

Equation 17.8.9

Therefore, one can determine its value by measuring the enthalpy change of a binding reaction at different temperatures. Plotting ΔH versus temperature would yield ΔC_P as its slope. The heat capacity of binding reflects the burial of polar and non-polar surfaces as a consequence of the binding reaction (Privalov and Makhatadze, 1992; Murphy and Freire, 1992; Gómez et al., 1995).

ΔC_P is usually determined by measuring the enthalpy of binding from 15° to 35°C at 5° intervals under identical buffer and pH conditions. Figure 17.8.7 shows the temperature dependence of the binding enthalpy of amprenavir and TMC-126, a clinical and an experimental inhibitor of HIV-1 protease, respectively (Ohtaka et al., 2002).

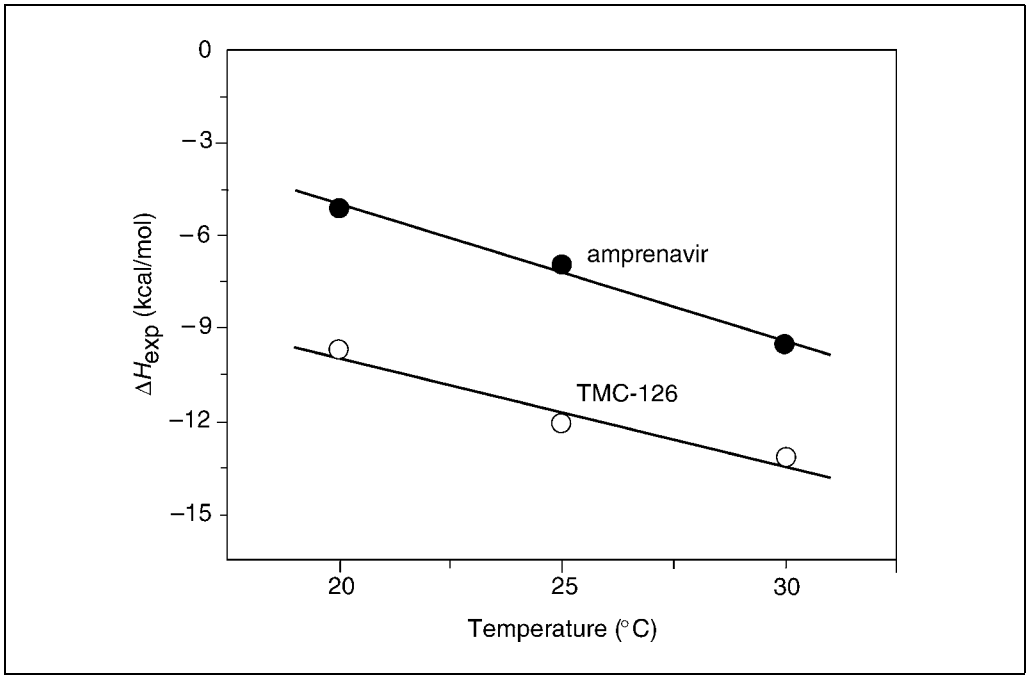

Figure 17.8.7 Temperature dependence of the binding enthalpy for amprenavir and TMC-126 binding to HIV-1 protease (Ohtaka et al., 2002). The experiments were done in 10 mM sodium acetate, pH 5.0/2%DMSO. The slope of the plot is equal to the heat capacity change upon binding and it is related to the burial of accessible surface areas from both the protein and the ligand. The values of the binding heat capacity for amprenavir and TMC-126 are −440 cal/K·mol and −350 cal/K·mol, respectively.

The heat capacity change of binding processes between a protein and a ligand is usually negative and <1 kcal/K·mol in absolute value. However, the binding of two macromolecules can be associated with a higher heat capacity change, which is indicative of a larger burial of solvent-accessible surface area upon binding or the structuring of some regions of the macromolecule.

ALTERNATE PROTOCOL 6

MEASURING PROTONATION/DEPROTONATION PROCESSES COUPLED TO BINDING

The change in the protonation state of certain residues involved in the binding process produces a proton transfer between the complex and the bulk solution. This phenomenon can be studied by detecting the heat effect produced by the protons exchanged between the protein and the buffer as a consequence of the inhibitor binding (Gómez and Freire, 1995; Baker and Murphy, 1996, 1997). In this case, the experimental enthalpy of binding, ΔH_{exp}, is the sum of the intrinsic enthalpy of the binding reaction, $\Delta H_{binding}$, independent of the buffer used and a term proportional to the enthalpy of ionization of the buffer (see Equation 17.8.10):

$$\Delta H_{exp} = \Delta H_{binding} + N_{H^+} \Delta H_{ion}$$

Equation 17.8.10

where the proportionality constant, N_{H^+}, is the number of protons that are exchanged between the complex and the bulk solution, and ΔH_{ion} is the ionization enthalpy of the buffer. Experiments are done under the same pH and temperature, using buffers with different ionization enthalpies. Figure 17.8.8 shows the protonation/deprotonation effect associated with the binding of amprenavir and TMC-126 to HIV-1 protease (Ohtaka et al., 2002). The coupling of protonation/deprotonation processes to the binding of a

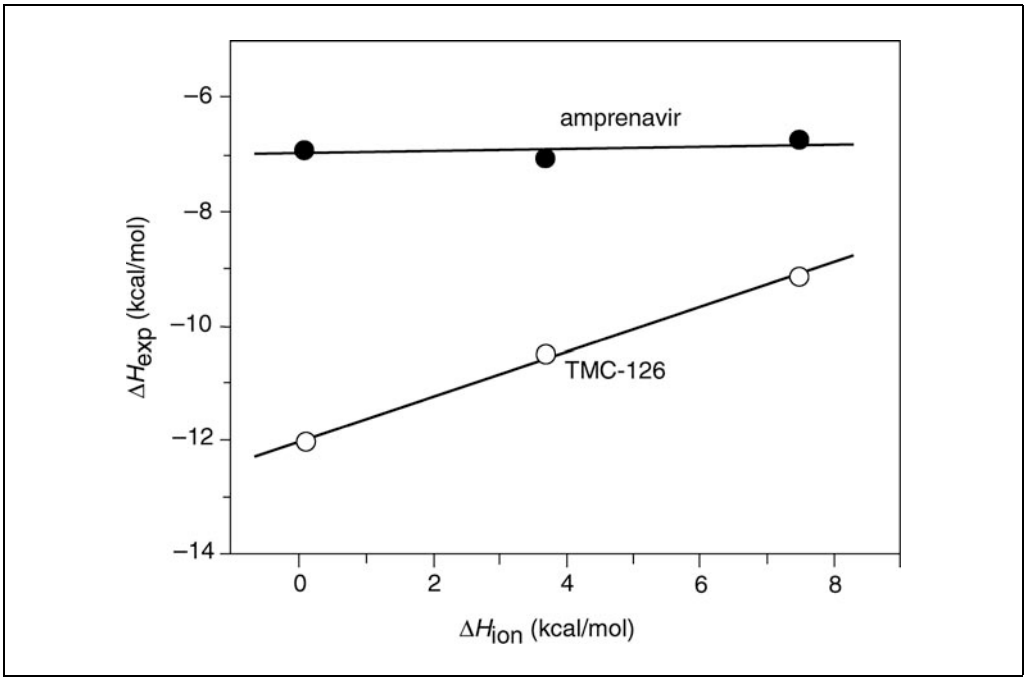

Figure 17.8.8 Dependence of the binding enthalpy for amprenavir and TMC-126 binding to HIV-1 protease on the ionization enthalpy of the buffer used in the experiment (Ohtaka et al., 2002). The experiments were done at pH 5.0 in 2% DMSO and 10 mM buffer concentration with buffers of different ionization enthalpy (acetate 0.12 kcal/mol, MES 3.72 kcal/mol, ACES 7.51 kcal/mol). The number of protons exchanged (slope) for amprenavir and TMC-126 is 0.02 and 0.39, respectively. The buffer-independent binding enthalpy (intercept with *y*-axis) for amprenavir and TMC-126 is −6.9 kcal/mol and −12.0 kcal/mol, respectively. Unlike in the case of amprenavir, where there is no net proton exchange between the ML complex and bulk solution upon binding, for TMC-126 there is a significant proton transfer.

ligand could make the experimental enthalpy measured directly, ΔH_{ion}, very different (even opposite) from the actual binding enthalpy, $\Delta H_{binding}$, if either N_{H^+} or ΔH_{ion} is large enough.

COMMENTARY

Background Information

Isothermal titration calorimetry (ITC) is a technique that directly measures the energetics associated with the binding of two components. ITC is the only biophysical method that directly determines the enthalpy (ΔH) associated with binding and provides a complete thermodynamic profile of the interaction, including binding affinity (K_a), number of binding sites (n), entropy change (ΔS), and Gibbs energy change (ΔG). ITC can be utilized for determining very low (10^3 M^{-1}) to very high binding affinities (10^{12} M^{-1}). Besides providing fundamental information about binding, ITC does not require immobilization of the binding components and takes very little time, unlike other techniques [e.g., surface plasmon resonance (*UNIT 17.6*) and ultra-centrifugation]. Moreover, since heat is universally generated or absorbed during any molecular interaction, ITC has become a more common and general detection method without the need of reporter labels. An ITC instrument has a simple setup consisting of two identical cells, a sample cell that contains a solution of one of the reactants and a reference cell that serves as a temperature reference. The instrument is equipped with a syringe that contains the second binding component and adds aliquots stepwise to the sample cell. In the last two decades, ITC has become an important technique in determining the binding energetics of various biochemical processes (Wiseman et al., 1989; Freire at al., 1990; Doyle, 1997; El Harrous and Parody-Morreale, 1997; Jelesarov and Bosshard, 1999; Leavitt and Freire, 2001; Ward and Holdgate, 2001; Ladbury, 2001), including protein-ligand binding, protein-protein binding, DNA-protein binding, protein-carbohydrate binding,

protein-lipid binding, and antigen-antibody binding. Moreover, with the ability to precisely determine the enthalpic and entropic components of binding, ITC has become an important component of the drug discovery process.

Thermodynamics of binding

Consider a binding reaction at equilibrium where a biological macromolecule M binds another molecule L (ligand; see Equation 17.8.11):

$$M + L \leftrightarrow ML$$

Equation 17.8.11

Assuming that there is a single binding site, the binding affinity or association constant, K_a, which is the inverse of the dissociation constant, K_d, determines the partition of the reactants into free and bound species, is given by (see Equation 17.8.12):

$$K_a = [ML]/[M][L]$$

Equation 17.8.12

The binding affinity is related to the free energy of binding, ΔG (see Equation 17.8.13):

$$\Delta G = -RT \ln K_a$$

Equation 17.8.13

where R is the gas constant and T the absolute temperature. The free energy of binding can be expressed in terms of the enthalpy (ΔH) and entropy (ΔS) of binding (see Equation 17.8.14):

$$\Delta G = \Delta H - T\Delta S$$

Equation 17.8.14

and therefore (Equation 17.8.15):

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S$$

Equation 17.8.15

As it can be seen from Equation 17.8.15, enthalpy (ΔH) and entropy (ΔS) are the two determinants of the binding affinity. The binding enthalpy primarily reflects the strength of interactions between the ligand and the target (non-covalent interactions, e.g., van der Waals, hydrogen bonds, electrostatics) relative to those existing with the solvent. On the other hand, the entropy change mainly reflects two contributions, changes in solvation entropy, ΔS_{solv}, and changes in conformational entropy, ΔS_{conf} (Lee at al., 1994; D'Aquino et al., 1996; D'Aquino et al., 2000). Other energetic contributions, such as those due to the loss of translational degrees of freedom are similar for all bimolecular associations and amount to 8 to 10 cal/K-mol (Murphy et al., 1994; Amzel, 1997, 2000). As the ligand binds to the macromolecule, desolvation occurs, and water molecules are released from the binding site and the ligand, producing a significant increase in degrees of freedom. This desolvation process is favorable to binding as it increases the entropy of the system. At the same time, however, the ligand and certain groups in the protein lose conformational freedom as they bind to each other (relative to their conformational degree of freedom in water). Therefore, this loss in conformational entropy, ΔS_{conf}, is usually unfavorable for the binding process, unless the binding reaction is coupled to the loss in structure of distal regions from the binding site. Since the enthalpy and entropy changes are the manifestation of different types of inter- and intra-molecular interactions, two ligands that exhibit different enthalpic and entropic contributions to the Gibbs energy of binding reflect different binding mechanisms, even if their binding affinities might be the same (Myzska et al., 1997; Parker et al., 1999; Velázquez-Campoy et al., 2000a,b; Velázquez-Campoy et al., 2001). Additional information and more advanced formalism and developments about binding thermodynamics can be found in the literature (Cantor and Schimmel, 1980; Wyman and Gill, 1990; Luque and Freire, 1998; Van Holde et al., 1998).

Applications

ITC has evolved to become a very useful technique in determining the energetics of a wide variety of chemical and biochemical interactions. Furthermore, ITC can be utilized as a standard technique in determining the precise binding affinities of components from nanomolar range to micromolar range and coupling of the binding reaction to protonation/deprotonation processes. The implementation of displacement titration experiments has extended range for affinity determination, as explained below.

Ligand binding energetics

The useful range for an accurate binding affinity measurement by standard ITC is between 10^4 and 10^8 M^{-1}. However, the practical range of binding affinities experimentally accessible can be expanded to 10^3 to 10^{12} M^{-1} by using an appropriate well-characterized secondary ligand (ITC displacement technique). This is particularly useful in the drug design

process, where the ability to accurately measure binding affinities in the sub-nanomolar range is crucial during the lead optimization stage. ITC displacement experiments will be discussed in detail in later sections.

Drug development

ITC has been utilized in the characterization and optimization of lead compounds as viable drug candidates as it provides complete information of the binding affinity of a ligand to a target in terms of its thermodynamic components ΔG, ΔH, ΔS, and ΔC_P. Current strategies for lead identification and optimization depend almost exclusively on binding affinities or Gibbs energy of binding. The Gibbs energy of binding is a result of a delicate balance between the enthalpic and the entropic contributions, and different combinations of ΔS and ΔH can give rise to the same ΔG values and, therefore, elicit the same binding affinity. A higher affinity can be achieved by two means: (1) obtaining a more favorable (negative) enthalpy change, i.e., improving ligand-protein interactions over those with the solvent, and (2) obtaining a more favorable (positive) entropy change, which in turn can be achieved by making a ligand more hydrophobic in order to maximize the solvation entropy, and/or pre-shaping the ligand in order to minimize the loss of conformational entropy. Traditional drug-design methods rely on hydrophobicity and pre-shaping compounds through the use of conformational constraints, therefore, giving rise to entropically optimized compounds (Todd et al., 2000; Velázquez-Campoy et al., 2002; Ohtaka et al., 2002). Such an approach exhibits several pitfalls, the most important ones being the lack of specificity, the susceptibility to mutations in the target, and the impossibility to achieve extremely high affinity. For example, in the case of HIV-1 protease inhibitors, there is not a single reported case of an entropically driven inhibitor with an affinity stronger than 0.3 nM (Todd et al., 2000; Ohtaka et al., 2002). Therefore, ITC can be utilized not only to characterize existing ligands but also to provide a step-wise guide in the design process by permitting a direct evaluation of the energetic effects of specific chemical modifications.

Evaluation of mutations effects

Genetic diversity and mutations associated with drug resistance are the two major causes of failure of drug therapy in various bacterial and viral diseases, e.g., HIV infection. These two phenomena involve changes in the drug binding sites of the target molecules. Since drug molecules developed under the traditional drug-design paradigm, based on the lock-and-key concept, are conformationally constrained and pre-shaped to the geometry of the target molecule, they cannot effectively adapt to changes in binding site geometry due to naturally occurring polymorphisms or drug resistance mutations (Velázquez-Campoy et al., 2002; Ohtaka et al., 2002). As a result, they lose significant binding affinity. Therefore, a major challenge in drug design is the integration of binding site heterogeneity in the design process. An ideal molecule would be one that is able to adapt to the variability in the target while simultaneously displaying high binding affinity and specificity. ITC being the only technique to provide the individual components of the Gibbs free energy of binding (ΔH, ΔS, and ΔC_P), is able to determine subtle changes in the binding process arising from polymorphisms or mutations in the target. Therefore, ITC can be utilized in the design process of an adaptive ligand that will overcome the current deficiency in the drug design process against variable targets (Velázquez-Campoy et al., 2001; Velázquez-Campoy and Freire, 2001; Freire, 2002; Nezami et al., 2003).

Critical Parameters and Toubleshooting

For a titration experiment to be successful and well designed, the following critical points should be considered.

1. ITC measures the global heat effect associated with the binding of two molecules. This includes the actual heat of binding (binding enthalpy) and all other heat effects originated from non-specific events (dilution of reactants, friction of injected liquid, etc.). Therefore, it is important to ensure that these contributions are minimized or considered in the analysis. For example, in the case of the dilution of reactants, the last peaks in the experiment after saturation can be an estimate of the heat effect due to such unspecific phenomena. Also, a titration of the reactant in the syringe into the buffer solution gives an estimation of the dilution effect of the titrant. In the case of friction of the injected liquid, small injection volumes (between 3 and 10 μl) and the recommended rate of injection (0.5 μl/sec) should be employed.

2. Regarding the possibility of aggregation or association of the reactants, the reactant with the lower solubility should be placed in the calorimetric cell.

3. As explained above, the appropriate range of concentrations and the proper ratio of ligand/macromolecule concentrations needs to be employed to guarantee completion of titration, reaching the saturation point in a reasonable number of injections (<30). If saturation is achieved in fewer injections (<5), then, the ligand/macromolecule concentration ratio should be lowered. On the contrary, if saturation is hardly reached, then the ratio should be increased.

4. A perfect match between the buffers has to be achieved to avoid spurious heat effects due to protonation of different species and mixing of different components that could be larger than the actual heat effect associated with the binding reaction. Accordingly, extreme care should be taken when using organic co-solvents (e.g., DMSO), because a mismatch in the concentration of these components between the solutions in the cell and the syringe will cause large heat effects.

5. From a practical point of view, it should always be emphasized that a clean cell and a perfectly straight syringe are decisive to avoid spurious results and to give excellent baselines with high signal-to-noise ratio.

Anticipated Results

Different titration experiments and representative examples are shown in each protocol.

Typical titration experiments in the range of moderate affinity ($K_a \sim 10^6$ M^{-1}) corresponding to exothermic and endothermic binding processes are shown in Figures 17.8.3 and 17.8.4, respectively. The following are several features that will indicate the quality of the experiment:

1. The curvature of the titration curve is appropriate to obtain a reliable estimation of the binding affinity.

2. The saturation point has been achieved and the final molar ratio is ~2.5 to 3.

3. There is a good signal-to-noise ratio. The data in Figure 17.8.3 is better than that of Figure 17.8.4 because the concentrations of reactants used in the titration are ten times higher. A good signal-to-noise ratio will guarantee small errors in the integration stage in data analysis.

4. In terms of thermal power between the peaks before and after the titration, there is a 50-fold decrease (5 μcal/sec compared to 0.1 μcal/sec, in absolute value) and a five-fold decrease (1 μcal/sec compared to 0.2 μcal/sec, in absolute value) in Figure 17.8.3 and 17.8.4, respectively. The standard deviation of the noise is 0.002 μcal/sec and 0.004 μcal/sec in Figure 17.8.3 and 17.8.4, respectively, much lower than the height of the peaks before and after saturation.

Time Considerations

The time required for a complete ITC experiment can be divided into different steps. Preparing sample solutions requires ~30 min. Running the ITC experiment takes ~2 to 3 hr. Cleaning the ITC instrument requires ~20 min. Analyzing the results takes ~30 min.

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