Video Article

Collecting Variable-concentration Isothermal Titration Calorimetry Datasets in Order to Determine Binding Mechanisms

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Abstract

Isothermal titration calorimetry (ITC) is commonly used to determine the thermodynamic parameters associated with the binding of a ligand to a host macromolecule. ITC has some advantages over common spectroscopic approaches for studying host/ligand interactions. For example, the heat released or absorbed when the two components interact is directly measured and does not require any exogenous reporters. Thus the binding enthalpy and the association constant (K_a) are directly obtained from ITC data, and can be used to compute the entropic contribution. Moreover, the shape of the isotherm is dependent on the c -value and the mechanistic model involved. The c -value is defined as $c = n[P]tK_a$, where $[P]t$ is the protein concentration, and n is the number of ligand binding sites within the host. In many cases, multiple binding sites for a given ligand are non-equivalent and ITC allows the characterization of the thermodynamic binding parameters for each individual binding site. This however requires that the correct binding model be used. This choice can be problematic if different models can fit the same experimental data. We have previously shown that this problem can be circumvented by performing experiments at several c -values. The multiple isotherms obtained at different c -values are fit simultaneously to separate models. The correct model is next identified based on the goodness of fit across the entire variable- c dataset. This process is applied here to the aminoglycoside resistance-causing enzyme aminoglycoside N-6'-acetyltransferase-II (AAC(6')-II). Although our methodology is applicable to any system, the necessity of this strategy is better demonstrated with a macromolecule-ligand system showing allostery or cooperativity, and when different binding models provide essentially identical fits to the same data. To our knowledge, there are no such systems commercially available. AAC(6')-II, is a homo-dimer containing two active sites, showing cooperativity between the two subunits. However ITC data obtained at a single c -value can be fit equally well to at least two different models a two-sets-of-sites independent model and a two-site sequential (cooperative) model. Through varying the c -value as explained above, it was established that the correct binding model for AAC(6')-II is a two-site sequential binding model. Herein, we describe the steps that must be taken when performing ITC experiments in order to obtain datasets suitable for variable- c analyses.

Protocol

1. Preparing stock solutions

1. Purify the macromolecule of interest. (In this case, aminoglycoside N-6'-acetyltransferase-II (AAC6'-II), is isolated as reported elsewhere.¹³)
2. Prepare 4 litres of dialysis buffer. (In this case we used 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, MW 238.3 g/mol), containing 2 mM ethylenediaminetetraacetic acid (EDTA, MW 292.2), at pH 7.5.)
3. Dialyze the protein The AAC(6')-II sample (5 mL at 400 μ M) used must be dialyzed in dialysis buffer (3 x 1.3 L). The final dialysis solution is kept to rinse the machine and to dilute samples.
4. Filter the final dialysis solution through a 0.45 μ m cellulose filter, and stored at 4°C. Will be referred to as 'running buffer'
5. Filter protein solution through a 0.2 μ m syringe filter which has been rinsed thoroughly with running buffer. Measure the final concentration of the protein using a standard assay (Bradford, Lowry, etc.) or UV absorbance. Store the protein to maximize long-term stability. (In the case of AAC(6')-II, this means storage at 4°C. AAC(6')-II does not retain activity after freezing and thawing).
6. Prepare 200 μ L of 25 mM stock solution of acetyl coenzyme A (AcCoA, the ligand, MW 809.57) by dissolving 4.0 mg in running buffer. Freeze at -78°C until ready to use.
7. All protein and ligand samples must originate from the same stock solutions, to minimize random sample-to-sample fluctuations in concentration. A single correction factor for the protein concentration across the entire variable- c dataset is adjusted in the analysis.¹⁰

2. Preparing ITC samples

1. Dilute the enzyme solution to a c -value of 64 with a final volume of 2 mL. (In the case of AAC(6')-II, this corresponds to 192 μ M.)
2. Quickly thaw the ligand (AcCoA) stock solution in ice water.
3. Prepare 0.5 mL of a AcCoA solution 10 times more concentrated than the number of binding sites on the protein, in this case 4 mM, by diluting 80 μ L of the AcCoA stock solution in 420 μ L of running buffer. Transfer into a pipette filling tube. Quickly return the AcCoA stock solution to -78°C.
4. Degas the protein and solutions under vacuum for 5 min at a temperature 1°C below the desired running temperature (19 °C).

3. Setting up the syringe¹⁴

1. Insert the injection syringe through syringe holder until the pre-mounted syringe clamp is at the same height as the holder.
2. Feed the second syringe clamp over the injection syringe until it is firmly pressed against the bottom of the syringe holder. Gently tighten the clamp with the provided 0.050" Ball Point Hex Drive.
3. Place syringe holder into the pipette holder.

4. Gently slide the pipette injector into the injection syringe. Make sure the plunger tip is fed directly into the hole of the syringe. Once fully inserted, screw the locking collar of the syringe holder into the pipette injector.

4. Loading the sample cell¹⁴

1. Wash sample cell with a minimum of 50 mL of running buffer, and remove any remaining liquid by using a long-needed 2.5 ml glass syringe.
2. Slowly draw a minimum of 1.8 ml of protein sample solution into the clean and dry long-needed 2.5 ml syringe. Be careful not to introduce any bubbles.
3. Carefully insert needle in sample cell and gently touch the bottom of the cell. Raise tip slightly (~1 mm) and gently inject AAC(6')-li solution into the cell until excess liquid is visible above the top of the sample cell.
4. Slowly raise the needle about 1 cm while ensuring liquid remains in the overflow. Quickly withdraw and inject a small amount of solution (~0.25 ml) to remove any trapped bubbles in the sample cell.
5. Remove all solution overflow. This is achieved by gently sliding the needle along side of overflow into the sample cell. The syringe tip will hit a ledge, this is the desire height for the running solution. Remove all liquid which sits above this line.

5. Loading injection syringe and initiating run¹⁴

1. Attach the plastic tube loading syringe into fill port.
2. Lower plunger tip to the top of the fill port.
3. Place the AcCoA solution in the pipette filling tube at the bottom of the pipette holder. The syringe tip should not touch the bottom of the filling tube.
4. Slowly draw the solution into the syringe until a small amount enters the tube of the loading syringe.
5. Close the fill port by lowering the plunger tip and click on the Close Fill Port button. Purge and refill, by clicking on the Purge->Refill button, 3 times to remove any air bubbles that may have become trapped during loading.
6. Remove the filling tube from the pipette holder and gently wipe the syringe tip.
7. Take pipette assembly and gently lower syringe into the sample cell. Proceed slowly as the syringe can easily bend, so great care is needed. Ensure the syringe is completely inserted by pressing down on base of locking collar.
8. Set the desired running temperature (in this case 20°C), and select a reference power that is slightly greater than the maximum injection heat flow expected (in this case 20µcal/sec).
9. Program the desired injection volumes and delays. (In this case, 28 injections were employed. The first injection had a volume of 2 µL with a 60 s delay. All subsequent injections had volumes of 10 µL with 330 s delays.)

6. Subsequent Runs

1. In all subsequent runs repeat steps 2-5, while decreasing AAC(6')-li and AcCoA concentrations by a factor of 2,4,8,16, and 32.

7. Data Analysis

1. Use a fitting procedure which globally fits all of the isotherms to a single set of binding parameters, as described previously¹⁰.

8. Representative Results

Representative data are shown in Figure 1. The shapes of the isotherms should vary with concentration. Sharper transitions are expected for higher c-values (i.e. higher protein and ligand concentrations) (Figure 2).

In the case of AAC(6')-li, the two-site sequential model gives a better fit than one describing two sets of identical, independent sites with adjustable stoichiometries.

Figure 1. Isotherms produced by titration of AcCoA (3.86 mM) into AAC(6')-li (192 μM). A) Raw ITC trace. B) Integrated values used for determining binding parameters (squares) with a 2-site sequential fit (-).

Figure 2. ITC isotherms for AcCoA titrated into AAC(6')-li at varying concentrations. The experimental data (open circles) were fit to a 2-sets-of-sites independent model (dashed magenta) and a 2-site sequential model (solid blue). The 2-site sequential model clearly gives better overall agreement. The concentrations employed were A) 6 μM, 0.25 mM, B) 12 μM, 0.25 mM, C) 24 μM, 0.5 mM, D) 48 μM, 1.0 mM, E) 96 μM, 1.9 mM, and F) 196 μM, 3.86 mM, for AAC(6')-li and AcCoA respectively.

Discussion

This analytical portion of variable-*c* fitting has been previously described in detail¹⁰. Here we report practical aspects of collecting variable-*c* datasets suitable for this approach. It is essential that all protein and ligand samples are drawn from the same stock solutions. Therefore it is important that sufficient stock solution is prepared initially to complete the entire series of experiments. This ensures the ratio of AAC(6')-li and AcCoA is constant among all experiments, and reduces random fluctuations in concentration on a sample-to-sample basis.

In this case, much care must be taken in the handling of the ligand. AcCoA is chemically unstable in solution so it is essential that the stock solution remains liquid for very short periods of time. Once the appropriate amount is aliquoted from the stock solution, the stock must immediately be refrozen. If this is not done, then the AcCoA may degrade over time and the concentrations will not be correct in later runs. Once aliquoted, AcCoA samples must be used immediately. In the case of proteins that are unstable, similar precautions will be necessary.

This procedure can be easily modified to study other complex systems.¹⁰ The concentrations used must be adapted to the binding constants of the specific system. A wide range of *c* values between about 1 and 1000^{8,10} is required across the dataset.

Disclosures

No conflicts of interest declared.

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