Video Article

Isothermal Titration Calorimetry for Measuring Macromolecule-Ligand Affinity

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Abstract

Isothermal titration calorimetry (ITC) is a useful tool for understanding the complete thermodynamic picture of a binding reaction. In biological sciences, macromolecular interactions are essential in understanding the machinery of the cell. Experimental conditions, such as buffer and temperature, can be tailored to the particular binding system being studied. However, careful planning is needed since certain ligand and macromolecule concentration ranges are necessary to obtain useful data. Concentrations of the macromolecule and ligand need to be accurately determined for reliable results. Care also needs to be taken when preparing the samples as impurities can significantly affect the experiment. When ITC experiments, along with controls, are performed properly, useful binding information, such as the stoichiometry, affinity and enthalpy, are obtained. By running additional experiments under different buffer or temperature conditions, more detailed information can be obtained about the system. A protocol for the basic setup of an ITC experiment is given.

Video Link

The video component of this article can be found at https://www.jove.com/video/2796/

Protocol

Isothermal titration calorimetry (ITC) is a well established technique that can determine all the thermodynamic parameters (affinity, enthalpy and stoichiometry) of a binding interaction in one experiment.[1] ITC works by titrating one reactant into a second reactant under isothermal conditions. The signal measured is the heat released or absorbed upon interaction (binding) of the two reactants. A series of injections are performed and the heat signal will approach zero as the limiting reactant becomes saturated. Fitting of the isotherm gives the thermodynamic parameters. Several reviews are available that describe the instrumentation as well as the math of data collection and analysis. [2,3] While other calorimeters are available (most notably the ITC_{200} with small volumes), here we describe a general protocol for the VP-ITC manufactured by MicroCal (now part of GE Healthcare).

1. Preparing Samples

1. In order to prepare the macromolecule and ligand in buffer, some potential issues need to be addressed. Accurate fits require proper concentrations of the macromolecule, the species that typically goes in the sample cell of the ITC, and ligand, the species in the injection syringe. Because some proteins aggregate at the high concentrations needed for the species in the injection syringe, most often the protein is loaded into the sample cell. The optimal macromolecule concentration is determined from "c", the product of the predicted affinity of the system, which can be estimated using orthogonal methods prior to using ITC, and the total macromolecule concentration, where $c = K_a*[M]$. Optimal values of c range from 10-1000, [1] though it is possible to get accurate data for weak-binding systems under specific experimental conditions with c-values below the lower limit.[4] Thus, the macromolecule concentration must be determined with this range of c values in mind (i.e., for a K_a of 10^6 M^{-1}, macromolecule concentrations of 10 to 1000 μM should be used). Prior knowledge of the binding affinity of the system can help minimize the protein used for ITC through better design of the ITC experiment. The concentration of the ligand should be large enough (7-25 fold more concentrated than the K_d for the weakest ligand binding site) so that saturation occurs within the first third to half of the titration. Accurate fitting of the data also requires saturation of the signal. For systems with higher binding affinity, a lower ligand concentration should be used to avoid saturation too early in the titration, which will give inaccurate fits. Once the heat of dilution control (i.e. titration of ligand into buffer) has been subtracted from the titration, the enthalpy at saturation should approach zero.
2. Because small molecule impurities can give rise to artifactual signals in the ITC measurements, it is best, if possible, that the macromolecule and ligand be exhaustively dialyzed against buffer. Alternatively, column chromatography, desalting spin columns, or buffer exchange centrifugal filters (for example, Centricons) can be used to change the buffer of the macromolecule. If the ligand is a small molecule, it can be prepared using the dialysis buffer after the macromolecule has been dialyzed or by dialyzing against dialysis membranes with cutoffs suitable for small molecules (i.e. 100-500 Da for a Spectra/Por Float-A-Lyzer). Differences in the buffer composition between the ligand and macromolecule solutions can lead to signal artifacts from the heat of the dilution of impurities in the samples. After preparation, check to make sure the pH of the buffer, macromolecule and ligand match (± 0.05 pH units) as artifacts in the enthalpy can arise due to buffer protonation effects.[5] Make sure to prepare enough of each species. For triplicate experiments and a dilution control, the amount of material needed will depend upon the ITC used. But, for most ITC instruments that have approximate 2 ml volume sample cells, at least 6-7 ml of

macromolecule solution will be needed, and can be conveniently prepared in 15 ml falcon tubes. For 300 µL injection syringes, 1-2 ml of solution should be adequate, and can be prepared in either falcon tubes or microcentrifuge tubes.

3. Dust, and other particulates, can cause artifacts in the baseline of the ITC thermogram. It is imperative that they be removed prior to running the experiment. After preparation of sample stock solutions, the samples should be centrifuged in microcentrifuge tubes for five minutes at 8000 to 14,000 RPM to pellet particles in the solution. Remove the supernatant, being careful not to disturb the pellet, and place it in a new falcon/microcentrifuge tube.

   If reductants are needed to maintain reduced cysteines, use low concentrations and fresh stocks of β-mercaptoethanol,TCEP (tris(2-carboxyl)phosphine) or dithiothreitol to minimize any artifacts due to reductant oxidation. Also, the presence of organic solvents in the buffer (common for some small molecule ligands that may need methanol or DMSO in order to be soluble) can cause signal artifacts. If organic solvents are needed for the ligand, then the macromolecule solution must also contain the same concentration to avoid any signal arising from the heat of dilution of the organic solvent. Slight differences in buffer composition between the ligand and macromolecule due to cosolvents, salts or pH are possible during preparation of the samples. It is best to check the dilution of the ligand into the sample buffer or the sample buffer into the macromolecule to ensure that heat signals arising from differences in the buffer content don't cause data arising solely from artifacts.

4. Check the concentrations of the macromolecule and ligand carefully using techniques suitable to your system (such as absorbance measurements, HPLC ,colorimetric assays, BCA assays for proteins, etc) to record their exact concentrations. Differences in the actual concentration and the concentration used to fit the isotherm will cause errors in the stoichiometry, enthalpy and binding affinity determined from the experiment. It is common to degas the samples to avoid signal artifacts due to air bubbles or release of dissolved gases during the titration, particularly at higher temperatures.

2. Setting up the Experiment

1. Make sure the sample cell and injection syringe are cleaned according to the manufacturer's protocol prior to loading the macromolecule and ligand. Rinse the sample cell two or three times with 1.8 ml of distilled water using the Hamilton syringe (use care with the Hamilton syringe as the barrel is easily broken or the needle bent). Next rinse the sample cell several times with 1.8 ml of buffer. Load the sample cell with 1.8 ml of macromolecule solution, being careful to avoid bubble formation.

2. Fill the reference cell with distilled water. For most buffers, distilled water is fine to use as the reference solution. However, for buffers with particularly high ionic strengths or osmolality, it is better to use the buffer as a reference.

3. Remove air bubbles from the reference and sample cells using the Hamilton syringe. Gently move the needle of the syringe up-and-down the sides of the cell knocking any bubbles that may be at the bottom of the cell and attached to the well to the top of the cell. Remove any excess volume from the sample and reference cells.

4. Attach a plastic syringe to the fill port of the injection syringe using tubing. Rinse the injection syringe with distilled water. Follow by a buffer rinse. Make sure the injection syringe is completely evacuated by drawing air through the system. Place the needle of the injection syringe into the ligand solution and draw the ligand solution into the injection syringe until the entire syringe is full. Continue drawing a little excess volume (approximately 50 µl or more) into the port and attached tubing. Immediately close the fill port of the syringe and detach the tubing and plastic syringe. Purge and refill the injection syringe two more times to remove any bubbles from the syringe. Remove the syringe from the ligand solution and wipe the side with a kimwipe to remove any drops, being careful not to touch the syringe tip to the kimwipe as this may remove volume from the syringe. Also, be careful not to knock or jar the syringe as this also can cause loss of volume from the syringe tip. Place the injection syringe into the sample cell.

5. Set up the parameters for running the ITC. For binding systems with strong heat signals, a large number of low volume injections will give more data points for fitting (e.g. 75 injections of 3 µl). For systems that have weak heat signals, a small number of large volume injections are preferable (e.g. 33 injections of 8 µl). Most commonly, fewer injections with higher injection volumes are used. It may take several titrations to optimize the conditions that are best for your system. It is important to note that the binding enthalpy can either be exothermic or endothermic, depending upon the system being studied. Unfortunately, some systems have low heat signals, making the heat of reaction difficult to determine. Problems with such systems can potentially be overcome by increasing the concentration of the macromolecule, changing the temperature of the experiment (depending upon the heat capacity of the binding system), and/or altering the pH or ionic strength of the buffer.

6. Also consider the time spacing between each injection. It is imperative that after each injection of ligand, the system is given time to equilibrate and the heat signal returns to baseline before the next injection occurs. For most systems, three to five minutes should be adequate. The time between injections should be increased for systems where the equilibration doesn't occur within five minutes.

7. Because there will be some mixing between the macromolecule and ligand solutions in the injection syringe once it is inserted into the sample cell, the first injection will give spurious results. It is best to use small volumes (e.g. 2 µl) for the first one or two injections (so they can later be discarded) and keep the subsequent injections at the desired values.

8. Choose the temperature of the experiment (with 25 °C being the most common, though temperatures between 2 and 80 °C can be used). It is best to choose a temperature that matches other experiments (binding, kinetics, etc.) performed on the ligand-macromolecule system. The ITC can be set up to equilibrate at a temperature different from the temperature of the experiment. If the experiment is going to be performed at a temperature more than 10 °C away from room temperature, then it is best to set the equilibration temperature for the instrument within 5 °C of the experimental temperature. This will decrease the time the instrument will take to reach the temperature of the experiment.

   The stirring speed of the syringe also needs to be considered. Stirring is necessary for adequate mixing of the ligand and macromolecule during the titration, but some proteins are destabilized by rapid stirring. For these cases, the stirring speed should be set at a relatively low rate. Once all the experimental parameters have been set up, then the experiment can be started.

9. Once the experiment has finished, the ITC can be cleaned according to the manufacturer's protocol. The solution in the sample cell at the end of the experiment can be kept if additional experiments on the macromolecule-ligand complex mixture are desired.

10. Repeat the titrations at least one or two more times to get reproducible data. Run a control where ligand is titrated into buffer in the sample cell to determine the heat of dilution for the ligand. For some systems where there is cooperative binding, additional information about the binding process can be gained by injecting the macromolecule into the ligand. The different injection orientations can give additional information, which may be helpful for global fitting.[6]

3. Analyzing the Data

1. Fitting of the data can be easily performed using macros in any data fitting program (usually supplied by the manufacturer along with the instrument). Load the first data file. Check the raw thermogram for any signs of air bubbles or other artifacts in the signal. If there are any artifacts (such as spikes in the baseline or peaks when there was no injection at that time point), then note these data points as they should be removed. Next, load the ligand dilution control data, and subtract the ligand dilution data from the binding isotherm. At this time, remove all spurious data points, including the first one or two data points of the titration where dilution artifacts occur (refer to step 7).

2. Select the data fitting model (one binding site, two/multiple binding sites, cooperative binding, etc) to be used to fit the data. The data can be fit with initial guesses of the fitting parameters, stoichiometry (n), enthalpy (ΔH) and binding affinity (K_a). If prior knowledge of the binding affinity, or other parameters, is known from orthogonal experiments, then these values can be entered. This helps avoid the fit becoming trapped in local minima during fitting. Once the data have been fit for the first titration, repeat steps 15 and 16 for the rest of the isotherms. Alternatively, the data can be fit globally using programs such as SEDPHAT.[6] SEDPHAT may be particularly helpful in global fitting of binary complex datasets (i.e. molecules A+B) in combination with ternary complex datasets (molecules A+B+C). For example, in binding of molecule C to an A-B complex, it is not always clear whether there might be any signal due to binding of C to A or of B to A (if A is not fully saturated).

3. To ensure that the ITC data are not artifactual, the binding affinity and stoichiometries obtained from ITC should be compared with an orthogonal method.[7,8] Additionally, the ITC enthalpy value can be compared to the enthalpy from a van't Hoff plot.[9] It can also prove helpful to use different concentrations of the ligand or macromolecule as the absolute value of the heat signal should increase with increasing macromolecule concentration. Artifacts are most likely to arise if the buffers in the cell and syringe are not matched. Another possibility for artifacts arises from impure samples.

4. We recommend that the user start with simple binary complex titrations for which there is previous information available on K_d and stoichiometry. Then, if additional ligands bind, the user can try more complicated ternary complex titrations, varying the ligand concentrations, and perhaps switching the syringe and cell components. A comparison of enthalpies for both paths to ternary complex formation should be additive as enthalpy is a state function.[10] Again, SEDPHAT[6] is likely to be quite useful here.
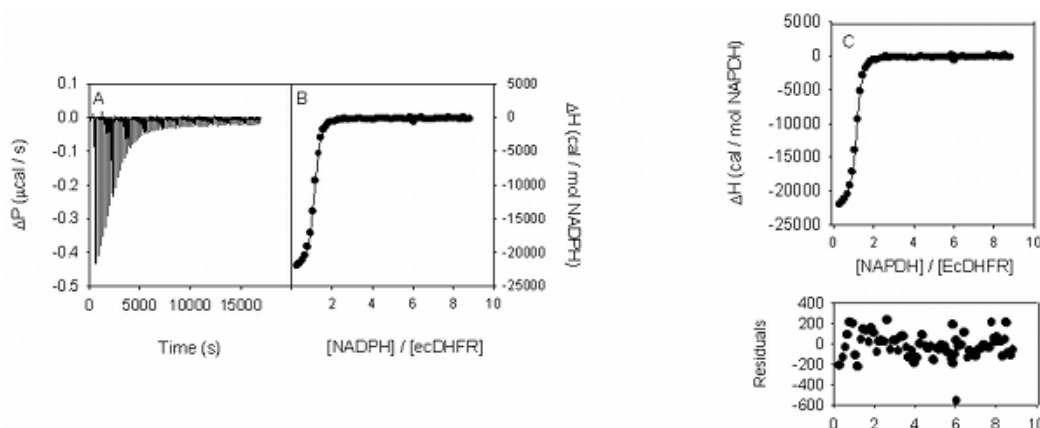
4. Representative Results:

Figure 1. A representative example of a well-behaved titration for the binding of the cofactor NADPH to *E. coli* chromosomal dihydrofolate reductase (ecDHFR). Panel (A) shows the raw thermogram; (B), the binding isotherm from the integrated thermogram fit using the one-site model in the Origin software; and (C) the fit of the isotherm using the one binding site model from SEDPHAT along with fit residuals. From the Origin software, using the one site binding model, n = 1.09 ± 0.02, K_d = 0.194 ± 0.001 µM, ΔH = -22.7 ± 0.4 kcal/mol, $T\Delta S$ = -13.1 ± 0.4 kcal/mol, and ΔG = -9.16 ± 0.01 kcal/mol. Fits of the data using Sedphat afford an n of 0.94 ± 0.01, a K_d of 0.195 ± 0.013 ΔM, a ΔH of -22.5 ± 0.2 kcal/mol, $T\Delta S$ of -13.39 kcal / mol and ΔG of -9.15 kcal/mol.

Discussion

ITC has been used extensively in studying ligand macromolecule interactions,[11] with studies looking at protein-ligand,[12] DNA-ligand[13] and RNA-macromolecule[14] studies. ITC can even be run with solid materials, such as nanoparticles, that form uniform suspensions.[15] Further, ternary systems, where one ligand is already bound to the macromolecule and a second ligand is titrated, can be used to determine the thermodynamics of, for example, binding of substrate to an enzyme-cofactor system.[7] Studies can also be performed for molecules with very high binding affinities that would normally exceed the detection limit of the ITC by performing competition binding assays with weaker binding ligands.[16] Information on weakly binding ligands can also be obtained by competition assays.[17] The role of water in binding can be explored by ITC,[18] along with the dependence of the enthalpy on solvent reorganization.[19] Recently, the thermodynamics of conformational change of DNAK variants were measured upon binding of ADP and ATP.[20] Protein-protein association can be studied by ITC, yielding information on hetero complexes[21] as well as on homo-association.[22] Temperature effects on the binding enthalpy will give the heat capacity of the binding event.[2] The number of protons absorbed or released upon binding can also be determined from experiments performed in buffers with different enthalpies of ionization.[5] If additional ITC studies are done at different pH values, then the pKa of the group involved can potentially be determined.[23]

To summarize, accurate measurements of the concentration of the macromolecule and ligand are imperative for a good ITC experiment. Sample concentrations also need to be within a proper range to get reliable data. Care should be taken with the buffer as small molecule impurities and pH mismatches will cause artifacts in the thermogram.

Disclosures

No conflicts of interest declared.

Acknowledgements

References

1. Wiseman, T., Williston, S., Brandts, J.F., & Lin, L.N. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Anal. Biochem*. **179**, 131-137 (1989).
2. Jelesarov, I. & Bosshard, H.R. Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition. *J. Mol. Recognit*. **12**, 3-18 (1999).
3. Velazquez-Campoy, A., Leavitt, S.A., & Freire, E. Characterization of protein-protein interactions by isothermal titration calorimetry. *Methods Mol. Biol*. **261**, 35-54 (2004).
4. Turnbull, W.B. & Daranas, A.H. On the value of c: can low affinity systems be studied by isothermal titration calorimetry? *J. Am. Chem. Soc*. **125**, 14859-14866 (2003).
5. Fukada, H. & Takahashi, K. Enthalpy and heat capacity changes for the proton dissociation of various buffer components in 0.1 M potassium chloride. *Proteins*. **33**, 159-166 (1998).
6. Houtman, J.C., *et al*. Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: application to adaptor protein complexes in cell signaling. *Protein Sci*. **16**, 30-42 (2007).
7. Bradrick, T.D., Beechem, J.M., & Howell, E.E. Unusual binding stoichiometries and cooperativity are observed during binary and ternary complex formation in the single active pore of R67 dihydrofolate reductase, a D2 symmetric protein. *Biochemistry*. **35**, 11414-11424 (1996).
8. Shenoy, S.R., *et al*. Multisite and multivalent binding between cyanovirin-N and branched oligomannosides: calorimetric and NMR characterization. *Chem. Biol*. **9**, 1109-1118 (2002).
9. Chopra, S., Lynch, R., Kim, S.H., Jackson, M. & Howell, E.E. Effects of temperature and viscosity on R67 dihydrofolate reductase catalysis. *Biochemistry*. **45**, 6596-6605 (2006).
10. Norris, A.L. & Serpersu, E. Interactions of Coenzyme A with the Aminoglycoside Acetyltransferase (3)-IIIb and Thermodynamics of a Ternary System. *Biochemistry*. **49**, 4036-4042 (2010).
11. Falconer, R.J., Penkova, A., Jelesarov, I., & Collins, B.M. Survey of the year 2008: applications of isothermal titration calorimetry. *J. Mol. Recognit*. **23**, 395-413 (2010).
12. Ababou, A. & Ladbury, J.E. Survey of the year 2005: literature on applications of isothermal titration calorimetry. *J. Mol. Recognit*. **20**, 4-14 (2007).
13. Buurma, N.J. & Haq, I. Advances in the analysis of isothermal titration calorimetry data for ligand-DNA interactions. *Methods*. **42**, 162-172 (2007).
14. Salim, N.N. & Feig, A.L. Isothermal titration calorimetry of RNA. *Methods*. **47**, 198-205 (2009).
15. De, M., You, C.C., Srivastava, S., & Rotello, V.M. Biomimetic interactions of proteins with functionalized nanoparticles: a thermodynamic study. *J. Am. Chem. Soc*. **129**, 10747-10753 (2007).
16. Velazquez-Campoy, A. & Freire, E. Isothermal titration calorimetry to determine association constants for high-affinity ligands. *Nat. Protoc*. **1**, 186-191 (2006).
17. Velazquez Campoy, A. & Freire, E. ITC in the post-genomic era...? Priceless. *Biophys. Chem*. **115**, 115-124 (2005).
18. Harries, D., Rau, D.C., & Parsegian, V.A. Solutes probe hydration in specific association of cyclodextrin and adamantane. *J. Am. Chem. Soc*. **127**, 2184-2190 (2005).
19. Chervenak, M.C. & Toone, E.J. A direct measure of the contribution of solvent reorganization to the enthalpy of binding. *J. Am. Chem. Soc*. **116**, 10533-10539 (1994).
20. Taneva, S.G., Moro, F., Velazquez-Campoy, A., & Muga, A. Energetics of nucleotide-induced DnaK conformational states. *Biochemistry*. **49**, 1338-1345 (2010).
21. Jelesarov, I. & Bosshard, H.R. Thermodynamics of ferredoxin binding to ferredoxin:NADP+ reductase and the role of water at the complex interface. *Biochemistry*. **33**, 13321-13328 (1994).
22. Burrows, S.D., *et al*. Determination of the monomer-dimer equilibrium of interleukin-8 reveals it is a monomer at physiological concentration. *Biochemistry*. **33**, 12741-12745 (1994).
23. Baker, B.M. & Murphy, K.P. Evaluation of linked protonation effects in protein binding reactions using isothermal titration calorimetry. *Biophys. J*. **71**, 2049-2055 (1996).