Application of Isothermal Titration Calorimetry in the Biological Sciences: Things Are Heating Up!

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During the last 15 years isothermal titration calorimetry (ITC) has come of age. There are now in excess of 2000 instruments sited in laboratories in more than 40 countries around the world. Research scientists in such diverse fields as biophysics, cell biology, pharmaceutical screening, and food research routinely investigate their systems of interest using ITC. Why is it that this methodology has sparked such enthusiasm and interest, and what use is the data obtained?

The dramatic advances in the field of structural biology in the last couple of decades fed a desire of biochemists to define molecular function and mechanism in ever increasing detail. Describing a biochemical process however, cannot be served by structure alone. A full understanding is only obtained with a quantification of the change of state of the system. In an equilibrium process, such as a biomolecular interaction, thermodynamic measurement provides quantification of the change in energy on going from the free to the bound state.

The ITC instrument (for reviews, see References 1–5) uses the extremely accurate measurement of heat as a probe for an interaction as it occurs. Knowing the concentrations of the interacting moieties allows the calculation of the observed change in molar enthalpy of the interaction, ΔH_{obs}. The term observed (denoted by the subscript obs) signifies that the quantity is not solely from the isolated events associated with forming a biomolecular interface (i.e., direct noncovalent bonds between the atoms of the interacting moieties), but also includes heat derived from perturbation of the solvent around the binding site, potential conformational changes occurring elsewhere in the interacting biomolecules (6), and direct formation of noncovalent bonds between other solutes such as ions or apolar compounds that may be incorporated as an ingredient of the bulk solvent. Since every biomolecular interaction has either an uptake or release of heat associated with it, the ITC is a universal detector of the occurrence of binding (at an appropriate temperature). The direct determination of the ΔH_{obs} negates the indirect calculation of this parameter using a van't Hoff-based method, which can be problematic over extended temperature ranges due to the influence of the change in heat capacity (i.e., the ΔH changes with temperature; see Equation 3). Furthermore, since the two components of an interaction can be titrated, the measured heat gives a direct readout of the extent of interaction at any given concentration regime (see Figure 1 and Reference 7 for experimental tutorial). As a result, the concentrations of free and bound molecules and hence the observed binding or dissociation constant, ($K_{B,obs}$ or $K_{D,obs}$, respectively; $K_B = 1/K_D$) can be determined. Armed with the ΔH_{obs} and the $K_{B,obs}$, a full thermodynamic description of the interaction can be elucidated at a given experimental temperature (T) based on the following relationships:

$$\Delta G_{obs} = -RT\ln K_{B,obs} \qquad \text{[Eq. 1]}$$

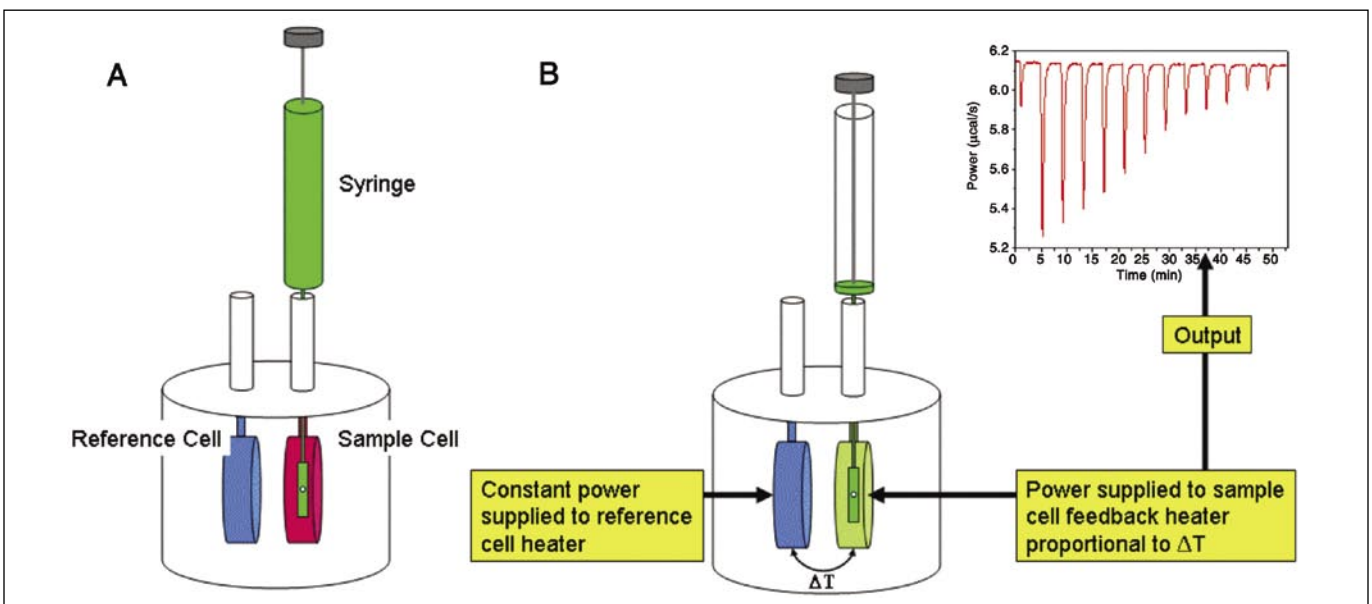

Figure 1. Schematic representations of isothermal titration calorimetry (ITC) instruments. (A) An ITC instrument prior to performing a titration. The sample cell and the reference cell as kept at the same temperature, which is typically 5°–10°C above the temperature maintained outside the jacket in which the cells are housed. The reference cell is always kept at the experimental temperature. One of the components of the interaction is placed in the syringe and the other in the cell. (B) A ITC instrument performing a titration. When an injection is made, the change in heat associated with binding (endothermic or exothermic) results in a change in temperature in the sample cell. A change in power (heat/s) is required to return the cells to identical temperatures (T) (i.e., $\Delta T = 0$). This change in power is recorded as a series of injections is made. In the raw data presented in the inset, each injection is accompanied by an interaction where heat is given out (exothermic). As the course of injections is completed, the binding sites on the sample in the cell are gradually saturated, and the exothermic effect becomes reduced. For details, see References 1–5 and 7.

Table 1. ITC Measurement of the Interaction of the Src SH2 Domain With a Series of Peptides

Peptide Sequence	$K_B \times 10^6$ M^{-1}	ΔG (kJ/mol)	ΔH (kJ/mol)	TΔS (kJ/mol)
Ile	10.80	-40.1	-38.7	1.4
Val	6.24	-38.8	-28.6	10.2
Glu	4.87	-38.1	-32.7	5.4
Trp	3.18	-37.1	-32.2	4.9
Asp	2.64	-36.6	-27.5	9.1

Data taken from Reference 11. All peptides have the sequence Ac-GluProGln(pTyr)GluGluXxxProIleTyrLeu-NH_2, where Xxx is the residue denoted in the first column of the Table. ITC, isothermal titration calorimetry; K_B, observed binding constant; ΔG, change in Gibbs free energy; ΔH, change in observed enthalpy; T, experimental temperature; ΔS, change in entropy.

from which the change in Gibbs free energy (ΔG_{obs}) is obtained (where R is the gas constant), and

$$\Delta S_{obs} = (\Delta H_{obs} - \Delta G_{obs})/T \quad \text{[Eq. 2]}$$

from which the change in entropy (ΔS_{obs}) can be ascertained. A further thermodynamic characterization of an interaction, the change in constant pressure heat capacity (ΔCp) can be determined from measurement of the ΔH over a range of temperatures since

$$\Delta Cp = d\Delta H_{obs}/dT \quad \text{[Eq. 3]}$$

Thus ITC has become the instrument of choice for thermodynamic analysis of biomolecular interactions, since it can provide the ΔH, ΔG, and the ΔS in one experiment. Furthermore, the experiment is rapid, typically taking less than an hour, there is no need for chemical modification or immobilization of any of the components, there is no size limitation on the molecules to be investigated, and the experiment can be conducted in turbid or colored solutions or in the presence of suspensions.

Why Use ITC?

The complete thermodynamic characterization of a bimolecular interaction can be obtained relatively easily using the ITC in one experiment. However, the assimilation of these data is not so straightforward. The underlying events contributing to a biological interaction are many fold and interlinked in a complicated manner. The observed thermodynamic parameters associated with forming a series of noncovalent bonds between the biomolecules in forming a complex cannot be divorced from those derived from breaking bonds as the solvent rearranges to accommodate the complex. The latter effect cannot currently be determined empirically. Therefore, it is reasonable to question how data derived from ITC can add to our understanding of biology. The answer to this question lies in the ability to separate out various contributions to binding using a combination of ITC experiments. The following serves exemplify this approach.

pH and Electrostatic Effects on Complex Formation

A single ITC experiment does not provide thermodynamic data for an isolated bond (or series of bonds). However, there are methods to deconvolve (or parse) various contributions occurring on formation of a complex such that a detailed picture of the energetics that drive the interaction can be determined (8–11). For example, the thermodynamic parameters associated with a protonation/deprotonation event(s) on forming a biomolecular complex can be determined by performing titrations over a range of pH. The dependence of the $K_{B,obs}$ on the pH can reveal what protonation events might be occurring on binding and their respective pK_a values (10,12). Furthermore, the relationship between the ΔH_{obs} and the change in enthalpies for the experiment performed in defined buffer solutions (with differing heats of ionization) can indicate the exact number of protons in exchange (10). A similar understanding of the electrostatic contribution to the formation of a complex can be determined by performing the ITC experiment over a range of salt concentrations. The uptake, or release of ions, can be observed in the dependence of the $K_{B,obs}$ on the concentration of salt (see Figure 2 and Reference 13).

The Correlation of Thermodynamics with Biomolecular Structure

Based on an empirically determined correlation of the ΔCp term with the change in surface area buried on forming a bimolecular interface, thermodynamic parameters can be directly related to high resolution structure, and prediction of one from the other is possible (14,15). ITC experiments performed at different temperatures provide an accurate direct determination of the ΔCp term (see Equation 3). It should be pointed out that this correlation should come with a warning that it can be misleading in cases, for example, where water molecules appear in the interface (13,16,17) or where significant conformational changes in one or both molecules accompany the binding (18).

Another important advantage of the full thermodynamic characterization provided by ITC is the additional level of information by which to distinguish binding events. In some instances,

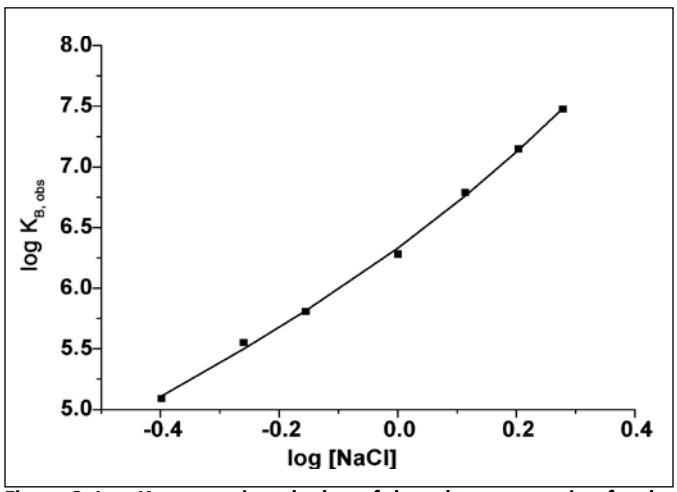

Figure 2. Log $K_{B, obs}$ against the log of the salt concentration for the interaction of the archaeal *Pyrococcus woesei* TATA binding protein (*Pw*TBP) with DNA. The dependence of the equilibrium binding constant, $K_{B,obs}$, on the concentration of a monovalent salt, [MX], can be determined using the relationship:

$\log K_{B,obs} = \log K_{ref} - A\log[MX] + 0.16B[MX]$

where A is the net number of ions released (a negative number) or taken up (a positive number) on forming the complex, B is the total number of water molecules released (a positive number) on forming the complex, and K_{ref} is a hypothetical binding constant at 1 M salt, in the absence of any contribution from release or uptake of water (i.e., B = 0). Binding studies on the *Pw*TBP DNA interaction, performed at high salt concentrations using isothermal titration calorimetry (ITC), reveal that the interaction occurs with a net uptake of two ions and the release of approximately 40 water molecules (for details, see Reference 13).

the K_B of a series of interactions with one biomolecule are similar or indistinguishable, however, the determination of the ΔH and ΔS terms allows a further level of discrimination. Although this may not provide a rigorous definition of the changes in noncovalent interactions on the atomic level, it can nonetheless be used as a guideline in understanding binding. For example, the interaction of a series of ligands with the SH2 domain from the protein Src show very similar affinities (between 2.64–10.8 M^{-1}), however the enthalpic and entropic changes that occur provide a clear level of discrimination between the interactions (see Table 1 and Reference 19). These data reveal that the tightest interaction has a significantly lower favorable (+ve) TΔS term and a more favorable (-ve) ΔH term. Whereas substituting the Ile residue for a Val dramatically improves the TΔS value, but the compensating ΔH is significantly less favorable. Based on the high resolution structural detail, it is possible to speculate that the placing of the smaller Val side chain in a pocket that accommodates the Ile results in fewer noncovalent bonds (less favorable ΔH, but allows the Val to occupy more configurational states; favorable ΔS). The subtle difference between these two peptides has a limited effect on the affinity but clearly has a dramatic effect on the way in which binding occurs, which is revealed by the ΔH and TΔS terms. Thus, although, it may not be possible to provide a full thermodynamic-structure correlation from an ITC experiment, it is possible to draw sensible conclusions from data by comparing systems where subtle changes have been imposed.

The Future of ITC

Technical advances in ITC instrumentation are likely to increase the viability of the technique in more research laboratories. Recent developments have largely centered on attempting to improve the throughput of samples such that ITC can be, at least partially, integrated into screening or drug development scenarios. Instrumentation that includes automated sample handling capability is currently available, and instruments with silicon chip-based heat sensors capable of analysis of small samples in a 96-well plate format are in development. The heat of interaction provides a signal that a desired molecule or "hit" from a screen has bound.

The real strength of ITC in the development of pharmaceuticals is to add a further level of information to the decision-making process. For example, in drug development, it commonly occurs that decisions have to be made as to which compounds to pursue as leads from a large number of "hit" compounds derived from initial screens of libraries. In a case where two or more compounds have similar affinities, it has been suggested that the compounds with the most favorable ΔH terms are likely to make the best lead compounds for further chemical modification. This is because the ΔH term corresponds to the energy associated with the net change in noncovalent bonds. A more favorable ΔH term suggests a better complementarity of bonds in the interface. Since improving the bonding in the biomolecule-drug interface is a major challenge, the initial enthalpy data is a useful tool to discriminate between compounds.

Upping the ante in the race to develop new drug compounds requires as much help in deciding which molecules to pursue as possible. As a result, the addition of thermodynamic data in the decision making process will become widely adopted. It is unlikely that ITC will in the foreseeable future be able to make a valuable contribution to the identification of hits in high-throughput screens due to instrumental limitations and possible requirements of materials. However, the integration of thermodynamic data at the decision making point in secondary screening and in optimization of rational design will be fundamental applications.

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