Isothermal Titration Calorimetry: Principles and Applications

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Abstract:

Molecular recognition is a fundamental phenomenon underpinning almost all aspects of biological processes. Associated energy changes result in release or absorption of heat that can be measured, providing valuable information about the interaction specific features. Isothermal titration calorimetry (ITC) is a label-free binding assay which measures the affinity, stoichiometry, and thermodynamics of molecular interactions from the reaction heat. It is considered the gold-standard technique owing to its unique capacity to provide a complete thermodynamic and even kinetic profile of the interaction in a single experiment, thus giving a detailed understanding of driving forces and underlying processes. Moreover, ITC can be used to characterize complex systems hardly amenable using other techniques and also enzyme catalyzed reactions. Consequently, ITC is being used to study a wide range of interactions mediated by proteins, nucleic acids, lipids, oligosaccharides and other natural or synthetic molecules, and has become a key component of drug discovery platforms.

Key words:

Isothermal titration calorimetry; molecular recognition; binding thermodynamics; binding kinetics; thermodynamic signature; drug design; enzyme kinetics; biomolecular interactions.

Key Concepts:

Molecular recognition *via* non-covalent interactions is of fundamental importance to most processes occurring within living organisms.

ITC provides a general, quantitative approach to analyze the thermodynamics and kinetics of such recognition events, and to characterize enzyme mediated reactions.

Parsing of the free energy of binding (ΔG) in its two components (ΔH and ΔS) provides new insights into the molecular nature of molecular interactions being studied.

The thermodynamic signature provided by ΔH and ΔS is fundamental to not only understand naturally occurring binding interactions but is particularly useful in drug discovery studies.

The kinetics of binding events is also central to understand not only physiological processes but also drug efficacy since equilibrium states often may not be established *in vivo*

Understanding the kinetic behavior of enzymes is important to understand biochemical pathways, and is also a yielding field for drug discovery and development, and biotechnology.

Introduction:

The unique importance of studying molecular interactions arises from the fact that nearly every physiological or pathological event, as well as therapeutic intervention, is triggered by or depends critically on non-covalent binding of two or more molecules in a stoichiometric manner. Eventually, complexes may react to form product/s when the receptor is an enzyme and the ligand a substrate. Over past few decades, an outburst of high resolution structures of biomolecular complexes have allowed a better understanding of *how* molecules interact but no *why* they do so, which implies achieving a quantitative description of the energetic forces and mechanisms governing complex formation or substrate transformation. Isothermal titration calorimetry (ITC) can provide the thermodynamic and kinetic data required for such understanding as it directly measures, as a function of time, the heat exchange associated to a chemical reaction triggered by the mixing of two or more components at constant pressure and temperature. As absorption or release of heat accompanies almost every chemical reaction, ITC is a quantitative analytical tool of general application. In addition, there is no need to chemically modify or immobilize the reactants or use extrinsic probes, and it is unaffected by the spectroscopic properties of the samples. Therefore, ITC can be applied to the study of complex systems hardly amenable by other techniques, including the use of physiological reagents and conditions. Thermodynamic information can in turn be used to interpret the data at molecular level, together with information from other techniques (e.g. X-Ray, NMR, MDS, statistical thermodynamics, etc.). All these issues are briefly reviewed in this article. Examples from literature of the different methodologies and applications for thermodynamic and kinetic studies are gathered together, with the aim of illustrating the current state-of-the-art of ITC and guiding unfamiliar readers to further explore specific subjects. Methodological aspects key to optimal design of ITC experiments, reliable data analysis or critical interpretation of results are specifically discussed and, lastly, practical tips are provided. **See also:** DOI: 10.1002/9780470015902.a0003010.pub3; DOI: 10.1002/9780470015902.a0027015; DOI: 10.1002/9780470015902.a0001341; DOI: 10.1038/npg.els.0001271; DOI: 10.1002/9780470015902.a0000716.pub2 for related readings.

ITC Operational Principle and Instrumentation

<insert> Modern commercial ITCs operate on the dynamic power compensation principle. They are composed of two identical (reference and sample) cells surrounded by an adiabatic jacket, and a syringe for titrant addition which also works as stirring device (**Figure 1a**). The cells are connected by sensitive thermocouple circuits that detect the difference of temperature between them, and between cells and jacket.

Heaters located in both cells are activated when required to maintain identical temperatures among all components. The feedback system continuously applies a fixed power to the reference cell which determines the baseline level, and the measured signal ($\mu\text{cal/s}$) is the compensating feedback power applied to the sample cell to maintain constant (close to zero) the temperature difference with the reference cell (**Figure 1b, top**). When a reaction is started by computer-controlled injection of the reactant in the syringe into the sample cell loaded with the other reactant, the reaction heat causes a difference of temperature between the cells, and the feedback system either lowers (exothermic reactions) or raises (endothermic reactions) the thermal power to compensate the temperature unbalance. Thermal equilibrium is restored when the reaction ends and the initial baseline is recovered. Protocols applied in ITC studies differ only in the way the experiment is planned and not in the way the instrument is used (Velázquez-Campoy *et al.*, 2004). References describing most common protocols are gathered in **Table 1**.

Improvements in modern ITCs have been in the direction of developing increasingly sensitive (0.01 - 0.02 μcal detection limit) easy-to-use instruments, which allow precise titrations using small amounts of samples (0.2 - 1.5 ml cell volume) in a wide range of temperatures (2 to 80 °C). Automated versions of the standard instruments are also available for high/medium throughput experiments. Interestingly, a custom-built, non-commercial nanocalorimeter in a 96-detector array format has been developed and validated for screening applications (Recht *et al.*, 2008; Recht *et al.*, 2016). Compared with conventional calorimeters, its lower volume (500 nl) and measure time (5-10 min) are partially offset by a lower sensitivity that narrows the range of equilibrium and rate constant experimentally accessible. **See also:** DOI: 10.1002/9780470015902.a0003010.pub3; DOI: 10.1002/9780470015902.a0027015.

Thermodynamic Characterization of Molecular Interactions

Principles

One of the oldest field of ITC application is the thermodynamic characterization of binding events. The strength of the interaction for a reversible association equilibrium between two molecules, M and L, that react to form the complex ML is described by the equilibrium association constant, K_a ,

$$K_a = \frac{[ML]}{[M][L]} = \frac{1}{K_d} \quad [1]$$

where [ML] is the concentration of complex, and [M] and [L] the concentrations of unbound reactants, and K_d de dissociation constant. Eqn. [2] relates K_a with the Gibbs free energy of binding (ΔG), that can be expressed in terms of the enthalpic ΔH and entropic ΔS contributions to the binding affinity (R is the universal gas constant and T the absolute temperature in Kelvin):

$$-RT \ln K_a = \Delta G = \Delta H - T\Delta S \quad [2]$$

In biological terms, L could be a drug, cofactor, inhibitor, metal ion, polypeptide, oligonucleotide, protein, oligonucleotide, carbohydrate or any other molecule that non-covalently interact with a specific site of a second molecule (usually, but not necessarily a protein, nucleic acid, lipid, polysaccharide or nanoparticle). **See also:** DOI: 10.1002/9780470015902.a0003010.pub3; DOI: 10.1002/9780470015902.a0001341.pub3.

Titration protocols

Typically, titration experiments are carried out by stepwise addition of the reagent in the syringe (L) into the reaction cell containing the other reactant (M). Each injection triggers the binding reaction and the heat flow is proportional to the thermal power. The area under each peak corresponds to the heat measured at the injection, Q_i , given by Eqn. [3],

$$Q_i = V \cdot \Delta H \cdot \Delta[ML]_{i+1} = V \cdot \Delta H \cdot ([ML]_{i+1} - [ML]_i) \quad [3]$$

where V is the cell volume and $\Delta[ML]_{i+1}$ the increment in complex concentration between consecutive injections. As full conversion of M into ML is approached, the size of the peaks decreases until only the amount of heat associated with other effects (ligand dilution, liquid friction, etc.) is observed, yielding the characteristic profile of titration experiments. Blank contribution should be measured by titrating L into buffer in the sample cell, or estimated from the late injections when full complex formation takes place before the end of titration. Some fitting programs can subtract a constant value to detract blank contribution although such option may reduce fitting parameter precision (Tellinghuissen, 2016). It is advisable to verify that the dilution heat of M is negligible by recording the heat of buffer injection into the cell loaded with M, and otherwise subtract its contribution from the experimental values. The instrument software integrates the area under each injection peak, subtracts the heat of blanks, if required, and produces the isotherm of binding shown in **Figure 1b (bottom)**, where the heat evolved in each injection, normalized per mole of L injected, is plotted as a function of the molar ratio $[L]_T/[M]_T$ (quotient between the total concentration of L and M in the cell). Using non-linear squares algorithms, the analysis software fits the integrated data to the appropriate binding equation to yield the best fitting values and probable errors for K_a (and then ΔG), ΔH , ΔS and the stoichiometry of binding (n). **Figure 1b** also indicates the thermodynamic properties yielded by different parts of the curve.

A continuous single-injection titration method (SIM), applicable to very rapid binding reactions, has been developed as an alternative to the incremental titration method (Markova and Hallén, 2004). In this procedure, particularly indicated at low ΔH values, only one slow, continuous injection of L is made from the syringe into the sample cell filled with M, as shown in **Figure 1c**. Using the instrument software, the raw thermogram (top plot) is filtered and corrected for the response time (τ) of the instrument and blank/s effects, the concentration continuous calculated, and the conventional plot of the normalized heat vs $[L]_T/[M]_T$ generated (**Figure 1c, bottom**).

The experiment can be completed in less total time than required for incremental titrations but the accuracy of the binding parameters obtained from well-designed multiple-injection experiments is usually higher. Nonetheless, the higher density of data points obtained from SIM titration can facilitate discrimination between different binding models.

Usually, when one of the reactant is a macromolecule and the other a small ligand, the macromolecule is loaded in the sample cell and the small ligand in the injection syringe, but this setting can be inverted, depending on the stability, availability and solubility of the reactants, and the nature of the event to be studied.

At present, only ITC allows a complete thermodynamic characterization of binding reactions in a single experiment. By performing measurements at different temperatures, the heat capacity change ($\Delta C_p = (\partial \Delta H / \partial T)_p$) associated with the binding reaction can also be obtained from the slope of the plot of ΔH vs. T . By contrast, determination of the binding parameters by other techniques requires measuring K_a at different temperatures in order to get ΔH by means of the van't Hoff equation (Eqn. [4]), and then ΔS (Eqn. [2]).

$$\frac{d \ln K_a}{d(1/T)} = - \frac{\Delta H_{vH}}{R} \quad [4]$$

Using buffers with different ionization enthalpies, ΔH_{ion} , allows to assess if ligand binding is linked to proton exchange, since the observed ΔH will linearly depend on ΔH_{ion} (Gómez and Freire 1995). **See also:** DOI: 10.1002/9780470015902.a0003010.pub3. DOI: 10.1038/npg.els.0003117.

Selection of reagent concentrations

Successful design of titration experiments should accomplish the following requirements: *i*) initial reactant concentrations must guarantee completion of the reaction at the end of titration, and *ii*) heat change should be within the calorimeter detection range. For one-to-one complexes or when M has a single set of identical sites, the shape of the titration curve is determined by a dimensionless parameter, c , defined as the product of K_a for the total concentration of sites in the sample cell ($c = K_a \cdot [M]_T \cdot n$; Wiseman *et al.*, 1989). At very high c values the binding isotherm loses its characteristic curvature and the slope tends towards verticality (**Figure 2a**), making estimation of K_a , but not of ΔH , unreliable. Conversely, at very low c values the isotherm appears as featureless which would make K_a and ΔH determination unreliable (see below). Accurate estimation of K_a apparently requires $1 \leq c \leq 1000$, the ideal range being $10 \leq c \leq 100$ (**Figure 2a**). In practice this impose upper (10^8 - 10^9 M⁻¹) and lower (10^3 - 10^4 M⁻¹) limits to K_a values directly appraisable by ITC.

On the other hand, measuring ΔH , K_a and n by deconvolution of titration data requires at least 10 separate injections to define the binding isotherm, and each injection heat must be, on average, 10 times the limit of detection to be precisely detected. So, the minimal total heat, Q_t , to be measured for a low volume ITC with a detection limit of 0.02 μ cal (see above) is 2 μ cal; *i.e.*

$$Q_t = 2 \cdot 10^{-6} \text{ cal} = n \cdot \Delta H \cdot V \cdot [M]_T = n \cdot \Delta H \cdot [M]_T (0.2 \cdot 10^{-3} \text{ L}) \quad [5]$$

For a 1:1 complex ($n = 1$) with a ΔH of -10 kcal/mol, solving Eqn. [5] for $[M]_T$ gives a minimum concentration of *ca.* 1 μM . This concentration will be in the experimental window for $K_a \geq 10^6 \text{ M}^{-1}$. At lower K_a values, M_T should be increased so that $1 \leq [M]_T \cdot K_a \leq 10^3$. For high volume instruments as the VP-ITC the minimal concentration is rather similar since the lower sensitivity of the instrument is almost compensated by the higher cell volume. In general, minimal M_T is determined by the instrument sensitivity for high-affinity ligands, and by the accessible K_a window for medium-affinity ligands. Nonetheless, better appraisals of thermodynamic parameters are achieved using higher M_T concentrations (heat signal will be larger, and c will approach the ideal 10^{-1} - 10^2 range). In such conditions, saturation of M can be almost achieved at a final molar ratio of *c.a.* 2.

Poor solubility of receptors and ligands is a common problem in ITC, more often when dealing with low affinity systems, which requires working at low c values. **Figure 2a** is commonly accepted as evidence for the failure of isotherm fitting at low c values, since the curve becomes an almost flat line with no distinctive features. However, this is only true for the interval of molar ratios displayed in the figure, which is insufficient to describe 90% of whole isotherm when $c < 1$. In such conditions, the saturation degree depends on K_a rather than on M_T . As a rule of thumb, free L concentration in cell must be $(10 \cdot K_d)$ to reach 90% saturation and $(100 \cdot K_d)$ for 99% saturation for non-cooperative binding. So, K_a and $(n \cdot \Delta H)$ can be accurately derived from titrations at low c values when: *i*) a sufficient part of the binding isotherm is monitored, *ii*) there is an adequate level of signal-to-noise in the data, and *iii*) the concentrations of L and M are accurately known. Additionally, the use of variable volumes (small at first and larger as the injection number increases) can yield a better distribution of heat, and so a better parameter precision (**Figure 2b**). The strong correlation existing between n and ΔH at $c < 1$, prevents a reliable determination of both parameters unless n is known by an independent appraisal (Turnbull and Daranas, 2003; Tellinghuisen 2016).

Experiment design for non-equivalent multi-site systems and other complex binding models is often a difficult task, the c parameter being meaningless for them. New tools have been implemented in different software to help select the best titration settings and the reagent concentrations in cell and syringe by simulation of ITC experiments. **See also:** DOI: 10.1002/9780470015902.a0003010.pub3.

Measuring the affinity of tight- and weak-binding ligands

Evaluation of tight binding can be carried out by competition with a weaker affinity ligand previously pre-bound to M (Sirgurskjold, 2000). In the conventional approach the binding parameters of the weakest ligand and ΔH of the high-affinity ligand are determined in separated experiments (**Figure 2c**). Ideally, concentration of the competing ligand $[B]_T$ should be selected so that $K_a/(K_B \cdot [B]_T) \cong 10^5 - 10^8 \text{ M}^{-1}$, K_a being the tight-ligand constant. Alternatively, simultaneous characterization of the binding of a high-affinity and a moderate-affinity ligand for the same receptor can be achieved either by *i*) titration of the tight-binding ligand into a solution containing the moderate-affinity ligand and the receptor present in excess (**Figure 2d**; Krainer and Keller, 2015), or *ii*)

titration of the receptor into a dilute mixture of the two competing ligands to overcome limitations imposed by poorly water-soluble compounds (**Figure 2e**; Krainer *et al.*, 2012). When a suitable competing ligand is available, the displacement protocol can also be applied to low affinity ligand characterization, by measuring the binding parameters of a good ligand in the absence and presence of the low affinity ligand (Zhang and Zhang, 1998; Velázquez-Campoy *et al.*, 2004). The possibility of using a competitor with ΔH opposite to the high- or low-affinity ligand has the added benefit of amplifying the calorimetric signal during the displacement experiments (Velázquez-Campoy *et al.*, 2001).

Competition protocols extend the K_a window to 10^2 - 10^{12} M⁻¹ and are particularly useful in supramolecular chemistry and in drug design, where the ability to accurately measure binding affinities in the sub-nanomolar range is essential (Velázquez-Campoy *et al.*, 2001). Affinities in the millimolar range are also critical during lead optimization stage, as high-affinity ligands can be constructed by tethering together complementary low affinity fragments (Fattori, 2004; Retch *et al.*, 2016), and many biological processes are mediated by low affinity interactions (*e.g.* carbohydrate-protein; carbohydrate-carbohydrate, cyclodextrin-guest).

Analysis of binding isotherms

The use of ITC in binding thermodynamic studies is now well established and there are excellent reviews in which a wide number of applications is provided and the thermodynamics behind the studied processes scrutinized. A selection of papers illustrating ITC applications in biology, supramolecular chemistry and natural products is gathered in **Table 2**. Models to fit or simulate ITC binding data available in the software provided by ITC manufactures (Origin and NanoAnalyse) include the one-site model, the two set of independent sites, sequential filling of binding sites, dimer dissociation, and the competition-binding model, and user-made models have been developed to analyze more complex processes (Velázquez-Campoy 2015). Several other packages including simulation and global fit of titration curves to a variety of binding models (SEDPHAT, AFFINImeter, *pytc*), or user-unbiased integration of data sets (NITPIC, AFFINImeter, *pytc*) have been developed. AFFINImeter and *pytc* also allow easy implementation of new fitting models, whereas SEDPHAT and AFFINImeter provide the species population along the whole titration (see **Table 1**).

Interpretation of binding isotherms in simple molecular terms is not always easy. Even for multi-sites systems the number of binding parameters can quickly exceed what can be confidently determined from a single data set or titration configuration. Therefore, global analysis of different experiments – at identical or varying conditions – to provide a single set of binding parameters is an efficient way of improving the reliability of fits, by reducing the number of freedom degrees in the fitting model (Zhao *et al.*, 2015; Muñoz & Piñeiro, 2018). Its use becomes increasingly important as the system complexity increases, as characterization of multi-component systems usually requires multiple titration experiments in different configurations. In such scenario, simultaneous and direct fitting of all data sets is often more accurate and stringent than the analysis with apparent binding constants extracted from independent analysis, and helps to elucidate complex binding mechanisms, as illustrated in **Figure 3a-d**. **See also:**

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DOI:

10.1002/9780470015902.a0027015; DOI: 10.1002/9780470015902.a0001341.pub3;
DOI: 10.1002/9780470015902.a0001340.pub2;

Thermodynamic binding signature

As it can be inferred from Eqn. [2], ΔH and ΔS determine the binding affinity. ΔH primarily reflects the strength of the interactions responsible for complex formation (e.g. hydrogen bonds, van der Waals interactions, etc.) relative to those existing with the solvent, whereas the entropy change mainly reflects variations in the solvation entropy (particularly important for hydrophobic groups) and in conformational entropy (lose of conformational freedom upon complex formation). ΔC_p of binding primarily reflects the burial of polar and non-polar surfaces due to the binding reaction (Gomez *et al.*, 1995; Schön and Freire, 2016). As a result, different types of interactions reflect themselves in different thermodynamic signatures (entropy and enthalpy changes; **Figure 4**), and two ligands that have different ΔH and $T\Delta S$ contributions will have different mechanism of binding, even if their binding affinities may be the same (Velázquez-Campoy *et al.*, 2004). ΔH and ΔS have been, therefore, acknowledged as critical determinants of important drug properties (Velázquez Campoy *et al.*, 2001; Klebe, 2015;); only intrinsic parameters of binding can be used in structure-activity relationship in drug development of mechanistic studies of modes of binding. **See also:** DOI: 10.1002/9780470015902.a0003011.pub2; DOI: 10.1002/9780470015902.a0002974.pub2.

Evaluation of Kinetic Constants from ITC Titration Experiments

Kinetic information on binding events is central to understand biological processes *in vivo*. However, little attention has been paid to this aspect until very recently. Since the heat produced or absorbed during each ligand injection – due to a small sequential perturbation of the equilibrium under study – is monitored as a function of time, titration raw data contain intrinsic kinetic information of the binding process. Nonetheless, ITC experiments have generally been designed to obtain only thermodynamic information. The availability of highly sensitive instruments with low response time (~ 3.5 to 10 – 12 s), together with the development of theoretical methods and public software (kinITC) to analyse the shape of the ITC peaks, have drastically reduced the barriers for retrieving kinetic data (k_{on} and k_{off} ; $K_a = k_{on}/k_{off}$) from bimolecular reactions ($M + L \rightleftharpoons ML$), even if they proceed through complex mechanisms (Burnouf *et al.*, 2012, Vander Meulen *et al.*, 2016). Recently, a methodological simplification (kinITC-ETC) applicable to one-step bimolecular processes has been implemented in the AFFINIMETER software (Dumas *et al.*, 2016; Muñoz *et al.*, 2019). Together with affinity data, kinITC-ETC determines k_{on} and k_{off} by measuring the time required to return to equilibrium at each injection as a function of $[L]_T/[M]_T$ ratio (**Figure 5a-d**). By doing titrations at several temperatures, ΔC_p , the van't Hoff enthalpy and entropy, and the enthalpy and entropy of activation ($\Delta H^\ddagger$ and $\Delta S^\ddagger$) can be derived as well. Of note, kinetic results will be correct only if the kinetic model used was correct. Besides, thorough cleaning of the instrument is essential to obtain reliable results as the τ value of the instrument strongly depends on the cleanness of the sample cell (Dumas *et al.*, 2016). Kinetic analysis of ITC data using

kinITC or kinITC-ETC compared to those performed with other biophysical techniques have been reported for a variety of systems, covering from simple ligand binding to folding processes triggered by ligand binding (Burnoud *et al.*, 2012; Van der Meulen and Butcher 2012; Dumas *et al.*, 2016; Muñoz and Piñeiro 2018). Noteworthy, the study of bacterial adhesin FimH binding to a collection of antagonists with a broad range of affinities highlighted how combination of thermodynamic and kinetic information helps to derive structural information on the mode of binding (Zihlmann *et al.*, 2018).

Enzyme Kinetic Characterization

ITC can be used to monitor the progression of enzyme catalyzed reactions and is emerging as a powerful tool for measuring the kinetics of challenging enzymes and precise conditions for specific enzyme activity (Sotoft *et al.*, 2010; Murphy *et al.*, 2010). In comparison with conventional methods, ITC allows using physiological and synthetic substrates, immobilised enzymes, or media that mimic physiological conditions, is as rapid and sensitive as spectroscopic techniques but do not require additional reagents, tags or secondary reactions (Reimund *et al.*, 2017). Enzyme activity can be titrated in two different ways: *i*) by successive injections of substrate, S, into the enzyme solution, allowing the baseline to equilibrate between injections (**Figure 1d**), and *ii*) by a single injection of S into the cell loaded with enzyme, E, (or viceversa), monitoring the signal change until all the substrate has reacted (**Figure 1e**).

The amount of heat, Q , associated with conversion of m moles of substrate S in product P after triggering the reaction by S injection is given by:

$$Q = m \cdot \Delta H_{app} = [P] \cdot V \cdot \Delta H_{app} \quad [6]$$

where ΔH_{app} is the molar enthalpy of product formation. Hence, the experimental signal (dQ/dt) generated by the enzymatic reaction allows to know the reaction rate, v , defined as the variation of product concentration with time $d[P]/dt$.

$$v = \frac{d[P]}{dt} = \frac{1}{V \cdot \Delta H_{app}} \cdot \frac{dQ}{dt} \quad [7]$$

In the multiple injection method, the shift in the power signal after injection i , (dQ_i/dt), is proportional to the substrate concentration $[S]$ in the cell, and subsequent injections take place when the signal has reached a steady state and prior to significant reaction of S (**Figure 6a**). The difference between the original baseline and the plateau attained after each injection is used to create a plot of the reaction rate vs $[S]$ (**Figure 6b**). ΔH_{app} , required for conversion of experimental rates (power units) to enzyme turnover, is determined in an independent experiment at higher enzyme concentration by allowing complete substrate depletion and then integrating the total heat evolved (**Figure 6c**):

$$\Delta H_{app} = \frac{1}{V \cdot [S]_{total}} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt} dt \quad [8]$$

As rates are measured at steady-state conditions, they do not depend on τ and the fit of the kinetic curves to standard equations would yield k_{cat} and K_m . This method can be used when $K_m > 10 \mu\text{M}$ and $[E] \ll [S]_T$.

In the single injection method, a continuous kinetic curve is generated by monitoring the thermal power as S is completely transformed (**Figure 1e**) and ΔH_{app} is calculated by integrating the total heat. At any given time, v can be calculated from the thermal power using Eqn. [7], and the remaining substrate concentration $[S]_{(t)}$ from the integral of the heat evolved (Eqn. [9]).

$$[S]_{(t)} = [S]_T - [P]_{(t)} = [S]_{\text{total}} - \frac{\int_{t=0}^t Q_{(t)}}{V \cdot \Delta H_{\text{app}}} \quad [9]$$

The impact of τ in the analysis of single injection data will be marginal if $K_m < 10 \mu\text{M}$ and $[S] \gg K_m$ (Todd and Gomez, 2001). At higher values of K_m or when $[S] \ll K_m$ the effect may be minimized by choosing experimental conditions where $0.1/\tau < (k_{\text{cat}} \times [E]/K_m) < 1/\tau$ and the reaction goes at least to 99% completion. Otherwise, raw data have to be mathematically corrected to remove the effect of the finite response time of the instrument on k_{cat} and K_m calculation (Transtrum et al., 2015).

The analysis of kinetic essays performed with and without competitive inhibitors is implemented in the software provided by ITC manufacturers, as well as calculation of K_m , k_{cat} and K_i (inhibition constant) for Michaelis-Menten kinetics. The τ value provided with the instrument is used for single-injection data correction. However, τ can vary with the experimental conditions (e.g. density of solutions), and the protocol and equations required to include τ as a fitting parameter for the Michaelis-Menten model have been reported (Hansen et al., 2011; Demarse et al., 2013).

Under appropriate conditions both methods provide a precise characterization of enzyme kinetics (Todd and Gomez 2001; Transtrum et al., 2015; Hansen et al., 2016), including fast evaluation of inhibitor/activators capacities and the investigation of their mechanism of action. Indeed, ITC is extremely powerful to characterize enzyme inhibitors, a central theme, for example, to lead generation and optimization in drug development (Recht et al., 2016). Remarkably, a simple and effective ITC assay, built on sequential single-injections with substrate and inhibitor premixed in the injection syringe, has been recently developed to completely characterize enzyme inhibition in a single experiment (Di Trani et al., 2018). Product inhibition, hard to evaluate in simple spectrophotometric assays, can be easily detected by ITC (Draczkowski et al., 2016). As in the thermodynamic studies, the use of buffers with different ΔH_{ion} allows to evaluate the net exchange of protons during catalysis (Todd and Gomez; 2001; Draczkowski et al., 2016). **See also:** DOI: 10.1002/9780470015902.a0000716; DOI: 10.1002/9780470015902.a0000714.pub2; DOI: 10.1002/9780470015902.a0000719.pub2; DOI: 10.1002/9780470015902.a0000865.pub3; DOI: 10.1038/npg.els.0000862; DOI: 10.1038/npg.els.0000600.

Experimental Remarks

- A clean cell and a perfectly straight syringe are decisive to avoid false results and to get excellent baselines with high signal-to-noise ratio.
- Incertitude in K_a , ΔH and n relies in accurate determination of reactant concentrations.
- Concentrations of reactants should guarantee reaching the saturation point in a reasonable number of injections.
- Both reactants have to be in identical solutions. Even small buffer mismatches can generate large dilution heats, which can mask the heat of ligand binding. Extreme care should be taken when using DMSO, glycerol, detergents or high salt concentrations.
- Use buffers in which ligand and macromolecule are stable and soluble. Be sure that buffer concentration can compensate for any pH effects during titration.
- Before ITC, dialyze reactants against the same dialysis buffer. Use the final dialysis buffer to solubilize small-ligands. Glycerol and detergents dialyze slowly and its use requires longer dialysis time.
- If DMSO is needed to solve a ligand, check ligand solubility by serial dilutions of 100% DMSO stock solution in buffer. Immediately before the experiment, add DMSO to the protein solution (many proteins are stable, in the short term, in up to 2-5% DMSO).
- Use TCEP or beta-mercaptoethanol rather than DTT if a reducing agent is needed. DTT can produce aberrant baselines.
- Baseline assignment is of capital importance for precision of experimental data and of derived parameters. Bias-free automated assignment of baselines and inclusion of peak integration-associated errors in fitting is recommended.
- If the heat of blanks varies perceptibly with the injection number, reaction heats should be corrected peak by peak, subtracting the blank integrated curve (or a function fitting blank heats) from the titration one.
- Experimental data should be analyzed using the simplest model that makes sense with prior knowledge of the system (e.g. stoichiometry).
- Errors provided by fitting methods reflect only the goodness of fit of a model to a particular set of results. Estimate of random error must be obtained from measurement repetition.

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Figure 1. (a) Schematic representation of an ITC instrument based on the dynamic power compensation principle, showing the sample (S) and reference (R) cells enclosed in the thermostated jacket, the injection syringe, and the computer controlled thermostatic, feedback and output systems. (b) Representation of a typical multi-addition experiment (exothermic binding) showing 2'CMP titration into RNase A (25 °C; 20 mM KAc, 20 mM KCl, pH 5.0). Raw thermogram (each peak corresponds to one 2'CMP injection and peak area represents the heat released at the injection; top plot) and binding isotherm (normalized heat effect per injection vs [2'CMP]_T/[RNase A]_T molar ratio; bottom plot). The solid line through the points is the best fit of results to the one-site model. The amplitude of the curve is proportional to ΔH , the inflection point gives n , and the slope K_a . (c) Binding experiment of 2'CMP to RNase A using the SIM method. The experimental signal is shown in the top panel and the continuous plot of normalized heats vs the total molar ratio in the bottom panel (30 °C; buffer as in (b)). The red line is the best fit of results to the one-site model. (d) Representation of a typical ITC enzymatic assay using the multiple-injection method. The thermal power deflection at a given injection (dQ_i/dt) is proportional to the reaction rate constant and the substrate concentration in the cell (top panel). The rate of product formation as function of [S], calculated from the top plot, is shown the bottom panel. (e) Thermogram of an enzymatic assay using the single-injection method. The thermal power was monitored until complete depletion of the substrate in the absence (solid line) and presence (dashed-dot line) of a competitive inhibitor (top plot), and continuous kinetic curves were generated by plotting the reaction rates measured in the absence (circles) and presence (triangle) of inhibitor vs the remaining concentration of substrate in the cell. Solid red lines in (d) and (e) represent the fit of the kinetic curves to the Michaelis-Menten model.

Figure 2. (a) Influence of the “c” value (plot label) on the shape of ITC isotherms for receptors with a single-set of identical sites (n). Simulations were performed assuming 100 μM of sites and the following K_a values (M^{-1}): grey (10^3); blue (5×10^3); magenta (10^4); green (5×10^4); red (4×10^5) and black (5×10^6). (b) ITC isotherm for titration of lactose (lac; 15 mM) into a lectin-like protein (364 μM in cell) at low “c” value. Titration was optimized by doing two sequential sets of injections. The red line is the fit of the isotherm with the best parameters ($K_a = 1.5 \times 10^3 \text{ M}^{-1}$ and $\Delta H = -7.06 \text{ kcal/mol}$, fixing $n=1$). (c) Simulated isotherms for a three-experiment displacement protocol, showing titration of the high-affinity ligand (HA-lig; 130 μM) into the protein (M; 10 μM) in the absence (lower plot) and presence (upper plot) of a lower affinity competitor (LA-lig; 200 μM), whose titration into M is shown in the central plot ($K_{\text{HA-lig}} = 2.2 \times 10^9 \text{ M}^{-1}$; $\Delta H_{\text{HA-lig}} = 3.1 \text{ kcal/mol}$; $K_{\text{LA-lig}} = 2.3 \times 10^6 \text{ M}^{-1}$; $\Delta H_{\text{LA-lig}} = 8.0 \text{ kcal/mol}$; $n = 1$). Red solid lines are the fits of the isotherms. (d) Simulation of a single-experiment displacement titration of HA-lig (130 μM in syringe) into M (10 μM) and LA-lig (5 μM) in cell; binding parameters were as in (b). Differences between competition isotherms in upper plot of (c) and (d) are due to full (c) and partial (d) saturation of M sites with LA-lig. (e) Simulation of a competition experiment for complete thermodynamic evaluation of poorly soluble tight ligands by single titration of M (400 μM in syringe) into a dilute mixture of HA-lig (20 μM) and LA-lig (7.4 μM) in cell ($K_{\text{HA-lig}} = 2.2 \times 10^9 \text{ M}^{-1}$; $\Delta H_{\text{HA-lig}} = 12.2 \text{ kcal/mol}$; $K_{\text{LA-lig}} = 2.8 \times 10^6 \text{ M}^{-1}$; $\Delta H_{\text{LA-lig}} = -7.4 \text{ kcal/mol}$; $n = 1$).

Figure 3. Global analysis of ITC isotherms from (a) dCRY (light responsive flavoproteine) titration into CaM (Ca²⁺/Calmodulin); (b) INAD (photo-receptor specific protein) titration into CaM; and (c) competitive titration of dCRY into CaM incubated with INAD. The AFFINImeter models applied to each isotherm in the global fitting are indicated in respective panels. (d) Species distribution plot calculated from the competition experiment. Simultaneous analysis of all data sets unveiled that CaM has two independent sites of different affinities for dCRY (K_d in nanomolar and micromolar ranges). The lower-affinity site showed also a weak interaction with INAD (adapted with permission from Zhao *et al.*, 2015, published in *Frontiers in Molecular Neurosciences*).

Figure 4. Dissection of the binding energetics of KNI-764 and clinical inhibitors Indinavir, Nefilnavir, Sequinavir and Ritonavir to the HIV-1 protease. Black bars, ΔG ; white bars, $-T\Delta S$; grey bars, ΔH (adapted from Velázquez-Campoy *et al.*, 2001, published in *Archives of Biochemistry and Biophysics*). Unlike the clinical inhibitors, which bind with unfavourable or slightly favourable enthalpy changes, binding of KNI-764, effective against resistant mutants, is strongly exothermic.

Figure 5. ITC data of a 1:1 binding interaction used for thermodynamic and kinetic analysis with kinITC-ETC. (a) The raw thermogram. (b) The processed thermogram after baseline correction and noise removal. (c) Binding isotherm resulting from integration of the processed thermogram as a function of $[L]_T/[M]_T$ (total molar ratio) with integration error bars. (d) Equilibrium time curve showing the equilibration time for each injection as a function of $[L]_T/[M]_T$. Solid lines in (c) and (d) are the results of globally fitting the binding isotherm and the time equilibration curve with kinITC-ETC. Best fitting parameters are shown in respective panels.

Figure 6. cAMP hydrolysis by CaM-dependent PDE1 (3',5'-cyclic nucleotide phosphodiesterase1) determined by ITC. (a) PDE1 (0.04 units) was allowed to reach thermal equilibrium at 37 °C in the presence of 3.8 μ M wild-type CaM. Then, serial injections of cAMP (4.7 mM) were made every 120 s. To avoid distortions due to dilution events, the power change associated with substrate addition at injection "i" was average over the 30 s immediately before next injection. (Inset) close-up view of baseline shift in the three first injections ($1 \times 1 \mu$ l; $2 \times 2 \mu$ l). (b) Rate vs cAMP concentration curves, obtained from the baseline shifts using Eqn. [7] and corrected for enzyme concentration dilution, were fit to the Michaelis-Menten equation (solid lines) to obtain k_{cat} and K_m from titrations carried out in the absence (black circles) and presence of CaM wild-type (black triangles) and of phospho-(Y)-mimetic CaM Y99D/Y138D double mutant (grey circles). CaM increases V_{max} without altering the K_m , the effect being higher for phosphorylated CaM wild-type. (c) The thermogram represent the instrument response produced by cAMP hydrolysis reaction. PDE1 (0.1 units) was loaded in the sample cell and 15 μ l of cAMP (4.7 mM) was injected and the reaction was monitored until the baseline returned to the initial level. Numerical integration of the area under the peak gave ΔH_{app} . Two additional injections were made at intervals indicated by the arrows; the decrease in the maximal deflection of the heat flow signal in successive injections was indicative of partial product inhibition (see Stateva *et al.* 2015 for details).

Table 1: Common protocols and software packages not provided by ITC manufactures

Protocol	Parameters	Reference
Direct titration		
Multi-injection titration	K_a (ΔG), ΔH , ΔS , n	Velázquez-Campoy <i>et al.</i> , 2004 Freyer and Lewis, 2008
Continuous isothermal titration (SIM)	K_a (ΔG), ΔH , ΔS , n	Markova and Hallén, 2004
Heterodimer formation	K_a (ΔG), ΔH , ΔS	Velázquez-Campoy <i>et al.</i> , 2004
Monomer-dimer equilibrium	K_a (ΔG), ΔH , and ΔS of dimerization	Burrows <i>et al.</i> , 1994
Displacement titrations		
Theoretical analysis of ligand competition by displacement ITC	K_a (ΔG), ΔH , and ΔS (both ligands)	Zhang and Zhang, 1998 Sigurskjold, 2000
Three-experiment evaluation of tight- and low-binding by ligand competition	K_a (ΔG), ΔH , and ΔS (both ligands)	Velázquez-Campoy <i>et al.</i> , 2004
Single-experiment evaluation of high-affinity hydrophobic ligands	K_a (ΔG), ΔH , and ΔS (both ligand)	Krainer <i>et al.</i> , 2012
Single-experiment displacement for tight binding evaluation	K_a (ΔG), ΔH , and ΔS (both ligands)	Krainer and Keller, 2015
Binding coupled processes		
Coupled proton transfer process	Number of protons exchanged (n_{H^+}); buffer-independent ΔH	Velázquez-Campoy <i>et al.</i> , 2004
Ligand-induced conformational change	Intrinsic binding parameters	Velázquez-Campoy, 2015
Binding-linked protein folding	Global thermodynamic parameters using a general thermodynamic model	Sahu <i>et al.</i> , 2016 Analysis software available at https://scholarsphere.psu.edu/downloads/5712mr21f
Kinetic analysis of ligand binding		
Thermodynamic and kinetic analysis of ITC experiments with kinITC-ERT (single-step binding)	K_a (ΔG), ΔH , and ΔS k_{on} (10^3 - $5 \cdot 10^5$ M ⁻¹ s ⁻¹), k_{off}	Muñoz <i>et al.</i> , 2019
Thermodynamic and kinetic analysis of ITC experiments with kinITC (single- and two-step processes)	K_a , ΔH , ΔG , ΔS , k_{on} , k_{off}	Burnoff <i>et al.</i> , 2012 Vander Meulen <i>et al.</i> , 2016 http://www-ibmc.u-strasbg.fr:8080/webMathematica/kinITCdemo/
Enzyme catalyzed reactions		
Measurement of enzyme kinetic constants; Evaluation of activity regulators	V_{max} (k_{cat}), K_m K_i (mechanism of action) K_i and IC50 values	Todd and Gomez, 2001 Freyer and Lewis, 2008 Drazckowski <i>et al.</i> , 2016
Collection and analysis of enzyme	Step-by-step conversion of	Demarse <i>et al.</i> , 2013

kinetic data using the single injection method	raw heat data into kinetic parameters of Michaelis-Menten model	
Complete characterization of enzyme inhibition in a single ITC experiment	k_{cat} , K_m , K_i and inhibition mode	di Trani <i>et al.</i> , 2018
Screening of fragment-based libraries for lead discovery using nanocalorimeter arrays	k_{cat} , K_m (substrates), K_a , K_i (competitive inhibitors) of medium/low affinity compounds	Recht <i>et al.</i> , 2016
Software for ITC analysis		
SEDPHAT http://sedfitsedphat.nibib.nih.gov/software (free download)	Global multi-method analysis of data from different ITC data sets or complementary biophysical disciplines using one single model	Zhao <i>et al.</i> , 2015 Brautigam <i>et al.</i> , 2016 Tutorials and help available on line
AFFINImeter https://www.affinimeter.com/site/itc/	Global thermodynamic analysis of ITC data. Easy user-definition of new binding models k_{on} and k_{off} (kinITC-ERT)	Dumas <i>et al.</i> , 2016 Muñoz and Piñeiro, 2018 Piñeiro <i>et al.</i> , 2019 Tutorials and model information available on line
NITPIC http://biophysics.swmed.edu/MBR/software.html (free download)	Automated and precise serial integration of unlimited number of ITC data sets	Keller <i>et al.</i> , 2012 Scheuermann and Baritigam, 2015
pytc (Pythom-based) http://github.com/harmslab/pytc (free download)	Global fit of multiple ITC data sets. User implementation of new binding models	Duvvuri <i>et al.</i> , 2018 Model information and tutorials available on line

Glossary

Activation energy In chemical kinetics, the activation energy (E_a or Gibbs energy of activation $\Delta G^\ddagger$) is the minimum energy required to start a chemical reaction and represents difference between the reactants and the activated complex or transition state. It determines the exponential factor of the Arrhenius equation for the temperature dependence of reaction rates ($k = A \exp(-E_a/RT)$, where k is the rate constant and A the preexponential term).

Activation enthalpy The minimum amount of enthalpy ($\Delta H^\ddagger$) needed to start a chemical reaction ($\Delta H^\ddagger = E_a - RT$).

Activation entropy The activation entropy ($\Delta S^\ddagger$) is the change in entropy when the reactants change from their initial state to the activated complex or the transition state. It determines the preexponential factor A of the Arrhenius equation.

Enthalpy of binding The enthalpy change (ΔH) accompanying the binding process. It is equal to the heat absorbed or released in the process when it takes place at constant pressure.

Entropy of binding The entropy change (ΔS) accompanying the binding process. Entropy is a measure of the disorder of a system.

Gibbs free energy of binding The change in the Gibbs free energy (ΔG) coupled to the binding process. It is equal to the energy exchange upon binding and determines the binding affinity ($\Delta G = -RT \ln K_a$), where R is the gas constant, T the absolute temperature and K_a the binding equilibrium constant).

Table 2. Selection of case studies and reviews illustrating ITC applications to analyze biomolecular interactions

System/Process	Summary	Reference
Enzyme-inhibitor	Thermodynamic, kinetic and structural parametrization of human carbonic anhydrase interactions towards enhanced inhibitor design	Linkuvienė <i>et al.</i> , 2018
Protein-metal ion	Special issues for ITC studies. Thermodynamics of complex formation. Competing and coupled reactions. Buffer protonation and metal-buffer complex thermodynamics	Quinn <i>et al.</i> , 2016
RNA-small molecule (alkaloid)	Advances in thermodynamic and structure-activity correlations of RNA-targeted drugs	Kumar and Basu, 2016
DNA-ligand	Advances in thermodynamic characterization of DNA-ligand interactions. Binding coupled events. Analysis of complex binding modes	Buurma and Haq, 2007
Protein-carbohydrate	Thermodynamics of bacterial lectin BC2L-A interaction with monosaccharides, disaccharides and bacterial oligo- and polysaccharides	Marchetti <i>et al.</i> , 2012
Protein-glycoconjugate multivalent interactions	Thermodynamics of lectin binding to multivalent synthetic carbohydrates, mucins and glycoproteins. Microscopic binding parameters. Negative cooperativity among glycan-epitopes	Dam <i>et al.</i> , 2016
Protein-polyelectrolyte (PE)	Thermodynamic analysis and driving forces of protein interaction with lineal PE and PE-modified nanoparticles as a guide for PE-based system application	Wang <i>et al.</i> , 2019
Monomer-dimer equilibrium	Interleukin-8 monomer-dimer equilibrium thermodynamics (K_{dim} , ΔH_{dim} , ΔS_{dim})	Burrows <i>et al.</i> , 1994
Binary and ternary protein multisite interactions	Evaluation of negative and positive cooperativity by global analysis of ITC titrations in different configurations to follow binary and ternary complex formation	Houtman <i>et al.</i> , 2007
Binding-linked equilibria (ligand-induced conformational change)	Evaluation of intrinsic binding parameters of homotropic and heterotropic model systems using binding polynomial	Velázquez-Campoy, 2015
Protein-terpene interactions	ITC-based multi-step binding model of testosterone to sex hormone binding globulin (SHBG)	Zakharov <i>et al.</i> , 2015
Binding-linked equilibria	Complete characterization of HIV aspartic protease inhibition by entothiapepsin. Net proton exchange; and buffer-independent thermodynamic parameters. Individual contributions of enzyme and inhibitor residues to total ΔG	Gómez and Freire, 1995

Recombinant proteins	Evaluation of protein quality (binding-active fraction). Effects of purification tags	Baranauskiene <i>et al.</i> , 2019, and references herein
Characterization of extremely tight ligand-receptor binding by competition ITC	Determination of HIV-1 protease inhibitor binding parameters ($K_a \sim 10^{10} \text{ M}^{-1}$) Evaluation of Zn^{2+} -dependent association of sensor protein RsrA to sigma factor (σ^R) ($K_a > 10^9 \text{ M}^{-1}$). Identification of a new mechanism of oxidative stress regulation	Velázquez-Campoy <i>et al.</i> , 2001 Rajasekar <i>et al.</i> , 2016
Characterization of low-affinity ligand-receptor binding by competition ITC	Determination of ligand binding parameters to tyrosine phosphatase 1B ($K_a \sim 10^3 \text{ M}^{-1}$)	Zhang & Zhang, 1998
Enthalpy screen of drug candidates	Thermodynamic profiling of drug candidates; binding driving forces	Schön and Freire, 2016 Klebe <i>et al.</i> , 2015
Titration in complex media (suspension of bacterial cell extract)	Thermodynamics of binding (K_a , ΔH , and $T\Delta S$). Assessment of site locus by ITC competition experiments	Kim <i>et al.</i> , 2017
Ligand-membrane protein interaction studies	Thermodynamic signature ($K_a \sim 10^2 \text{ M}^{-1}$, ΔH , $T\Delta S$ and n). Challenges of experimental setup and data analysis	Rajaratnam and Rösger, 2014
Drug partition and translocation in model lipid membranes	Kinetics and thermodynamics of chlorpromazine interaction with lipid bilayers. Intrinsic partition coefficients (K_p , ΔH and ΔS)	Martins <i>et al.</i> , 2012
Surfactant micellization; association with biopolymers and colloidal particles	Micellization thermodynamics. Binding models. Association mechanisms	Loh <i>et al.</i> , 2016
Biomolecule-nanoparticle (NP)	Main driving forces for NP-biomolecule interactions. Relative effects of different NP properties on binding	Huang and Lau, 2016
Natural product interactions	Overview of ITC applications to investigate natural product interactions with proteins, nucleic acids, oligosaccharides and other receptors	Callies and Hernández Daranas, 2016
Receptor-agonist interaction in living cells	Characterization of metabotropic glutamate receptor (mGluR1a) interaction with agonist (DHPG); evaluation of number of binding sites per live cell	Ikeda <i>et al.</i> , 2013

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