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ITC in the post-genomic era...? Priceless

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Abstract

The information available after decoding the genome of the human species and many others is opening the possibility of new approaches to target thousands of protein interactions critical for a continuously increasing list of genetic and infectious diseases and pathologies, and to understand complex regulatory pathways and interaction networks describing cell function and interrelation. There is a need for a reliable technique offering the capability of measuring accurately macromolecular interactions (e.g. protein/ligand, protein/protein, protein/nucleic acid) in the laboratory. Compared to other analytical techniques, isothermal titration calorimetry (ITC) exhibits some important advantages for characterizing intermolecular interactions and binding equilibria. ITC is suitable for characterizing both low affinity interactions (e.g. protein network regulation and natural ligands) and high affinity interactions (e.g. rational drug design). Considering the advanced technological level reached as well as the outstanding quality of the information accessible through this technique, ITC is expected to play a very prominent role in the next years in the areas of rational drug design and protein network regulation.
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1. Introduction

Many experimental techniques can be used to study binding processes. Some of them (e.g. dialysis, ultracentrifugation) rely on the direct determination of the different species present in the system (free macromolecule, free ligand and macromolecule/ligand complex), whereas others (e.g. spectroscopy and calorimetry) measure a signal that it is proportional to the advance of the reaction (formation of macromolecule/ligand complex). Due to practical reasons (amount of sample and time needed, mainly), the second group constitutes the preferred one in biophysics and biochemistry labs. Calorimetry entered a golden age in the early nineties, when, due to technological and methodological advances in the field, it was possible to measure the tiny amount of heat associated with the non-covalent interaction between biological molecules [1–5].

Calorimetry measures directly the heat associated with a given process, which, at constant pressure, is equal to the enthalpy change in that process, ΔH. Due to sensitivity and accuracy reasons, the calorimeters used to characterize binding processes belong to the category of titration calorimeters with dynamic power compensation operating at constant temperature (isothermal titration calorimeter, ITC). A detailed description of the instrument and the technique can be found in the literature [1–9]. Briefly, the macromolecule solution is located inside the sample cell and the ligand solution in the injector syringe. A feedback control system supplies thermal power continuously to maintain the same temperature in both reference and sample cells. Any event taking place in the sample cell, usually accompanied by heat, will change the temperature in that cell and the feedback control system will modulate the power supplied in order to minimize such temperature imbalance. A sequence of injections is programmed and the ligand solution is injected periodically into the sample cell.

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The value of the association constant (or binding affinity), K_a, governs the equilibrium, i.e. the partition between the different species (free and bound). The change in composition inside the sample cell after each injection triggers the binding reaction and the rearrangement of populations leading to the formation of complex. The system will pass through different equilibrium states, differing in composition, as the sequence of injections proceeds. The heat associated with each injection is proportional to the increase in complex concentration (advance of the reaction) and it is calculated integrating the area under the deflection of the signal measured (amount of heat per unit of time provided to maintain both cells, sample and reference, at the same temperature). At the end of the experiment, saturation of the macromolecule is reached. Applying non-linear regression using the appropriate model in the data analysis procedure, it is possible to estimate the association constant or binding affinity, K_a, the binding enthalpy, ΔH, and the stoichiometry, n, in a single experiment. Spectroscopic techniques in which the signal is also proportional to the advance of the reaction (e.g. absorbance, fluorescence intensity, molar ellipticity or chemical shift) are only able to determine the association constant in a single experiment, requiring additional experiments and assumptions to estimate the enthalpy of binding.

Calorimetry is unique in that it is capable of measuring simultaneously the association constant and the enthalpy of binding. Therefore, it is possible a complete thermodynamic characterization of the binding process, that is, the evaluation of the main thermodynamic potentials associated with the binding process, using basic thermodynamic relationships:

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

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

where ΔG is the Gibbs energy change, ΔS is the entropy change, R is the gas constant and T is the absolute temperature. Enthalpy and entropy are temperature-dependent thermodynamic potentials:

$$\Delta C_P = \left(\frac{\partial \Delta H}{\partial T}\right)_P = T\left(\frac{\partial \Delta S}{\partial T}\right)_P \tag{2}$$

where ΔC_P is the heat capacity change upon binding.

2. ITC: estimation of the enthalpy of binding

Heat is the direct observable in calorimetry. This fact is the root for calorimetry's strong and weak points. First, being heat a universal signal, reporter labels are not required to follow the reaction advance (making possible to use natural non-modified ligands). However, only overall non-mechanistic information about the binding process is available. Extreme care must be taken in preparing samples, conducting experiments, analyzing data and discussing results, since any additional or spurious heat-generating process will contribute to the experimental output. And second, enthalpy is directly measured without any additional assumption. When using spectroscopic techniques, experiments at different temperatures have to be performed in order to estimate the binding enthalpy from the temperature dependence of the association constant, the van't Hoff relationship:

$$\frac{\partial \ln K_a}{\partial\left(\frac{1}{T}\right)} = -\frac{\Delta H}{R}$$

$$\frac{\partial^2 \ln K_a}{\partial\left(\frac{1}{T}\right)^2} = \frac{T^2 \Delta C_P}{R} \tag{3}$$

This approach exhibits two main drawbacks. First, due to practical reasons, it is not always possible to cover a broad temperature range when estimating the association constant. Second, although enthalpy and entropy are strongly dependent on temperature, the Gibbs energy usually exhibits a weak temperature dependency over the experimentally accessible temperature range (see Fig. 1). This enthalpy/entropy compensation phenomenon makes very difficult to obtain accurately first and second derivatives of the association constant (related to the enthalpy and heat capacity of binding), both readily available via calorimetry. And third, the determination of the binding affinity and the Gibbs energy of binding are accompanied of certain error. These difficulties have led to discrepancies between the enthalpy measured calorimetrically (calorimetric enthalpy) and spectroscopically (van't Hoff enthalpy) that can be explained by errors and inaccuracies inherent to the non-calorimetric methods [10–14]. Let us take the temperature derivative of the two contributions to the Gibbs energy, the enthalpy and the entropy:

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

$$\left(\frac{\partial T\Delta S}{\partial T}\right)_P = \Delta C_P + \Delta S \tag{4}$$

If we consider now that binding entropy changes larger than ± 30 cal/K·mol are unusual and heat capacity changes values between -200 and -500 cal/K·mol are very common (i.e. normally the entropy change is less than 10% of the heat capacity change and ΔS is negligible compared to ΔC_P), we can conclude the two contributions to the Gibbs energy share approximately the same temperature factor, ΔC_P, thus canceling out the temperature dependency in ΔG. On the other hand, the temperature

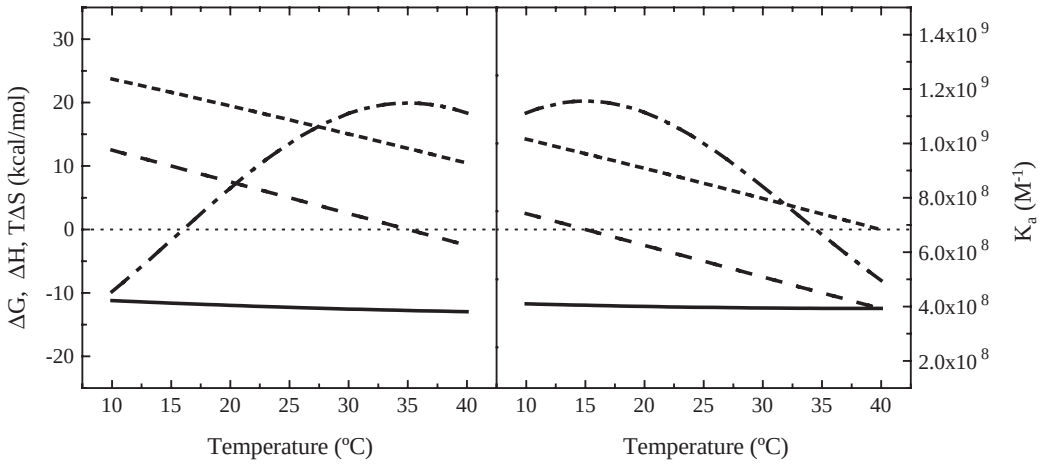

Fig. 1. Temperature profile of the Gibbs energy ΔG (solid line), the enthalpy ΔH (dashed line), the entropy $T\Delta S$ (dotted line) and the affinity K_a (dashed-dotted line) for a macromolecule/ligand binding characterized by: (left) K_a(25 °C)=10^9 M^{-1}, ΔH(25 °C)=5 kcal/mol, ΔC_P=−0.5 kcal/K · mol; (right) K_a(25 °C)=10^9 M^{-1}, ΔH (25 °C)=−5 kcal/mol, ΔC_P=−0.5 kcal/K · mol. Although enthalpy and entropy exhibit a marked temperature dependency (determined by the heat capacity change upon binding), the Gibbs energy is almost flat and hardly changes (less than 2 kcal/mol) in the 10–40 °C interval, making very difficult the estimation of the binding enthalpy and heat capacity changes.

derivative of the Gibbs energy change is the minus entropy change:

$$\left(\frac{\partial \Delta G}{\partial T}\right)_P = -\Delta S \tag{5}$$

and the second derivative is related to the heat capacity change:

$$\left(\frac{\partial^2 \Delta G}{\partial T^2}\right)_P = -\frac{\Delta C_P}{T} \tag{6}$$

Therefore, a value for the entropy change of 30 cal/K · mol would indicate that raising or lowering the temperature 10 °C will only change the Gibbs energy in 300 cal/K · mol. Besides, it is not rare to find that the inversion temperature for the binding entropy (temperature at which the binding entropy is zero) is close to room temperature, and, therefore, the Gibbs energy change at room temperature would be in the vicinity of a minimum (its second derivative is usually positive), showing a very shallow temperature profile. Fig. 1 illustrates the difficulty to extract enthalpy and heat capacity values from the temperature dependence of the Gibbs energy of binding.

3. ITC: measuring intermolecular interactions

As stated above, calorimetry is able to provide the complete energetic characterization of a binding process: ΔG, ΔH and ΔS. It is conceptually useful to express the Gibbs energy of binding in terms of certain energetic contributions in the following form [15,16]:

$$\Delta G = \Delta H - T\Delta S_{SOL} - T\Delta S_{CONF} - T\Delta S_{R,TR} \tag{7}$$

where ΔS_{SOL} is the solvation entropy (entropy change associated with the change in solvation of the binding molecules upon binding), ΔS_{CONF} is the conformational entropy (entropy change associated with the change in the conformational space of the binding molecules upon binding) and $\Delta S_{R,TR}$ is the roto-translational entropy (entropy change associated with the change in the configurational space of the binding molecules upon binding).

The enthalpic term reflects the energetic contribution of many individual interactions (hydrogen bonds, van der Waals, electrostatic, polar and dipolar interactions, etc.) between the two binding molecules, considering the interactions with the solvent as a reference and, therefore, it reflects also the solvation enthalpy. It is negative (favorable) if the interactions between the binding molecules overcompensate the interactions of the individual molecules with the solvent; otherwise, it will be positive (unfavorable). The solvation entropy represents the gain in degrees of freedom of the water molecules that prior to the binding are localized in the surface of the binding molecules and are released to the bulk solvent upon binding. It is usually positive (favorable) and huge, since ligands have a greater percentage of non-polar than polar surface area buried upon binding. The burial of solvent-exposed molecular surface area upon binding is also the main contribution to the heat capacity change upon binding. The conformational entropy, as well as the roto-translational entropy, is usually negative (unfavorable), because there is a loss of degrees of freedom due to a reduction in the number of accessible conformations and configurations for both molecules upon binding; however, the former can be modulated by modifying structurally the binding molecules, whereas the latter is essentially the same in any binding event and cannot be manipulated.

The partition of the Gibbs energy of binding according to Eq. (7) indicates that enthalpy and entropy reflect different

interactions of very distinctive nature. However, it is not unusual the situation in which the introduction of a particular modification in any of the binding partners in order to improve the binding affinity in one direction (e.g. enthalpically, introducing a functional group able to establish an additional hydrogen bond) leads to an opposite counterbalancing effect in the opposite direction (entropically, maybe a bigger loss in conformational degrees of freedom upon binding or a lower hydrophobic desolvation), leaving the binding affinity unchanged (again the enthalpy/entropy compensation phenomenon). Traditionally, organic and medicinal chemists have optimized the binding affinity (that is, have made ΔG more negative) manipulating the entropic contribution: making ligands more hydrophobic and structurally constrained. That way, having the right shape and geometry for the binding site, the hydrophobic nature of the ligand is the driving force for directing it to the target. This is one of the reasons why many libraries are biased having a vast majority of hydrophobic compounds. The enthalpic term is more difficult to modulate and requires a very fine knowledge of the individual intermolecular interactions (van der Waals, electrostatics, hydrogen bonds, etc.) and the delicate, sometimes subtle, energetic balance involving the interaction network between bound molecules and the interaction network between free molecules and water. Therefore, considering that very high affinity can only be achieved if both enthalpy and entropy contribute favorably to the Gibbs energy of binding, enthalpically driven ligands possess better potentiality towards optimization than entropically driven ligands, because it is easier to improve entropy than enthalpy [17]. Besides, a favorable enthalpy contribution is an indication of specific interactions between binding partners and a good way to ensure ligand specificity, selectivity and adaptability. On the other hand, an unfavorable enthalpy contribution is an indication of non-specific interactions between the binding partners, making very difficult to provide specificity, selectivity and adaptability [17–28].

Calorimetry is an experimental technique providing only global information (change in thermodynamic potentials upon binding), with no possibility a priori for obtaining detailed (atomic or sub-molecular) knowledge about the origin or physical localization of the individual interactions. However, it is possible to modify either the experimental conditions (e.g. pH, ionic strength) or the functional groups in the binding molecules (e.g. mutant proteins or structurally related ligands) in order to shed light into which are the driving forces and the structural determinants of the interaction. This is the basis of performing quantitative-structure-activity-energetic-relationship studies, leading calorimetry to a privileged position as a fundamental tool for evaluation and decision-making steps in rational ligand optimization.

Fig. 2 shows two typical ITC experiments. RNase A is titrated with two structurally related inhibitors, 2′ CMP (cytidine monophosphate) and 3′ CMP, differing only in the location of the phosphate group. Both the sequence of injections (upper panel) and the differential heat plot or titration plot (lower panel) are depicted. The binding enthalpy is very similar in both cases (enthalpy can be inferred from the plot as the intercept with the y-axis at zero molar ratio), but the binding affinity is significantly different (affinity is related to the steepness of the titration plot at the inflection or neutralization point). This example illustrates the ability of this technique to measure very accurately the affinity of binding in addition to the possibility of measuring the enthalpy of binding, and the fact that small modifica-

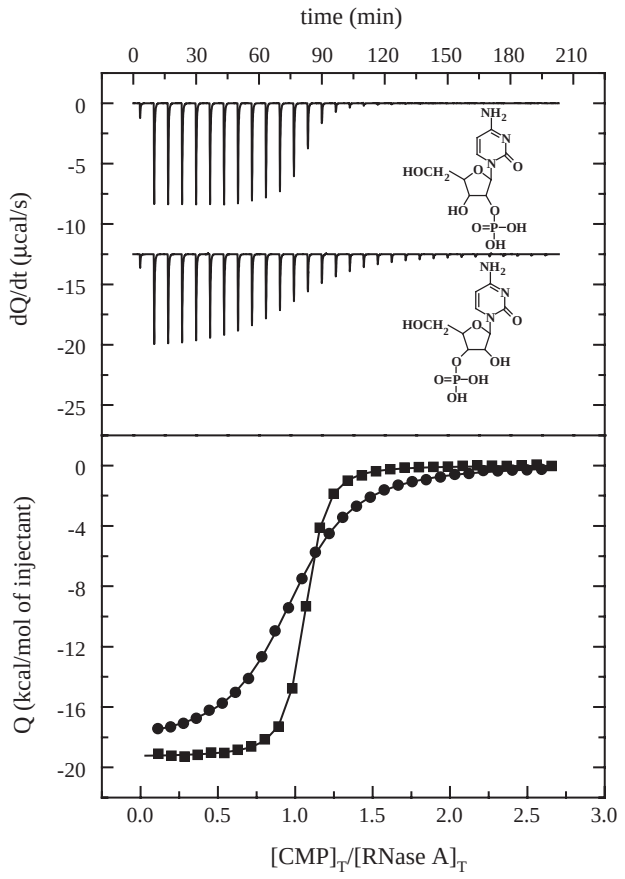

Fig. 2. Calorimetric titrations of RNase A with 2′ CMP and 3′ CMP (in 15 mM potassium acetate, pH 5.5, 25 °C). The signal recorded directly in each experiment, thermal power applied to maintain a constant temperature in the sample cell during the sequence of injections, is shown in the upper panel (the plot for the titration of 3′ CMP has been shifted down for clarity reasons). The integrated heat plots are shown in the lower panel (squares for 2′ CMP and circles for 3′ CMP). The thermodynamic binding parameters (binding affinity, binding enthalpy and stoichiometry) are obtained through non-linear regression of the experimental data. Changing the position of the phosphate group in the inhibitor (2′ to 3′) causes a 10-fold reduction in binding affinity ($2.0 \cdot 10^6 \pm 0.2 \cdot 10^6$ M^{-1} for 2′ CMP and $2.1 \cdot 10^5 \pm 0.2 \cdot 10^5$ M^{-1} for 3′ CMP) that can be visually detected comparing the steepness in the two plots. However, the binding enthalpy is similar for both ligands (-17.8 ± 0.2 kcal/mol for 2′ CMP and 18.1 ± 0.2 kcal/mol for 3′ CMP). Such affinity drop can be explained by less favorable binding entropy in the case of 3′ CMP. These two experiments illustrate the outstanding ability to determine affinity and enthalpy of binding in a single experiment, and the possibility to discriminate accurately small differences in affinity, Gibbs energy, enthalpy and entropy of binding.

tions in the ligand may cause significant changes in the thermodynamic parameters.

4. ITC: determining low, moderate and high binding affinity

Any technique employed for studying binding process suffers a limitation regarding the affinity range experimentally accessible. It is directly related to the concentration of macromolecule used in a typical experiment. Thus, it has been established that, in order to get reliable estimates of the binding affinity, the following relationship must be satisfied [2]:

$$0.1<K_a[M]_T<1000 \quad (8)$$

where $[M]_T$ is the total concentration of macromolecule titrated in the cell. This condition ensures that the curvature in the titration plot is appropriate (see Fig. 3): not so low that the titration plot is almost linear or so high that the titration plot is almost rectangular. Therefore, if the typical concentration of macromolecule is in the order of 10 μM (common in ITC experiments), then affinity values within the range 10^4-10^8 M^{-1} can only be reliably estimated. Lowering or raising the macromolecule concentration will raise or lower the limits for the affinity, accordingly.

Three cases may be considered: very low ($K_a<10^4$ M^{-1}), moderate (10^4 $M^{-1}<K_a<10^8$ M^{-1}) and very high affinity ($K_a>10^8$ M^{-1}). The moderate affinity case is the ideal one, from the experimental point of view, since binding affinity, binding enthalpy and stoichiometry can be determined independently and simultaneously in a single experiment without any assumptions or dependency in the estimated parameters. In the very high affinity case, both the binding enthalpy and the stoichiometry can be determined very accurately, but the binding affinity is not reliable. Finally, in the very low affinity case, neither one of the parameters can be obtained reliable without introducing additional assumptions or compromising the quality of the data analysis.

Traditionally, very low/high affinity systems have been transformed into moderate affinity systems changing the experimental conditions (e.g. changing the temperature or the pH), in order to alter the affinity towards values within the experimentally accessible range [29,30]. However, this method presents some limitations: temperature or pH changes might compromise the thermodynamic or kinetic stability of certain biological molecules, the changes in binding affinity are of limited extension, linkage equations are needed to extrapolate (sometimes with significant errors) to the intended experimental conditions, and some additional information is required (e.g. pK_a of ionizable groups involved in the proton transfer process coupled to the binding of the ligand).

In the last years, the displacement method, used extensively in spectroscopy, has been applied successfully in calorimetry when dealing with very high or very low affinity systems [31–33]. The basis of the method is the following fact: the alteration of the binding properties of a ligand when another competing ligand is present. It should be noted that the binary equilibrium macromolecule/ligand implies solving a quadratic equation, whereas the introduction of a second competing ligand turns the problem into a ternary equilibrium requiring solving a cubic equation, problem whose analytical solution may be even considered rather easy. However, more complex systems might involve solving high order polynomial equations, with cumbersome or impractical analytical solution (e.g. ligand that promotes dimerization in a protein exhibiting monomer–dimer equilibrium requires solving a quartic equation). On the other hand, numerical methods present three advantages in general: (a) they usually require only a small piece of

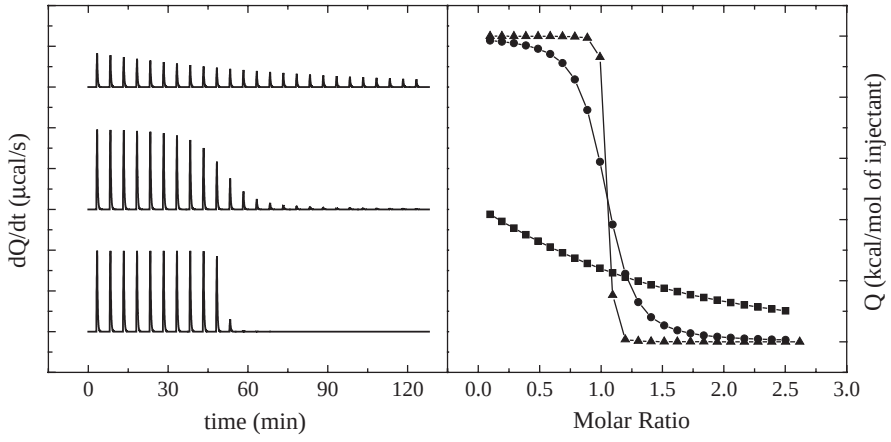

Fig. 3. Illustration of the effect of the binding affinity value on the shape of a titration curve. The plots represent three titrations simulated using the same parameters (concentrations of reactants and binding enthalpy), but different binding affinities. Low (squares), moderate (circles) and high affinity (triangles) binding reactions are shown, corresponding to $K_a[M]_T$ equal to 1 ($K_a=10^4$ M^{-1}), 100 ($K_a=10^6$ M^{-1}) and 10,000 ($K_a=10^8$ M^{-1}), respectively. In order to obtain accurate estimates of the association constant, an intermediate case is desirable ($1<K_a[M]_T<1000$).

programming code; (b) if the equations involved exhibit "good behavior" (e.g. derivatives do not vanish within the interval of interest), the convergence to the sought solution is quite fast starting from a reasonable initial guess, without the need for discriminating which of the possible solutions has physical meaning; and (c) complex problems, impracticable by analytical methods, are accessible. In what follows, the application of this approach to calorimetry is outlined briefly. Models corresponding to stoichiometry 1:1 are considered, but generalizations for 1:n or other stoichiometric ratios are easily made.

The heat evolved after any injection through the ITC experiment is proportional to the amount of complex macromolecule/ligand formed, that in turn, depends on the composition (total concentration of molecules) before and after that injection and the equilibrium constants or association constants determining the partition between free and bound species through the mass action law. Thus, the starting point would be determining the composition of the solution inside the calorimetric cell before and after any injection, i.e. the concentration of free and bound molecules given the total concentration of the different molecules (known variable) and the equilibrium constants; from that, any change in the different species is easily calculated. Let $\{[M_p]_T\}_{p=1,\ldots,n}$ be the total concentration of the different molecules present in the solution (p=1,. . .,n), after injection i and $\{K_{a,q}\}_{q=1,\ldots,m}$ the equilibrium constant governing each equilibrium in the solution (q=1,. . .,m). Making use of the mass conservation principle and the mass action law it is possible to express the concentration of any of the free species $\{[M_p]\}_{p=1,\ldots,n}$ as a function of total concentrations and equilibrium constants:

$$f_p\left([M_p]; \{[M_p]_T\}_{p=1,\ldots,n}, \{K_{a,q}\}_{q=1,\ldots,m}\right) = 0 \quad (9)$$

Therefore, it is possible to obtain numerically the solution to this equation, $[M_p]^*$, applying the Newton–Raphson algorithm:

$$[M_p]^* = \lim_{k\to+\infty} [M_p]_k$$

$$[M_p]_0 = \alpha$$

$$[M_p]_{k+1} = [M_p]_k - \frac{f_p([M_p]_k)}{f_p'([M_p]_k)} \quad \text{while}$$

$$\left|[M_p]_{k+1} - [M_p]_k\right| \geq \varepsilon \quad (10)$$

where f_p' is the derivative of the function f_p with respect to the variable $[M_p]$. It is recommended to use α=0.5$[M_p]_T$ as the initial value to start the iterative process and $\varepsilon=10^{-15}$ M as the convergence criterion. According to many simulated situations, the Newton–Raphson method applied to binding equilibrium equations provides very fast results and convergence is reached in less than 10 iterations. Once this solution is obtained, the concentration of all species present is calculated using again the mass conservation and mass action law equations. There is one detail left: how to compute the total concentration of any molecule before and after any injection, that is, the known variables $[M_p]_T$. If the calorimeter operates at constant cell volume, the total concentration of a molecule M_p placed in the calorimetric cell after injection i is:

$$[M_p]_{T,i} = [M_p]_0\left(1 - \frac{v}{V}\right)^i \quad (11)$$

where $[M_p]_0$ is its initial concentration in the cell, v is the injection volume and V is the calorimetric cell volume. And the total concentration of a molecule M_p injected in the calorimetric cell is:

$$[M_p]_{T,i} = [M_p]_0\left(1 - \left(1 - \frac{v}{V}\right)^i\right) \quad (12)$$

where $[M_p]_0$ is its concentration in the syringe.

5. ITC: moderate affinity

The usual titration scheme is appropriate for determining affinity and enthalpy of binding. The syringe contains the ligand solution and the macromolecule solution is placed in the calorimetric cell. This system corresponds to a binary equilibrium (n=2, m=1). Although this system can be solved analytically, we will employ the numerical approach. After any injection i, from the mass conservation principle:

$$[M_1]_{T,i} = [M_1]_i + [M_1M_2]_i$$

$$[M_2]_{T,i} = [M_2]_i + [M_1M_2]_i \quad (13)$$

and the mass action law:

$$K_a = \frac{[M_1M_2]_i}{[M_1]_i[M_2]_i} \quad (14)$$

it is possible to write the equations for the free concentration of each molecule as a function of the total concentrations and the equilibrium constant:

$$[M_1]_i^2 K_a + [M_1]_i(1 + K_a[M_2]_{T,i} - K_a[M_1]_{T,i}) - [M_1]_{T,i} = 0$$

$$[M_2]_i^2 K_a + [M_2]_i(1 + K_a[M_1]_{T,i} - K_a[M_2]_{T,i}) - [M_2]_{T,i} = 0 \quad (15)$$

Since these two expressions are equivalent (they reflect the symmetry of the system), let us continue using the first expression in Eq. (15), which can be expressed:

$$[M_1]_i^2 a + [M_1]_i b + c = 0 \quad (16)$$

and the numerical solution can be obtained following this iterative procedure:

$$[M_1]_{k+1,i} = [M_1]_{k,i} - \frac{[M_1]_i^2 a + [M_1]_i b + c}{[M_1]_i 2a + b}$$

$$[M_1]_{0,i} = \frac{[M_1]_{T,i}}{2} \tag{17}$$

And now it is possible to calculate $[M_1M_2]$ after injection i:

$$[M_1M_2]_i = [M_2]_{T,i} \frac{K_a[M_i]_i}{1 + K_a[M_1]_i} \tag{18}$$

and the heat, q_i, associated with injection i:

$$q_i = V\Delta H\left([M_1M_2]_i - [M_1M_2]_{i-1}\left(1 - \frac{v}{V}\right)\right) \tag{19}$$

This expression is employed in the data analysis procedure to estimate the binding affinity, K_a, and the binding enthalpy, ΔH, as adjustable parameters by non-linear regression. This is the type of analysis performed in cases similar to those illustrated in Fig. 2.

6. ITC: extreme affinity

The usual titration scheme has to be modified in order to be able to determine the affinity and enthalpy of binding of a weak or very potent ligand. We will consider two competing ligands of different potency and the system corresponds to a ternary equilibrium (n=3, m=2). The more potent ligand, A, is placed in the syringe and the weaker ligand, B, in the calorimetric cell together with the macromolecule, M. Although this system can be solved analytically, we will employ the numerical approach again. After any injection i, from the mass conservation principle:

$$[M]_{T,i} = [M]_i + [MA]_i$$

$$[A]_{T,i} = [A]_i + [MA]_i$$

$$[B]_{T,i} = [B]_i + [MB]_i \tag{20}$$

and the mass action law:

$$K_{a,A} = \frac{[MA]_i}{[M]_i[A]_i}$$

$$K_{a,B} = \frac{[MB]_i}{[M]_i[B]_i} \tag{21}$$

it is possible to write the equation for the free concentration of macromolecule as a function of the total concentrations and the equilibrium constants:

$$[M]_i^3 K_{a,A}K_{a,B} + [M]_i^2(K_{a,A} + K_{a,B} + K_{a,A}K_{a,B}([A]_{T,i} + [B]_{T,i} - [M]_{T,i})) + [M]_{T,i}(1 + K_A[A]_{T,i} + K_{a,B}[B]_{T,i} - (K_{a,A} + K_{a,B})[M]_{T,i}) - [M]_{T,i} = 0 \tag{22}$$

which is also symmetrical respect to the two ligands and it can be expressed:

$$[M]_i^3 a + [M]_i^2 b + [M]_i c + d = 0 \tag{23}$$

and the numerical solution can be obtained following this iteration procedure:

$$[M]_{k+1,i} = [M]_{k,i} - \frac{[M]_i^3 a + [M]_i^2 b + [M]_i c + d}{[M]_i^2 3a + [M]_i 2b + c}$$

$$[M]_{0,i} = \frac{[M]_{T,i}}{2} \tag{24}$$

Now it is possible to calculate [MA] and [MB] after injection i:

$$[MA]_i = [A]_{T,i} \frac{K_{a,A}[M]_i}{1 + K_{a,A}[M]_i}$$

$$[MB]_i = [B]_{T,i} \frac{K_{a,B}[M]_i}{1 + K_{a,B}[M]_i} \tag{25}$$

and the heat, q_i, associated with injection i:

$$q_i = V\Delta H_A\left([MA]_i - [MA]_{i-1}\left(1 - \frac{v}{V}\right)\right) + V\Delta H_B\left([MB]_i - [MB]_{i-1}\left(1 - \frac{v}{V}\right)\right) \tag{26}$$

where ΔH_A and ΔH_B are the binding enthalpy of ligands A and B, respectively. This expression is employed in the non-linear regression data analysis procedure in order to determine ΔH_A and $K_{a,A}$ or ΔH_B and $K_{a,B}$ as adjustable parameters. The strategy and the formalism are the same in the two cases, very high or very low affinity determination, but there is a subtle difference as it is explained below.

If we are interested in characterizing a very high affinity ligand (K_a>10^8 M^{-1}), then a moderate affinity ligand (10^4 M^{-1}<K_a<10^8 M^{-1}) is used as competing ligand. Three titrations are performed (Fig. 4): (1) direct titration of high affinity ligand A into the macromolecule solution, from which only the binding enthalpy ΔH_A can be obtained; (2) direct titration of moderate affinity ligand B into the macromolecule solution, from which both the binding affinity $K_{a,B}$ and the binding enthalpy ΔH_B can be obtained; and (c) displacement titration of potent ligand A into a

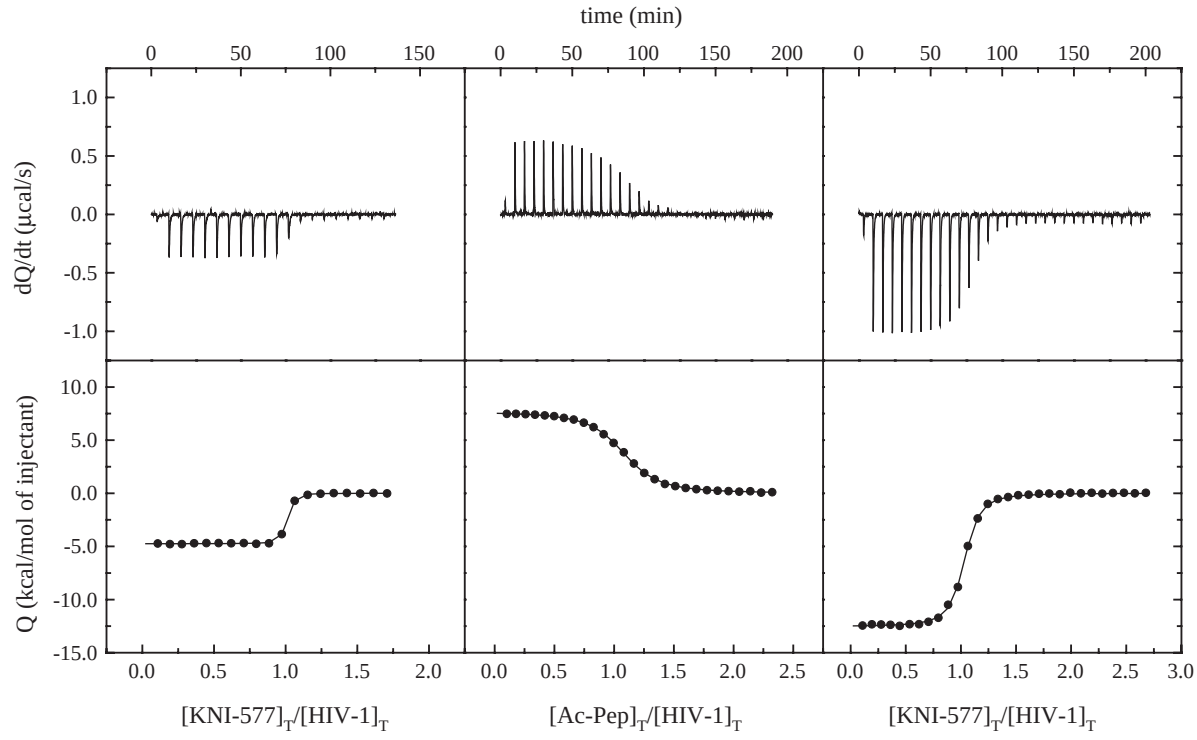

Fig. 4. Determining very high binding affinity. Experimental scheme aimed at determining the thermodynamic parameters for the binding of KNI-577 to HIV-1 protease. Three titrations are shown: (left) KNI-577 binding to HIV-1, (center) acetyl-pepstatin binding to HIV-1 and (right) KNI-577 binding to HIV-1 pre-bound to acetyl-pepstatin. In the first titration, the only binding parameter estimated is: ΔH=4.7±0.2 kcal/mol, because affinity cannot be reliably determined. In the second titration, the estimated parameters are: K_a=2.3 · 10^6±0.2 · 10^6 M^{-1}, ΔH=8.0±0.2 kcal/mol. Applying the displacement analysis to the third titration yields the following the following parameters for the binding of KNI-577 to HIV-1 protease: K_a=4.9 · 10^9±0.3 · 10^9 M^{-1}, ΔH=4.7±0.2 kcal/mol.

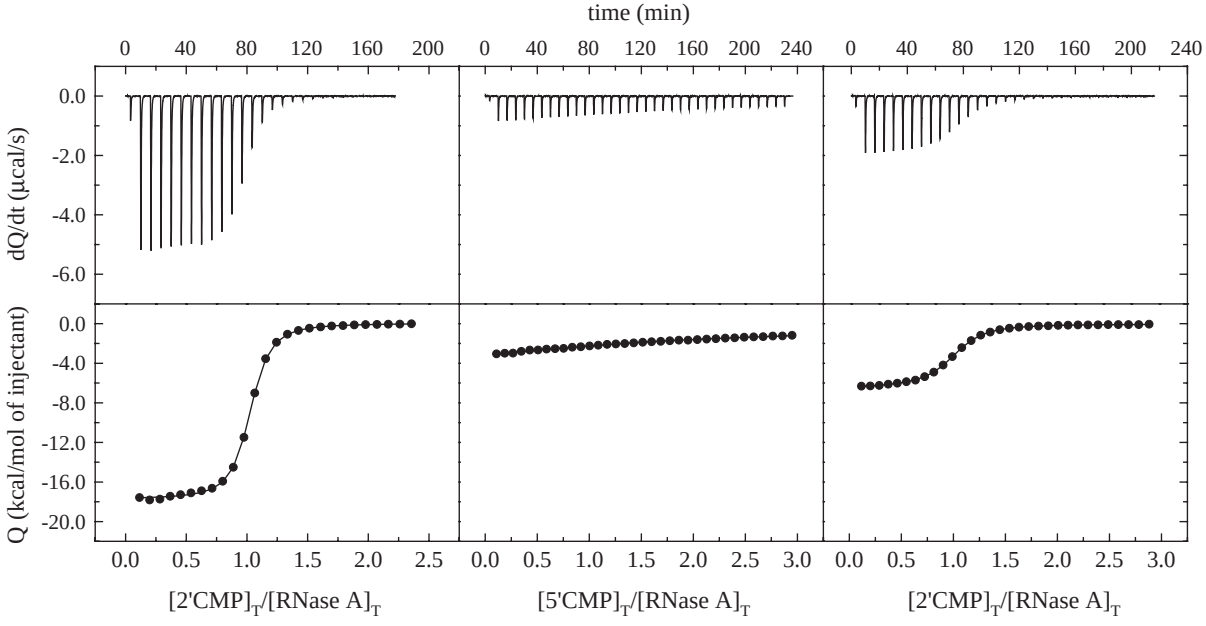

Fig. 5. Determining very low binding affinity. Experimental scheme aimed at determining the thermodynamic parameters for the binding of 5′ CMP to RNase A. In the case of the CMP inhibitors of RNase A, a change in the position of the hydroxyl group from 2′ to 5′ position causes a dramatic decrease in the strength of the interaction, thus, illustrating the effect of minor modifications in the ligand on the thermodynamic parameters of binding. Three titrations are shown: (left) 2′ CMP binding to RNase A, (center) 5′ CMP binding to RNase A and (right) 2′ CMP binding to RNase A pre-bound to 5′ CMP. In the first titration, the binding parameters are: K_a=2.0 · 10^6±0.2 · 10^6 M^{-1} and ΔH=−17.8±0.1 kcal/mol. It is not possible to obtain a reliable value for either the affinity or the enthalpy of binding from the second titration. Applying the displacement analysis to the third titration yields the following thermodynamic parameters for the binding of 5′ CMP to RNase A: K_a=4.2 · 10^3 M^{-1}, ΔH=−16.3 kcal/mol.

solution of macromolecule and ligand B. The binding parameters obtained in the second titration, ΔH_B and $K_{a,B}$, are included in the non-linear fitting procedure for the last titration experiment, in order to estimate the binding parameters of the high affinity ligand, ΔH_A and $K_{a,A}$.

If we are interested in characterizing a very low affinity ligand ($K_a<10^4$ M^{-1}), then a moderate affinity ligand (10^4 $M^{-1}<K_a<10^8$ M^{-1}) is used as competing ligand. Three titrations are performed (Fig. 5): (1) direct titration of moderate affinity ligand A into the macromolecule solution, from which the both the binding affinity $K_{a,A}$ and the binding enthalpy ΔH_A can be obtained; (2) direct titration of low affinity ligand B into the macromolecule solution, from which neither the binding affinity $K_{a,B}$ nor the binding enthalpy ΔH_B can be reliably obtained; and (3) displacement titration of moderate affinity ligand A into a solution of macromolecule and ligand B. The binding parameters obtained in the first titration, ΔH_A and $K_{a,A}$, are included in the non-linear fitting procedure for the last titration experiment, in order to estimate the binding parameters of the potent ligand, ΔH_B and $K_{a,B}$.

7. Conclusions

ITC provides more information than spectroscopic techniques, usually more accurate information and, very important, extremely useful and valuable. Whereas the Gibbs energy is the thermodynamic potential which determines if a given binding process is thermodynamically favored or not and the strength of the interaction, its partition between enthalpy and entropy is equally important. Since these two terms reflect energetic contributions of very different nature, if two binding processes are characterized by the same Gibbs energy but very different partition between enthalpy and entropy, they correspond to different binding modes and the main underlying driving intermolecular interactions are different. For that reason, the binding affinity (or the Gibbs energy) is only "half of the story". Traditionally, attention has only been paid to binding affinity and screening and optimization protocols have been designed bearing in mind only the affinity of binding as a criterion, overlooking the importance of enthalpy and entropy as descriptors. ITC allows a further classification of ligands: according to their binding affinity and according their enthalpy/entropy balance. Entropically driven ligands, characterized by a huge, favorable entropic contribution do not bind because they interact favorably with the target (as it would be in the case of favorable enthalpic interactions), but because they have the right shape and geometry and they are very hydrophobic (as it is reflected in their huge favorable entropic contribution). Moreover, since shape and hydrophobicity represent non-specific interactions, it is obvious that this would lead to a reduction in affinity when the target binding site is distorted by mutations (even conservative ones), thus, appearing the phenomenon of drug resistance. In addition, enthalpic and entropic ligands exhibit different potential towards optimization: enthalpic ones already interact favorably and specifically with the target and they could be easily modified adding some hydrophobic groups or structural constraints, whereas entropic ones need to be redesigned energetically and little room for hydrophobic or conformational optimization is left. It has been shown how ITC can be used for characterizing very broad affinity systems (K_a from 10^{-2} to 10^{12} M^{-1}). Given the exceptional capabilities (very broad affinity determination range, universal detection system with no reporter labels required, possibility of using optically dense solutions, exhaustive thermodynamic information accessible, applicability to complex systems, etc.) for extracting very useful thermodynamic information and characterizing binding processes, it is expected a more prominent role for ITC in the fields of drug discovery and cell biology when thermodynamic methods are totally integrated with many other experimental methodologies.

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