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The Use of Isothermal Titration Calorimetry in Drug Design: Applications to High Affinity Binding and Protonation / Deprotonation Coupling

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Isothermal Titration Calorimetry (ITC) plays a central role in the characterization and optimization of lead compounds as viable drug candidates. ITC is the only technique that permits a complete experimental characterization of the binding affinity of a ligand in terms of its thermodynamic components ΔG, ΔH, ΔS and ΔC_p. In addition, ITC is ideally suited to characterize coupled processes like protonation/deprotonation reactions. One of the major challenges for ITC has been the analysis of ligands with high binding affinities. This issue becomes a serious problem in drug design, where one of the main goals is to optimize the binding affinities of lead compounds to nanomolar or subnanomolar levels. These binding affinities have been traditionally considered to be beyond the useful range of calorimetric analysis. In this paper we will discuss the implementation of ITC experimental designs aimed at characterizing very high affinity binding processes ($K_a > 10^9\ M^{-1}$) and apply them to the characterization of HIV-1 protease inhibitors. We will also discuss the characterization of protonation/deprotonation coupling to the binding reaction.

1. Introduction

Structure-based drug design is one of the new frontiers in the post-genomic era. With the completion of the Human Genome Project about 50,000 genes encoding for as many proteins will be identified. Many of these proteins will be targets for drug design. In addition, the entire genomes of several pathogens have also been completed and many more will be available in the near future. Many of the proteins identified from genomic information of pathogens will also become targets for drug development against a wide variety of infectious diseases. These extraordinary developments underline the need for design strategies that accurately address the issues of binding affinity, specificity, and selectivity.

Existing strategies for lead identification and optimization rely almost exclusively on binding affinities. However, the binding affinity, $K_a = \exp(-\Delta G/RT)$, is determined by the Gibbs energy of binding, ΔG, which in turn is determined by the enthalpy, ΔH, and entropy, ΔS, changes ($\Delta G = \Delta H - T\Delta S$). In principle, many combinations of ΔH and ΔS values can give rise to the same ΔG value and, therefore, elicit the same binding affinity. Isothermal titration calorimetry plays a unique role in drug design because it is the only technique with the ability to measure the individual components of the Gibbs energy of binding (ΔH, ΔS, ΔC_p). As such it can be used not only to characterize existing ligands but to guide the design process, as it permits a direct evaluation of the energetic effects of specific chemical modifications. Most literature discussions about isothermal titration calorimetry deal with the initial stages of the drug development problem; i.e. when the binding affinities are still low and readily measurable by direct calorimetric titration. As the design process gets under way, new generations of compounds are created, exhibiting higher binding affinities with each successive iteration. At some stage in the optimization process the binding affinity

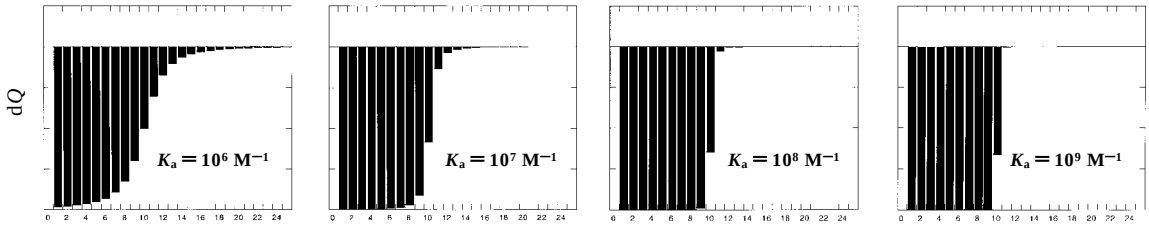

Fig.1 Simulated ITC experiments for increasing values of the association constant, K_a. Each panel shows the amount of heat released for each ligand injection. The following parameters were used in the simulations: $\Delta H = -8$ kcal mol^{-1}, protein concentration in cell 1 mg mL^{-1} (MW = 14,000), ligand concentration in syringe 1 mg mL^{-1} (MW = 1,000), injection volume = 10 μL, cell volume = 1.4 mL.

exceeds the appropriate range for direct calorimetric titrations. At this point, new experimental designs need to be implemented in order to accurately measure binding affinities by ITC. In this paper, we will discuss the application of ITC to tight binding reactions ($K_a > 10^9$ M^{-1}) and to the characterization of coupled processes like protonation/deprotonation.

2. Results and Discussion

2.1 High Affinity Binding

In drug design, it is customary to begin the optimization process with lead compounds exhibiting binding affinities in the micromolar range ($K_a = 10^6$ M^{-1}). These compounds are chemically optimized in order to achieve binding affinities in the nanomolar or subnanomolar range ($K_a > 10^9$ M^{-1}). Along this path, the ability of ITC to directly measure binding affinities becomes severely compromised as illustrated in **Fig.1**. A practical rule of thumb to determine this limit is given by the value of the product between the total macromolecule concentration and the binding affinity ($[\mathbf{M}]_{Total} \times K_a$). This quantity needs to be between 1～1000 for accurate binding constant determination as discussed by Wiseman *et al*.[1] As shown in **Fig.1**, as the binding constant increases, the titrations become progressively stoichiometric and the binding affinity cannot be determined: all the ligand injected into the reaction cell binds to the protein until saturation is achieved. Under those conditions, however, the binding enthalpy can be determined very accurately even though the binding constant cannot be measured.

In drug design, the practical limits for direct ITC binding constant determination are rapidly exceeded as new compounds with higher binding affinities are synthesized. Once this situation is reached, an equally powerful experimental design can be utilized: titration competition experiment. Even under conditions in which the binding affinity is too large to be accurately measured by direct calorimetric titrations, it can be measured in competition experiments with a well characterized inhibitor of lower affinity. This is an ideal situation in drug development since low affinity inhibitors always become available during the optimization process. The mathematics of binding competition has been presented by Wang and Sigurskjold.[2,3]

If the titration of the high affinity inhibitor is made in the presence of a weak inhibitor in the reaction cell (non-titrating inhibitor), the apparent thermodynamic parameters (apparent binding constant, K_{app}, and apparent binding enthalpy, ΔH_{app}) depend on the concentration of the weak inhibitor and are related to the intrinsic parameters by the following equations:

$$K_{app} = \frac{K_a}{1 + K_{a,1}\ [L_1]} \quad (1)$$

$$\Delta H_{app} = \Delta H_{bind} - \Delta H_{bind,1} \frac{K_{a,1}\ [L_1]}{1 + K_{a,1}\ [L_1]} \quad (2)$$

where K_a and $K_{a,1}$ are the association constants for the high affinity (titrating) and low affinity (non-titrating) inhibitors; ΔH_{bind} and $\Delta H_{bind,1}$ are the binding enthalpies for the high affinity (titrating) and low affinity (non-titrating) inhibitors and $[L_1]$ is the free concentration of the non-titrating inhibitor. If the concentration of the non-titrating inhibitor is much higher than the protease

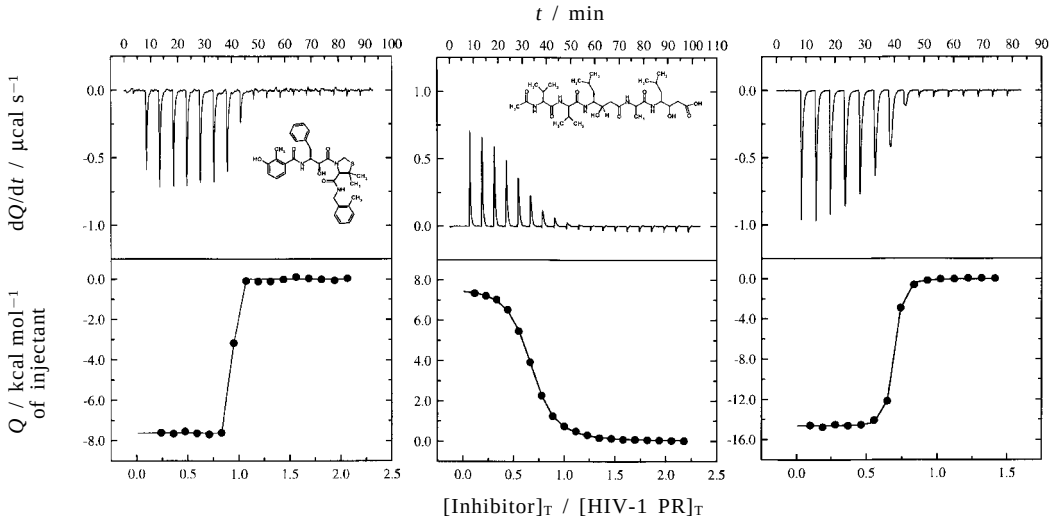

Fig.2 Calorimetric titrations of wild type HIV-1 protease with the inhibitor KNI-764 (left), the inhibitor acetyl-pepstatin (center) and with KNI-764 in the presence of acetyl pepstatin (displacement titration) (right). These experiments were performed at 25 ℃ in 10 mM acetate, pH 5.0, 2 % DMSO. For each experiment the reactant concentrations were: left: protease＝20 μM, KNI-764＝300 μM,; center: protease＝20 μM, acetyl pepstatin＝300 μM; right: protease＝20 μM, acetyl pepstatin＝200 μM, KNI-764＝250 μM.

concentration then $[L_1]$ is well approximated by its total concentration. Furthermore, if the displacement experiment is performed in the presence of saturating concentration of the weak inhibitor, then equation 2 reduces to:

$$\Delta H_{app} = \Delta H_{bind} - \Delta H_{bind,1} \quad (3)$$

It must be noted that if the high affinity and low affinity inhibitors have binding enthalpies of opposite sign, then the calorimetric signal will be amplified improving the signal-to-noise ratio. A total of three titration experiments need to be done in order to determine the association constant for the tight binding inhibitor: Individual titrations with each inhibitor in order to measure $K_{a,1}$, $\Delta H_{bind,1}$ and ΔH_{bind}, and a displacement titration in which the weak inhibitor is present at a constant concentration in the reaction cell.

We will illustrate the implementation of a competition titration design with the calorimetric characterization of the HIV-1 protease inhibitor KNI-764. This inhibitor developed by Kiso and collaborators[4-7] is a second generation protease inhibitor characterized by an inhibition constant, Ki, in the picomolar range. Direct calorimetric titrations (**Fig.2** left) indicate that this inhibitor binds with a negative enthalpy and that the binding affinity is well beyond the reach of direct calorimetric measurement.

In our experimental design, acetyl pepstatin is used as the non-titrating weak inhibitor. The binding of this inhibitor to the HIV-1 protease is well characterized.[8] The experimental conditions were set such that the total concentration of acetyl pepstatin (200 μM) is about 10-fold higher than the total protein concentration (20 μM) in the reaction cell. As a result, the free concentration of the non-titrating inhibitor is 180 μM at the beginning of the experiment and remains approximately constant throughout the entire experiment due to the compensating effects of displacement and dilution after each injection of the high affinity inhibitor. The binding of acetyl pepstatin to the HIV-1 protease is endothermic. Therefore, this inhibitor is very appropriate for this kind of competition calorimetric experiments: the calorimetric signal due to the displacement of acetyl pepstatin by KNI-764 is even more exothermic than the one measured in a direct binding experiment of KNI-764 without competition.

Fig.2 illustrates the three experiments necessary for the thermodynamic characterization of the tight binding

inhibitor. The left panel shows the titration of KNI-764 into the protease; The central panel, the titration of acetyl pepstatin into the protease; and the right panel, the titration of KNI-764 into the protease/acetyl pepstatin mixture. It is clear in the figure that the displacement titration produces a larger signal (due to the displacement of an endothermic inhibitor by an exothermic one) and a heat binding isotherm (right panel, bottom) with sufficient curvature to allow determination of the apparent binding affinity. The competition experiments were performed at pH 3.8 and 5.0 using buffers with negligible ionization enthalpy (formate and acetate buffers, respectively) in order to minimize the effect of the proton release on the binding enthalpy. From these and similar experiments at different pH values we determined binding constants for KNI-764 of 1.7×10^{10} M^{-1} and 3.1×10^{10} M^{-1} at pH 3.8 and 5 respectively.

2.2 Coupling of the Binding Reaction to Protonation/ Deprotonation Processes

The extent and contribution of protonation/ deprotonation processes to the binding of a ligand can be assessed by the determination of the pH-dependence of the binding enthalpy and by the use of buffers with different enthalpies of ionization.[9,10] In a binding process certain ionizable groups in the binding site and in the ligand may experience a change in their pK. As a result, and depending on the pH of the experiments, the binding reaction will be coupled to a protonation/deprotonation process. The physical evidence for the coupling between binding and proton transfer (proton linkage) is the pH-dependence of the binding thermodynamic parameters (enthalpy, association constant, Gibbs energy, entropy) and also the dependence of the measured or apparent binding enthalpy (ΔH_{app}) on the ionization enthalpy of the buffer in which the experiments are made.[9] If the binding reaction absorbs or releases protons, those protons will be given or taken by the buffer used in the experiments and the measured enthalpy change (ΔH_{app}) will be a function of the ionization enthalpy of the buffer:

$$\Delta H_{app} = \Delta H_{bind} + n_H \Delta H_{ion} \quad (4)$$

where ΔH_{app} (buffer-dependent and pH-dependent), is the sum of two terms: the reaction enthalpy, ΔH_{bind}, independent of the buffer used in the experiment (but pH-dependent) and another term representing the contribution of the proton ionization of the buffer, ΔH_{ion}, which is multiplied by n_H, the number of protons that are released (or absorbed if n_H is negative) by the buffer. A plot of ΔH_{app} versus ΔH_{ion} provides the simplest and most straightforward way of determining if the reaction under study is coupled to a protonation/deprotonation process.

If it is determined that the binding reaction is coupled to protonation/deprotonation then a more detailed analysis of n_H and ΔH_{bind} is required. This analysis requires a combination of measurements at different pH values and buffers with different ionization enthalpies. The pH dependence of the thermodynamic parameters is analyzed in terms of standard proton-linkage equations[10,11]:

$$n_H = \sum_i \left(\frac{10^{pK_i^c - pH}}{1+10^{pK_i^c - pH}} - \frac{10^{pK_i^f - pH}}{1+10^{pK_i^f - pH}} \right) \quad (5)$$

$$\Delta H_{bind} = \Delta H^0_{bind} + \sum_i \left(\left(\frac{10^{pK_i^c - pH}}{1+10^{pK_i^c - pH}} - \frac{10^{pK_i^f - pH}}{1+10^{pK_i^f - pH}} \right) \Delta H_{p,i} + \frac{10^{pK_i^c - pH}}{1+10^{pK_i^c - pH}} \Delta\Delta H_{p,i} \right) \quad (6)$$

$$K_a = K_a^0 \prod_i \left(\frac{10^{pK_i^c - pH}}{1+10^{pK_i^f - pH}} \right) \quad (7)$$

where pK_i^c and pK_i^f are the pK values for the i ionizable group in the ligated (complex) and in the unligated species (free), ΔH^0_{bind} is the buffer-independent and pH-independent binding enthalpy (fully deprotonated species), $\Delta H_{p,i}$ and $\Delta\Delta H_{p,i}$ are the protonation enthalpy and change in the protonation enthalpy for the i ionizable group in the protein or in the ligand, respectively, and K_a^0 is the pH-independent association constant (fully deprotonated species). The difference between the pK values after, pK_i^c, and before the binding, pK_i^f, reflects the change in the population of the protonated and deprotonated species for each ionizable group upon binding and the character of the contribution (favorable or not) to the global affinity and enthalpy of each single proton transfer.

Fig.3 shows the n_H and ΔH_{bind} values (equation 4) determined for the HIV-1 protease inhibitor KNI-

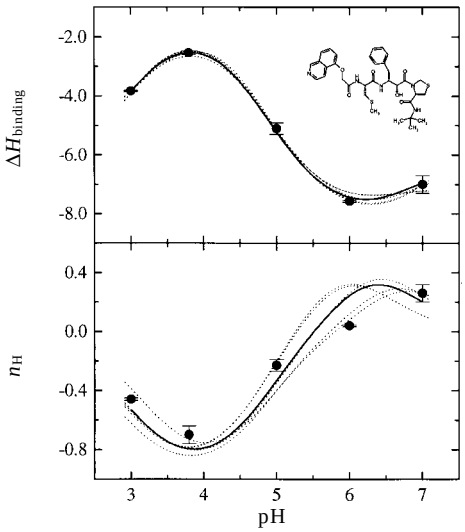

Fig.3 Binding of the inhibitor KNI-272 (insert) to the HIV-1 protease. The buffer independent binding enthalpy (top panel) and the change in the number of protons associated with the complex upon binding (bottom panel) as a function of pH. The solid lines correspond to the pH dependence calculated with the average values for the set of parameters obtained by global non-linear least squares of the data. The dotted lines correspond to the curves calculated with the parameter values obtained with individual fits.

272 at different pH values.[11] These quantities are a function of the pK and protonation enthalpies of the groups involved in both the free and bound forms, as well as the intrinsic enthalpy of the reference state, which corresponds to the fully deprotonated species. The n_H and ΔH_{bind} can be fitted globally by non-linear least squares regression analysis in terms of equations 5 and 6 as described in.[11] The data could be fitted well to a minimum of two ionization sites with no improvement in the goodness of the fit if a larger number of sites were included. Several alternatives were considered in the analysis in order to evaluate the robustness of the fit. These alternatives included fixing some of the fitting parameters to some specific values. Since one of the groups undergoing protonation/deprotonation appears to be an aspartic residue, the protonation enthalpy of one of the free groups was set to -1.3 kcal mol^{-1} which corresponds to the protonation enthalpy of a solvent exposed free carboxylic group.[12]

The analysis was consistent with the presence of two ionization sites with pK values of 6.0 and 4.8 in the free protein and 6.6 and 2.92 in the complex. Interestingly, the site characterized by the pK values of 6.0 and 6.6 is the one associated with the protonation enthalpy of a carboxylic group, strongly suggesting that this group is one of the aspartates in the aspartyl dyad. It has been concluded before that one of these groups has a pK $\sim$6 in the free state and that this pK increases to higher values when bound to KNI-272.[13,14] The second ionizable group has an apparent pK of 4.8 in the free protein and 2.92 in the complex. This group is estimated to have a protonation enthalpy of -6.3 kcal mol^{-1} in the uncomplexed system suggesting that a group different than an aspartyl moiety is contributing to the protonation thermodynamics. One plausible candidate is the nitrogen in the aromatic isoquinoline ring of the inhibitor which has a pK value and a protonation enthalpy very close to the pK and ionization enthalpy values obtained by the non-linear least squares fitting for the uncomplexed system. Together, these data strongly suggest that the two groups undergoing protonation/deprotonation are one of the aspartates in the aspartyl dyad and the aromatic isoquinoline nitrogen in KNI-272.

According to the non-linear least squares analysis, the estimated buffer- and pH-independent binding enthalpy is -6.3 kcal mol^{-1}, indicating that the binding of KNI-272 is intrinsically exothermic and not due to the coupling of the binding process to a protonation/deprotonation reaction.

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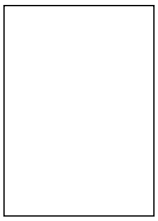

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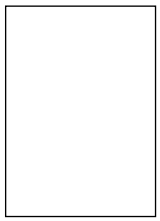

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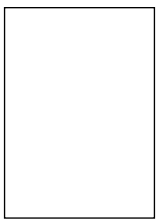

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