TA Instruments – Application Note

Enthalpy Screening by Isothermal Titration Calorimetry

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There are situations in which it is necessary to measure the binding enthalpy of a relatively large number of compounds for which binding affinities or IC_{50}'s have already been estimated using other approaches. In drug discovery for example, high throughput screening (HTS) is usually the first step in the identification of potential lead compounds. HTS often produces a number of hits that need to be ranked in terms of their quality as potential drug candidates. Recently, different metrics have been proposed in order to evaluate the drug-like quality of lead compounds. Among them, LipE has achieved widespread prominence. LipE or lipophilic efficiency is defined as LipE = pK_d – LogP, where pK_d is the negative logarithm of the dissociation constant (sometimes the inhibition constant, K_i, or the IC_{50} are used) and LogP, the logarithm of the partition coefficient of the compound between n-octanol and water (see for example http://en.wikipedia.org/wiki/LiPE). LipE essentially quantitates the proportion of the binding affinity that originates from forces other than hydrophobicity. LipE is just the difference between the logarithms of the binding potency and hydrophobicity. The higher the LipE value the better the quality of the compound. In our laboratory, we have discovered that LipE is proportional to the binding enthalpy. The more favorable the binding enthalpy the higher the LipE value. A polar group that establishes a strong hydrogen bond contributes favorably (exothermically) to the binding enthalpy, whereas a polar group that becomes buried from the solvent but does not establish a hydrogen bond will contribute unfavorably (endothermically) to the binding enthalpy. Retrospectively, this is a somewhat expected result as binding dominated by hydrophobicity is entropically driven while strong specific interactions like hydrogen bonds result in favorable contributions to the binding enthalpy. These results emphasize the advantage of knowing the thermodynamic signature of drug candidates as the enthalpy/entropy character of a compound can be used prospectively in lead optimization [1]. An example of the enthalpy LipE correlation is shown in **Figure 1** for a series of protease inhibitors.

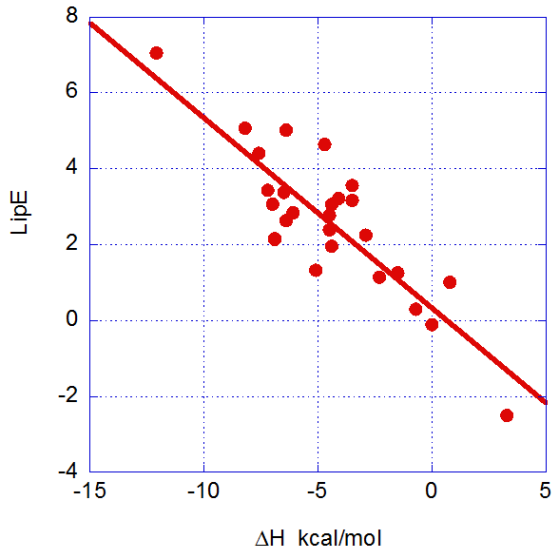

Figure 1. Correlation between LipE and binding enthalpy for 26 HIV-1 protease inhibitors belonging to the same chemical scaffold. It is clear in this plot that the compounds with the more favorable enthalpy are characterized by the higher LipE values. Since ITC data can be used in a prospective way [1], it is apparent that ITC can play a critical role in drug design.

The results in **Figure 1** are in agreement with previous observations indicating that enthalpically driven compounds should be preferred over entropically driven ones [2]. A highly polar compound will have a small LogP but only high affinity if the polar groups establish strong hydrogen bonds and contribute favorably to the binding enthalpy. Measuring the binding enthalpy provides an immediate account of the enthalpic efficiency of the polar functionalities in a compound and delineate optimization strategies. The LipE/enthalpy observations provide additional support to the idea that the availability of protocols for rapid and accurate binding enthalpy measurements is highly desirable.

In this Application Note, we present an ITC screening protocol aimed at rapidly measuring binding enthalpies for a large number of compounds using small amounts of protein. The premise of ITC screening is that binding constants, inhibition constants or IC_{50}'s have already been estimated. It is not required that the estimates are very precise as they are only used to calculate the ligand concentration to be used in the experiment.

The idea in enthalpy screening is to inject a small amount of protein into the calorimeter cell filled with an excess concentration of ligand. Under those conditions all the protein that is injected will bind to the compound and the enthalpy change will simply be equal to the reaction heat divided by the amount of injected protein.

The Experimental Setup. The first task is the determination of the appropriate compound concentration to fill the calorimeter cell. As a rule of thumb, if the compound concentration is 100-fold larger than its K_d, K_i or IC_{50}, close to 99% of the injected protein will bind. This concentration is more than adequate for accurate enthalpy determinations. If it is 10-fold larger 90% will bind. For compounds with affinities in the low micromolar range a concentration of 100µM will suffice. In our work, we routinely use buffer containing 2% DMSO and if necessary 4% DMSO in order to properly dissolve the compounds. Obviously, the protein needs to be in the same buffer and with the same concentration of DMSO as the compounds.

The protein concentration in the delivery syringe can be set anywhere between 10 - 5000µM. Considering that the dilution factor is about 100, the final protein concentration in the reaction cell will range between 0.1 – 50µM. Depending on whether the standard cell (~1000µL volume) or low volume cell (200µL volume) instrument is used, the amount of protein in the cell will range between 100 pmoles – 50 nmoles and 20 pmoles – 10 nmoles for the large and small cell instruments, respectively.

Figure 2 illustrates the data that is obtained with the enthalpy screening protocol using the NanoITC low volume instrument. The details of the experiment are summarized in the figure legend. Depending on the protein concentration in the syringe, typical injection volumes range anywhere between 1 and 5µL. Smaller injection volumes are preferable since they allow for the measurement of a larger number of compounds without refilling the syringe. For the example in the figure 2.9µL injections were used. First, aliquots of the protein solution are injected in triplicate to the buffer solution in order to determine the protein dilution heat effects (upper curves in the figure). This measurement is performed only once, usually at the beginning of the screen. The same volume of protein is injected also in triplicate to the calorimeter cell containing excess compound (lower curves in the figure). The binding enthalpy is calculated by subtracting the heat of dilution and dividing the result by the amount of protein injected according to the formula:

$$\Delta H_{binding} = (Q_{pr \rightarrow inhib} - Q_{pr \rightarrow buf}) \mu cal/\mu moles_{prot}$$

The binding enthalpy determined by using the screening protocol for the example in the figure is ($\Delta H_{binding}$) = -2.68±0.02 kcal/mol which compares favorably with the binding enthalpy determined with a standard titration (-2.0±0.1 kcal/mol). This procedure can be repeated with additional compounds without refilling the injection syringe and without exhaustively cleaning the reaction cell, thus allowing for the rapid evaluation of several

compounds. This procedure permits binding enthalpy determinations in no more than ten minutes per compound.

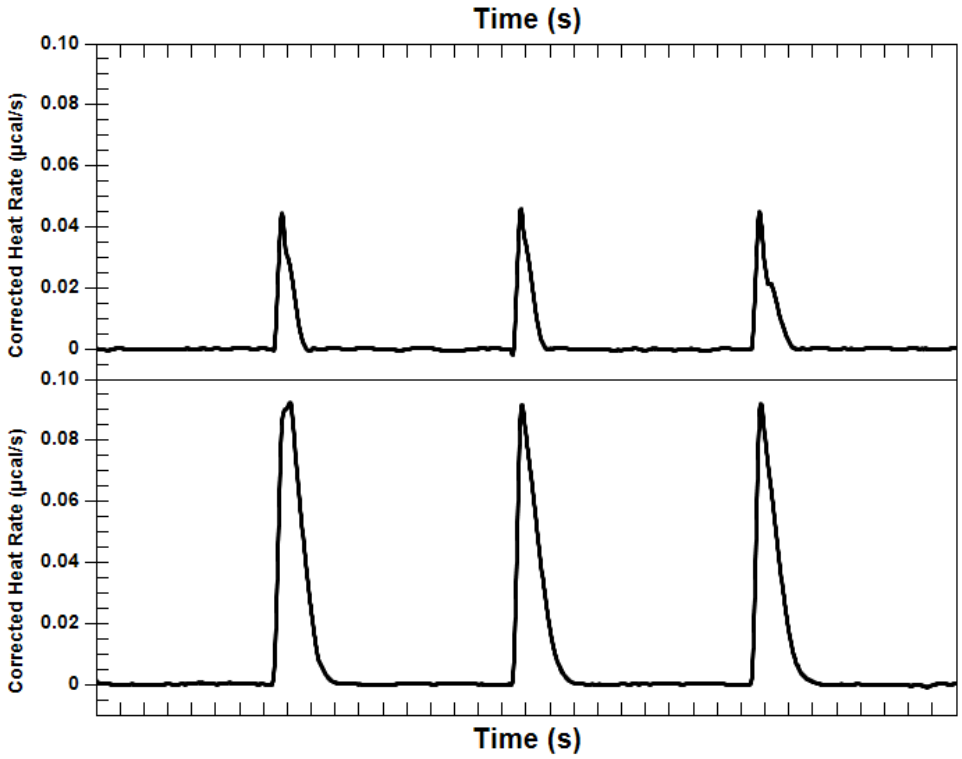

Figure 2: (Lower Panel) Serial injections of TEM-1 β-Lactamase (protein) into 3-benzyloxycarbonyl-5-nitrophenylboronic acid (compound). The titration syringe was loaded with 405 µM TEM-1 and the calorimetric cell filled with 600 µM 3-benzyloxycarbonyl-5-nitrophenylboronic acid. Each injection was 2.91 µL into a 170 µL calorimetric cell. All samples were in 50 mM sodium phosphate, pH 7.0, 2% DMSO. The titration was performed in a NanoITC LV (TA Instruments) at 25 °C. The injection rate was set to 10 ms, which corresponds to 1.43 µL / second. Following an initial delay of 360 seconds, injections were spaced 500 seconds apart. Raw data was analyzed using the NanoAnalyze application from TA Instruments, with resultant areas converted to calories. The first injection released -4.43 µcal, the second injection released -4.51 µcal and the third injection released -4.42 µcal yielding an average of -4.45±0.05 µcal. The binding affinity (Kd) for this compound has previously been determined to be 6.94 µM.

(Upper Panel) Serial injections of TEM-1 β-Lactamase into 50 mM sodium phosphate, pH 7.0, 2% DMSO. Each injection was 2.91 µL of 405 µM TEM-1 into buffer following the same procedure as of protein into compound. The first injection released -1.29 µcal, the second injection released -1.32 µcal and the third injection released -1.27 µcal. From these three experiments the heat of protein dilution is determined as -1.29 ±0.025 µcal. This average value is subtracted from the average of the heats obtained when the protein is injected into buffer containing compound.

The enthalpy screen permits a rapid approximation to the thermodynamic signature by combining affinity (K_d), inhibition constant (K_i) or IC_{50} data with the results of the enthalpy screen. This process involves two steps:

1) Estimate ΔG from K_d, K_i or IC_{50}
   $\Delta G \approx -RT \ln(1/K_d)$ or $\Delta G \approx -RT \ln(1/K_i)$ or $\Delta G \approx -RT \ln(1/IC_{50})$

2) Use ΔH from Enthalpy Screen to estimate -TΔS
   $-T\Delta S = \Delta G - \Delta H$

Figure 3 represents the thermodynamic signature for the compound in the example presented here. This procedure can be rapidly applied to a large number of compounds in order to evaluate their quality as potential drug candidates.

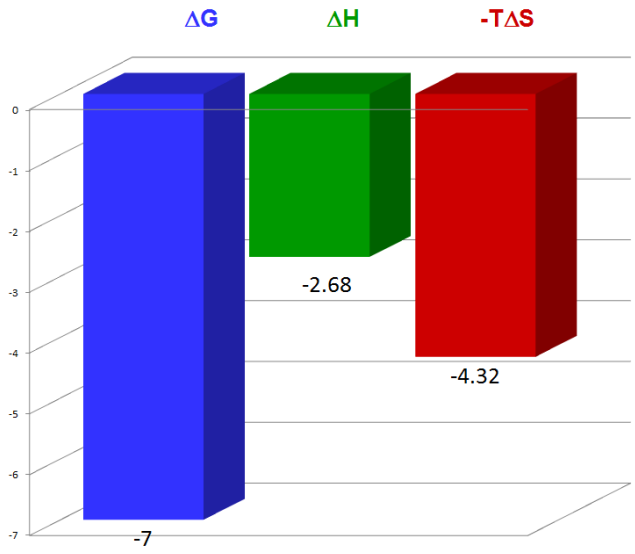

Figure 3: Thermodynamic signature for 3-benzyloxycarbonyl-5-nitrophenylboronic acid binding to TEM-1 β-Lactamase obtained by combining K_d data with the results of the enthalpy screen in **Figure 2**.

1 Freire, E. (2009) A thermodynamic approach to the affinity optimization of drug candidates. *Chem Biol Drug Des* 74 (5), 468-472

2 Freire, E. (2008) Do enthalpy and entropy distinguish first in class from best in class? *Drug Discov Today* 13 (19-20), 869-874