TA Instruments – Application Note

Discovery and Characterization of Inhibitors of Protein/Protein Interactions by ITC.

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The inhibition of protein-protein interactions is a major goal in the therapy of different pathological conditions including cancer, inflammation, autoimmune diseases, diabetes, osteoporosis, infection, etc. Since protein-protein interactions play a critical role in biological signaling, the identification and optimization of molecules that inhibit those interactions is a major research objective in the pharmaceutical industry. The number of targets of interest is continuously increasing and range from a vast number of cell surface receptors, such as EGFr, TNFr, and IGFr to other proteins involved in signaling and regulation (*1, 2*). In the case of HIV infection, for example, the first event is the binding of the viral envelope glycoprotein gp120 to the cell surface receptor CD4 (*3, 4*). Until now, biologics, i.e. monoclonal antibodies or recombinant versions of ligand proteins and/or soluble regions of the receptors, have defined the therapeutic arsenal aimed at targeting protein/protein interactions. The identification of small molecules that accomplish the same goals has become a new frontier in drug research.

Isothermal Titration Calorimetry (ITC) plays a critical role in the identification and characterization of inhibitors of protein/protein interactions (PPI). The identification of PPI inhibitors is fundamentally different to the identification of enzyme inhibitors for which inhibition assays are relatively easy to implement using a variety of approach. For PPI, functional or cell based assays do not reveal the molecular target(s) of molecules identified as active. On the other hand, binding assays alone cannot tell if a binder is also a PPI inhibitor. For PPI inhibitors it is necessary to implement an assay that measures directly the association between the two proteins and its inhibition by the inhibitor candidates. This is the assay where ITC excels.

The identification of PPI inhibitors by ITC requires:

1. Measuring the binding of the two proteins. This experiment is performed once and serves as the reference.
2. Measuring the binding of the two proteins in the presence of a fixed concentration of the inhibitor candidates.

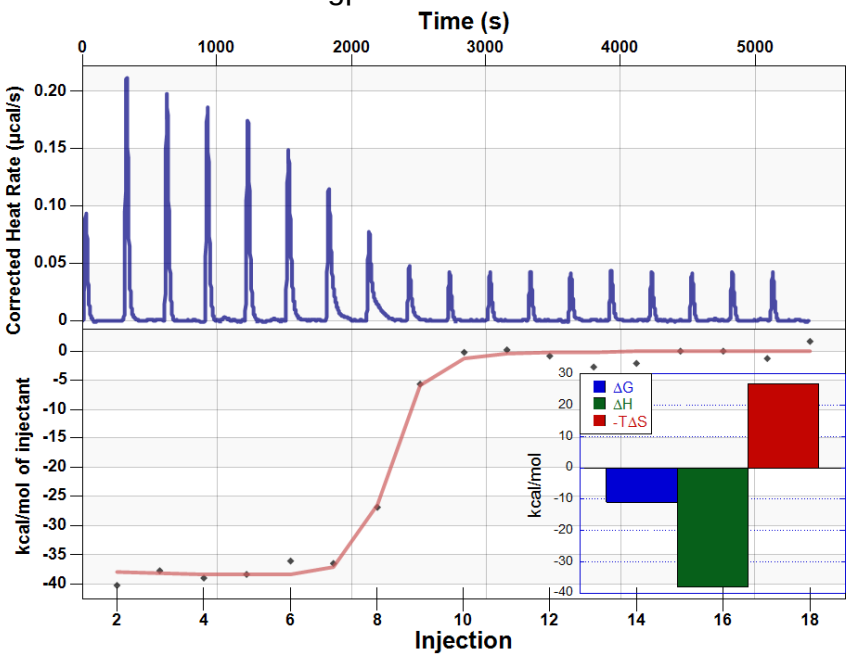
3. The characterization of those molecules that score favorably in the PPI inhibitors screen is performed by directly measuring the binding of the selected compounds to the target proteins.

Measuring Protein/Protein Binding

In this Application Note we will use the binding of the envelope glycoprotein of HIV-1 gp120 to the soluble form of the cell surface receptor sCD4 as an example.

The reaction cell of the Nano ITC Low Volume (LV) (TA Instruments, New Castle, DE) is filled with 0.17 mL of 4.5 µM of gp120. The injection syringe is filled with a 45 µM solution of sCD4. These protein solutions are equivalent to 0.25 and 2.0 mg/mL respectively. Injection volumes are 2µL in all experiments presented here. In general, with the current instrument precision the protein concentrations can be reduced 3-fold and still obtain accurate results. All protein solutions are in PBS (Roche Diagnostics GmbH, Mannheim, Germany), pH 7.4 with 2 % DMSO. **Figure 1** shows the titration of sCD4 into gp120. It is consistent with an association constant, K_a, of 1.2×10^8 M^{-1} or equivalently a dissociation constant $K_d = 1/K_a$ of 8.3 nM. Furthermore, the binding enthalpy, ΔH, is -38.0 kcal/mol and the entropy contribution to the binding Gibbs energy, –TΔS, is 27.0 kcal/mol (1 cal = 4.184 joules). The thermodynamic signature in the inset provides a visual representation of the magnitude of those contributions to binding.

Figure 1. ITC titration of sCD4 into gp120



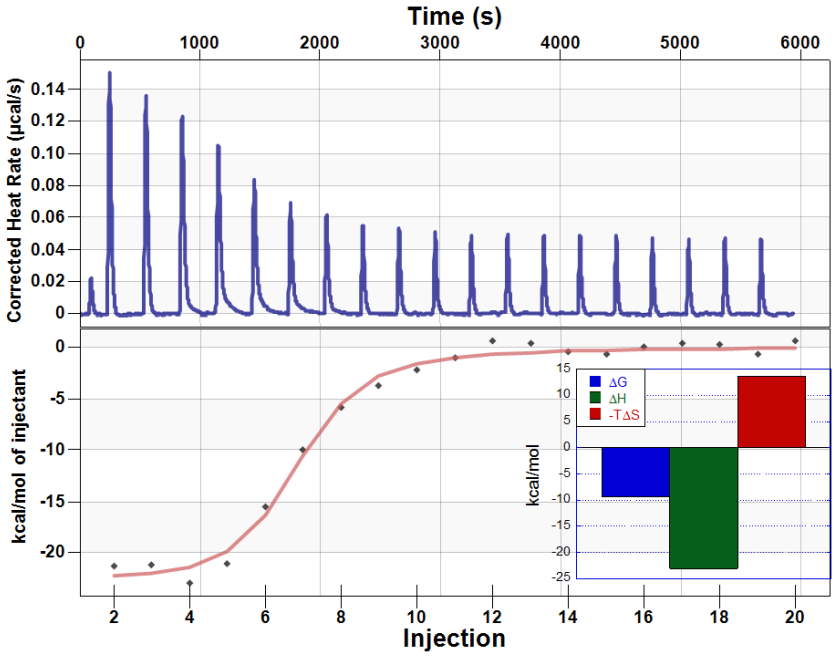
The binding of sCD4 to gp120 is characterized by large favorable enthalpy and large unfavorable entropy changes, indicative of a binding reaction associated with a large structuring process. In this case, the binding of sCD4 triggers the folding of intrinsically disordered domains in gp120 (*5, 6*). Protein/protein binding not associated with large refolding processes are characterized by favorable enthalpy and entropy changes.

Screening for Inhibitors

The identification of PPI inhibitors is accomplished by performing the same experiment shown in **Figure 1** except that the reaction cell also contains a fixed concentration of an inhibitor candidate. Since initial leads are usually active in the low micromolar range, a good concentration is in the hundreds micromolar range. If a compound inhibits the protein/protein interaction, it will be observed as a decrease in the observed or apparent binding affinity, K_{app}. The magnitude of the decrease can be expressed in terms of the ratio $A = K_{app}/K_a$. If A = 1, then the compound has no effect on the protein/protein interaction. If A<1, then the compound has an inhibitory effect. If all the compounds are screened at the same concentration, then the parameter A suffices to rank them in terms of their inhibitory potency. Sometimes a compound is found that exhibits an A value greater than one. This compound actually increases the binding affinity acting as an agonist of the protein/protein interaction. While most of the time drug developers are searching for inhibitors, we should emphasize that this technology also allows for the identification of PPI agonists.

Figure 2 shows a titration similar to the one shown in **Figure 1** except that the reaction cell also contains 200µM of NBD-556, a small molecular weight (MW = 337.84) low affinity sCD4/gp120 inhibitor (*5, 6*). This experiment is consistent with an apparent association constant, K_{app}, of 7.7×10^6 M^{-1} or equivalently an apparent dissociation constant $K_{d,app} = 1/K_{app}$ of 130 nM, indicating that the presence of the compound significantly reduces the affinity of sCD4 for gp120. The apparent binding enthalpy is -23.0 kcal/mol and the apparent entropy contribution to binding is 13.6 kcal/mol. The thermodynamic signature for this experiment is also displayed in the inset. Most importantly, the A value is 0.064 indicating that this compound is a PPI inhibitor.

Figure 2. ITC titration of sCD4 into gp120 in the presence of 200µM NBD-556



Characterization of PPI Inhibitor

The experiments in **Figures 1** and **2** identify a compound as a PPI inhibitor. A more complete characterization of the compound is obtained by measuring its binding thermodynamics to the target protein. If the target protein is not known, separate ITC experiments with each of the two proteins need to be performed. The A parameter is related to the affinity of the inhibitor to the target by the following equation:

$$A = \frac{(1+\beta K_I[I])}{(1+K_I[I])} \quad (1)$$

where β is the degree of competitiveness of the inhibitor. If β = 0 the inhibitor is absolutely competitive, i.e. either the inhibitor or the protein is bound but not both. If β = 1 the compound does not affect the binding affinity of the protein. This situation can be observed for allosteric inhibitors of protein signaling in which binding of the inhibitor does not affect the binding of the two proteins. For small molecular weight

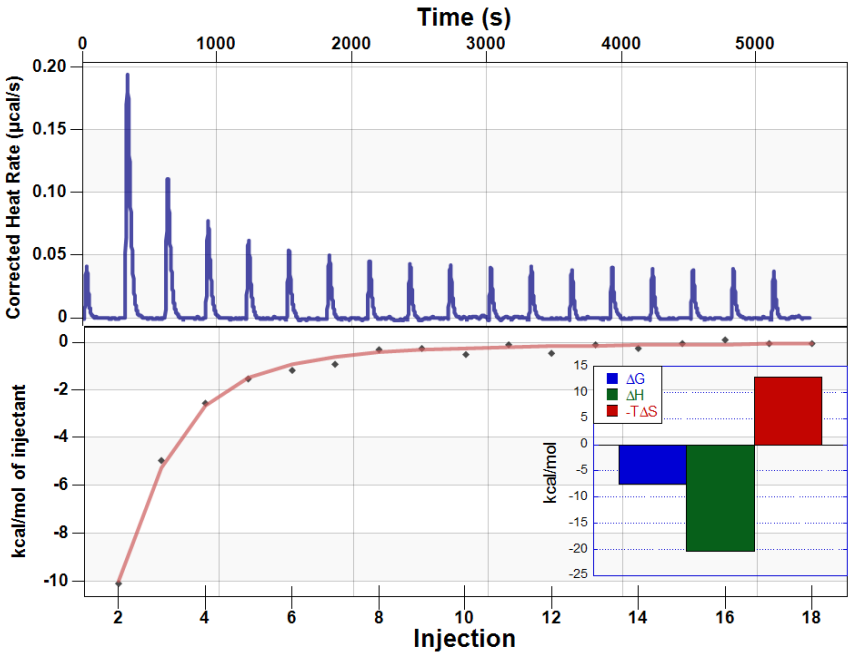
compounds β can assume a value between 0 and 1 (*7*). This situation is possible because the binding footprint of a small molecule is very small when compared to the entire protein/protein interface which can make it possible for both molecules to bind simultaneously. The presence of the small molecule can be thought off as a mutation that lowers the affinity but not abolishes the binding of the protein for its partner. The parameter β can be calculated from the ITC data by rearranging equation 1:

$$\beta = \frac{A(1+K_I[I])-1}{K_I[I]} \quad (2)$$

where A is obtained for the experiments in **Figures 1** and **2** and K_I from the ITC titration of the inhibitor into the target protein.

Figure 3 shows the ITC titration of NBD-556 into gp120 which is the target protein in this particular example. In this experiment, the reaction cell contained 5 uM gp120 and was titrated with 2 µL injections of a 300 µM NBD-556 in the syringe. This experiment is consistent with an association constant, K_I, of 3.3×10^5 M^{-1} or equivalently a dissociation constant $K_{d,I} = 1/K_I$ of 3.0 µM. The binding enthalpy is -20.4 kcal/mol and the entropy contribution to binding is 12.9 kcal/mol. The thermodynamic signature for this inhibitor is also displayed in the inset. Equation 2 indicates that the binding of NBD-556 is characterized by a β value of 0.05 which is characteristic for a moderately competitive inhibitor. The optimization of protein/protein inhibitors requires maximization of the binding affinity and modulation of the degree of competitiveness, β, in order to develop more or less competitive inhibitors according to the specific design needs. The results presented here demonstrate the unique capability of ITC to guide the optimization of protein/protein inhibitors.

Figure 3. ITC titration of the small molecular weight inhibitor NBD-556 into gp120.



Conclusions

The inhibition of protein/protein interactions is a major frontier in the pharmaceutical and biotechnological industries. The identification and optimization of protein/protein inhibitors require accurate measurements of their binding affinity as well as the efficiency with which they compete with the target protein. ITC is uniquely suited to perform this task as it can provide both the binding affinity and the degree of competitiveness of an inhibitor. Contrary to traditional enzyme inhibitors in which the degree of inhibition is proportional to binding affinity, for protein/protein inhibitors binding affinity is not sufficient. Due to the large size of the protein/protein binding footprint when compared to the size of a ~500 MW molecule, inhibitor optimization also requires tracking of the degree of competitiveness since binding affinity alone does not reflect inhibitor potency. The experiments presented here demonstrate the critical role of the Nano ITC LV in the development of protein/protein interaction inhibitors.

References

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