

Investigating protein-ligand/drug interaction – An introduction to nano differential scanning fluorimetry (nDSF) and Isothermal titration calorimetry (ITC)

Once a drug target has been identified, it is of particular importance that potential drug candidates exhibit high affinity for their targets, since they have to compete against natural ligands or substrates *in vivo*. Therefore, assays that enable us to characterize the interaction of drug and targeted structure will be integrated already early in the process of drug development as part of the structure-activity-relationship (SAR) analysis. In our group, we use two different methods to investigate protein-ligand or protein-drug interactions. The first method, well suited as screening method for ligand/drug-binding, is nano differential scanning fluorimetry (nDSF). The second, more laborious but also more informative on the binding process, is Isothermal titration calorimetry (ITC).

1. Nano differential scanning fluorimetry (nDSF)

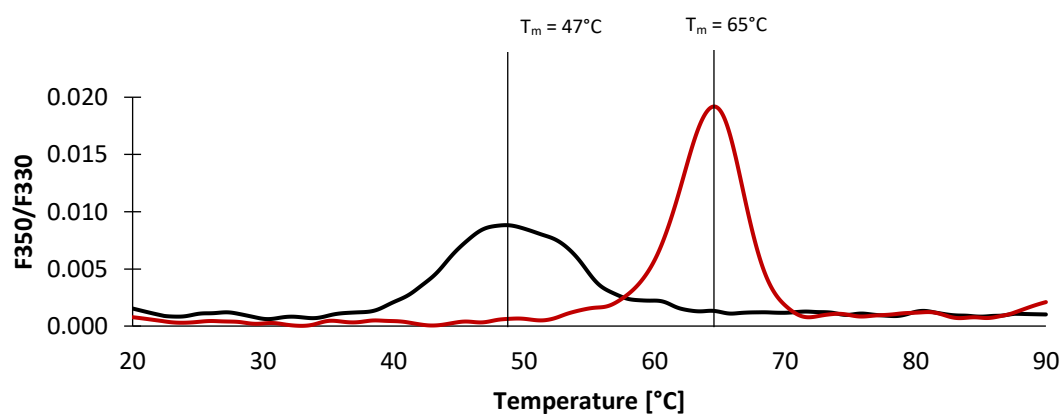


Fig. 1: Melting curves of protein 1 before (black) and after (red) incubation with 1mM ligand. Fluorescence at 350 and 330nm has been determined during thermal denaturation of the protein and the first derivative of the ratio F350/F330 is shown.

Nano differential scanning fluorimetry (nDSF) enables us to screen for drug/ligand interactions as it allows us to monitor protein stability based on its melting temperature T_m . T_m is defined as the temperature, in which half of the protein is denatured and the other half

is still in its native state. Ligand binding usually leads to higher stability of the protein and therefore shifts T_m to higher degrees (Fig. 1).

nDSF is a method that does not require labeling of your samples. During the run, the intrinsic fluorescence of aromatic residues in the protein, especially tryptophanes, is measured. Emission spectra of tryptophanes are highly dependent on the surrounding environment. Since they are hydrophobic amino acids, tryptophanes are usually buried within the protein. During unfolding, they might become surface exposed, which leads to a shift of the emitted light to higher wavelengths (red-shift) (Fig. 2, upper panel). The opposite (blue-shift) is also possible and often observed for tyrosines (Fig. 2, lower panel). nDSF detects fluorescence at 330 and 350nm. A red shift leads to a stronger decrease of fluorescence intensity at 330nm than 350nm, whereas a blue shift, in turn, leads to a decrease of fluorescence at 350nm. When we calculate the ratio of the fluorescence intensity at both wavelengths ($F(350)/F(330)$), and plot it against temperature, we obtain a sigmoidal curve with increasing $F(350)/F(330)$ for the red shift and decreasing $F(350)/F(330)$ for the blue shift (Fig 2, second panel from right). Most common illustration of the data is the first derivative of this curve with $t=T_m$ at its maxima or minima (Fig. 1, 2).

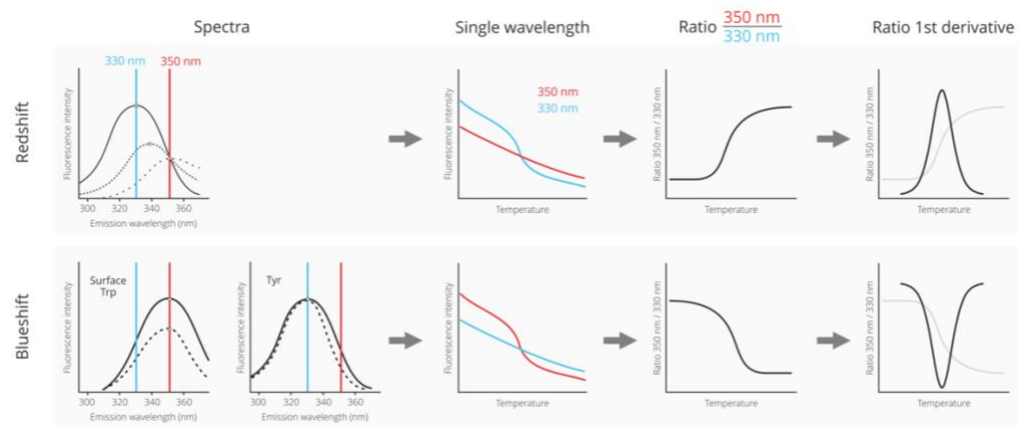


Fig. 2: Thermal denaturation profiles determined by nDSF are dependent on fluorescent behavior of amino acids during the unfolding process. A red shift of fluorescence (upper panel) is characterized by decreasing fluorescence intensity at 330nm whereas intensity at 350nm is nearly not affected. For a blue shift (lower panel), the opposite is observed and fluorescence intensity stronger decreases at 350nm. Note that in this case the ratio of F_{350}/F_{330} decreases during thermal unfolding (second figure from the right) and the first derivative is orientated downwards (right). Image taken from <https://2bind.com/nanodsf/>, accessed 30.6.21.

2. Isothermal titration calorimetry

nDSF is therefore a good option to quickly evaluate if your ligand/drug candidate interacts with your drug target. A strong interaction usually leads to a higher shift of the melting temperature T_m than a weaker interaction, but the accurate determination of binding affinity or thermodynamic signature is not possible with nDSF. To gain this information, we use Isothermal titration calorimetry (ITC). In ITC, heat changes during ligand/drug-binding to a protein are monitored over time. During the experiment, ligand/drug is titrated in defined volumes into a protein sample. When the ligand/drug binds to the protein, heat will be released (exothermic process) or absorbed (endothermic process). This change in heat will be detected by the instrument, because it permanently compares the temperature in the sample cell with the temperature of a reference cell (Fig. 3, left). The energy that is needed to re-gain the same temperature in both cells will be measured for each injection and plotted as a function of time (Fig. 3, right). After a certain number of injections of ligand or drug, all binding sites of the protein are loaded and no further heat changes are detected.

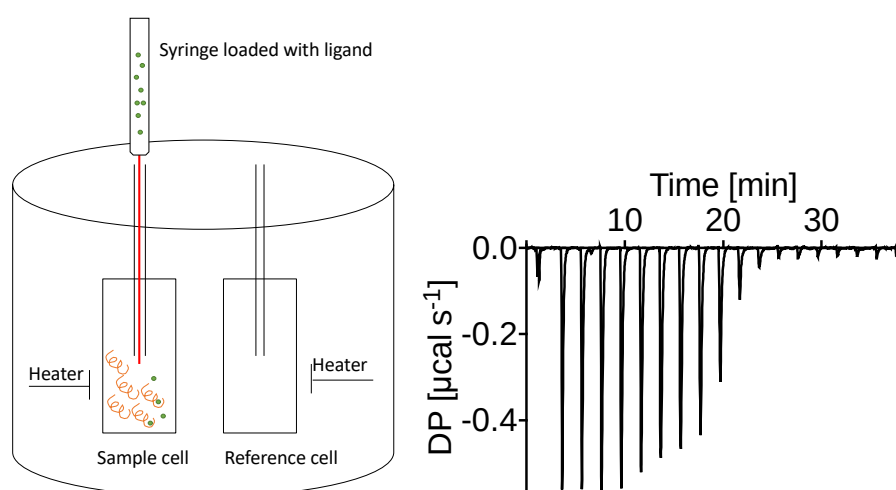


Fig. 3: Schematic representation of an ITC experiment set up. The sample cell is loaded with protein (orange) and ligand (green dots) is injected from a syringe in defined volumes and with a certain delay between the single injections. Heat differences (difference power DP[cal s⁻¹]) between sample cell and reference cell will be detected and plotted against time (right).

From ITC experiment, one can directly derive binding constant K_d , enthalpy ΔH and N-value:

1. K_d is the ligand concentration, at which half of the protein is saturated and can be drawn from the inverse of the slope of the sigmodal curve ($K_d = 1/\text{slope}$; Fig. 4, left)

$$K_d = \frac{[L][P]}{[LP]}$$

The lower the value, the less ligand is needed to saturate the protein and the higher the binding affinity of the protein for the titrated molecule.

2. ΔH [kcal/mol] is the total amount of heat released during the binding process and will be calculated as heat per mole of ligand. The value is directly measured during the experiment as it is the sum of the integrated peak area, drawn for every injection (Fig. 4). A negative ΔH means heat release during the binding process, positive ΔH means heat absorption.
3. N-value approximates the ratio of bound ligand molecules per protein molecule. It strongly depends on indicated ligand and protein concentrations as well as on the active portion of the protein sample that is capable of ligand binding.

Gibbs free energy (ΔG) and binding entropy ($T\Delta S$) are derived indirectly from the experiment as they can be calculated from the following equations:

$$\Delta G = R \times T \times \ln(K_d) \quad (\text{with } R = \text{ideal gas constant and } T = \text{temperature})$$

$$\Delta G = \Delta H - T\Delta S$$

Thus, ITC gives us detailed information on energy changes during ligand binding. The knowledge can be used to improve a first hit compound, not only in terms of binding affinity but also considering its thermodynamic profile (s. exercises).

For visualization: https://www.youtube.com/watch?v=o_lPWcWKNXI

3. Sample preparation and conducting the experiment

ITC requires high amounts of pure protein that is completely ligand-free, hence in a so-called APO version. We therefore express our protein of interest in *E. coli* and purify it from the cells under non denaturing conditions. To remove any pre-bound ligand molecules coming from *E.coli* we denature the purified protein in urea, separate it from small molecules and refold it again. After refolding and removal of unfolded protein aggregates by size exclusion chromatography, the protein is ready to use. Ligands that will be tested for binding have to be prepared at very accurate concentration and in the same buffer as your protein to avoid buffer mismatches (dilution leads to heat release which increases your off set (Fig. 4)). As soon as protein and ligand are prepared, both can be loaded onto a MicroCal PEAQ-ITC (Malvern) as shown in the video: <https://www.youtube.com/watch?v=Jy9xWy5N298>

You can now measure if and how strong protein and ligand/ drug interact.

4. Data interpretation

If your ligand/ drug binds to your protein, an integrated software of the instrument will plot the binding curve (Fig. 4, left) and the thermodynamic profile (Fig. 4, right). In the upper panel of the left figure, the normalized raw data is shown. Each injection gives a single peak, in which difference power (DP) – hence, the energy that is needed to adjust temperatures of sample and reference cell after injection – is plotted against time. Binding curves (Fig. F, lower

panel) are than obtained by integrating the area each peak fills to calculate the amount of heat released per mole of injected ligand. This heat released per mole of ligand is defined as enthalpy ΔH . Together with ΔH , we obtain K_D directly from the curves as it equals the slope of the binding curve in the inflection point. Stoichiometry of ligand to protein (N under optimal conditions) is gained from the x-value of the inflection point.

On the right, the thermodynamic signature of the reaction is shown. Along with enthalpy ΔH (green), entropy ($-T\Delta S$) (red) favors or counteracts ligand binding. The sum of both values gives the free binding energy ΔG (blue).

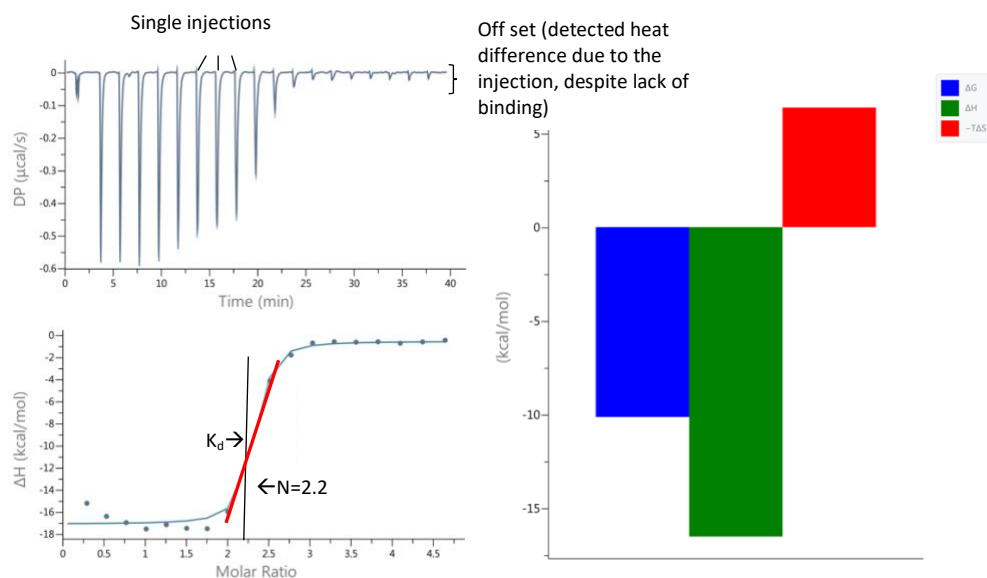
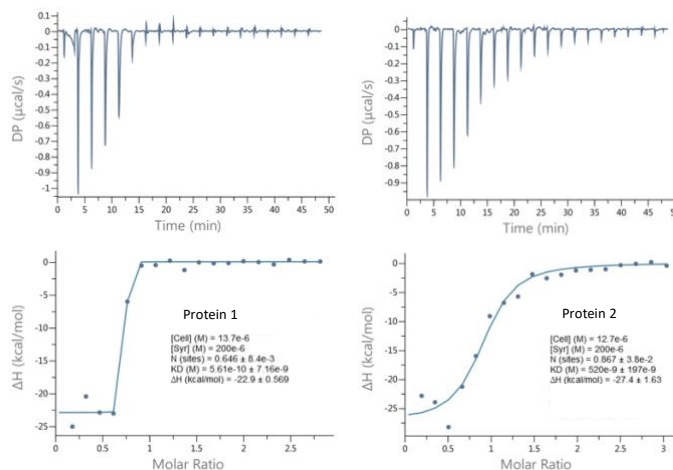


Fig. 4: Binding data obtained from an ITC experiment. On the left, a sigmoidal binding curve (lower panel) calculated from the normalized raw data (upper panel) as described in the text is shown. On the right, the thermodynamic signature of the binding event is shown as bar plot, corresponding to the color code on the right.

4. Exercises:

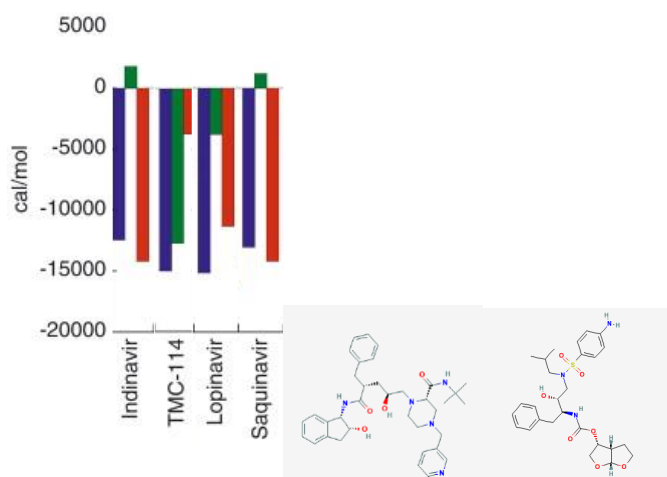
1. Compare the two curves below.

- Which protein has higher affinity for its ligand?
- Calculate ΔG and $-\Delta S$ for both samples and plot the thermodynamic signature as in Fig. 4 (you can use excel or prism for that).
- Describe the binding mode: is the binding more enthalpically driven or entropically driven? What are probably the main protein-ligand interactions in a crystal structure?
- Evaluate the quality of the data: Which curve would you trust more than the other and why?
- Design an experiment that would increase the quality of the “worse” curve. You can change the following parameters:
 - Protein concentration
 - Ligand concentration
 - Injected volume of ligand
 - Spacing between injections
 - Temperature (at which the experiment runs)



- In which phase of drug development do we need nDSF and ITC? What else is important in this phase?
- What are the requirements of a good drug?

4. Below, you see the thermodynamic profiles of four HIV protease inhibitors:
- Try to bring them into historic order. Give an explanation for your decision.
 - Calculate K_d for all four drugs ($T=25^\circ\text{C}=298,15\text{K}$; $R=1,985 \text{ cal K}^{-1} \text{ mol}^{-1}$)
 - What are the main changes/ improvements?
 - Beneath the thermodynamic profiles, the structure of the oldest and the “newest” (of the four) is shown. Which structure belongs to which thermodynamic profile? Give an explanation for your decision.



Source: Ohtaka et al., *Progr. Biophys. Mol. Biol*, 2005 and Freire, *Drug discovery today*, 2008

- What contributes to better enthalpy? What makes better entropy? What is easier to improve and why?
- What is the limit of entropy (value)? What is the problem of strongly hydrophobic drugs (at least three different arguments)?
- What are other methods to determine ligand binding affinity?

8. Draw (in excel or prism) two hypothetical thermodynamic profiles (ΔG , ΔH and $-T\Delta S$) that would lead to a 100fold higher affinity than Indinavir has for the HIV protease (start your calculation from the K_d value of Indinavir)
9. What are the limitations of ITC? How will your data be influenced when ligand or protein concentration are not determined accurately?