**Experiment:** Determination of thermodynamic parameters and affinity by isothermal titration calorimetry (ITC) using the example of trypsin – ligand binding.

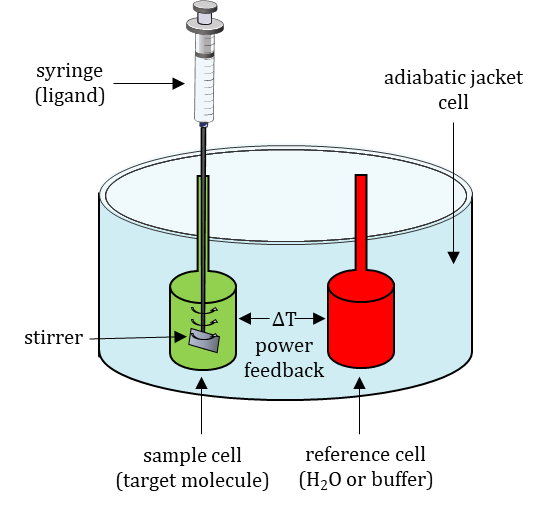
Introduction

Isothermal titration calorimetry (ITC) is a biophysical method that measures the change in heat that occurs when two or more molecules interact. In this experiment, this technique will be used to study the interaction of the serine protease trypsin with different inhibitors.[1-3]

Device and principle of operation

The device is constructed from two adiabatically encased cells, a reference cell and a sample cell. The reference cell contains water (or buffer), while the sample cell is filled with one of the binding partners, in this experiment trypsin. The temperature in both cells can be regulated and, *via* a feedback mechanism, the power required to heat the sample cell can be adjusted so tht the temperature of the sample cell is adapted to the reference cell temperature (**Figure1**).

Figure 1. Schematic layout of an ITC device.

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During an ITC experiment, the binding partner in the sample cell (here the protein) is titrated with the second binding partner (ligand) under stirring. In this experiment the inhibitors benzamidine or leupeptin are titrated against trypsin. After each injection, the temperature within the sample cell changes as heat is released or absorbed by the binding event. The ITC instrument measures the time-dependent increase or reduction of power (µW) needed to match the temperature of the sample cell to the reference cell. By plotting the measured power against the experimental time, a thermogram consisting of several spikes is obtained. (**Figure 2A**). Each spike represents a single injection of the ligand and the area under the spikes corresponds to the amount of heat absorbed or released by the injection. If the amount of heat of each injection, determined by integration, is plotted against the molar ratio of the binding partners, an ideally sigmoidal curve, is obtained (**Figure 2B**).

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Figure 2. Data evaluation of an ITC experiment. A Schematic representation of a thermogram obtained by ITC. B Schematic representation of a binding isotherm determined by ITC.

From this isotherm, the stoichiometry *n* can be determined based on the inflection point. The bond enthalpy Δ*H* is determined on basis of released heat from the first injections where virtually every added ligand binds to the target molecule. The dissociation constant *K*D from the slope at the inflection point. Using these values, with Equations 1 and 2, the Gibbs energy Δ*G* and the binding entropy Δ*S* can be calculated.

(1)

(2)

The thermodynamic binding profile is usually represented as a bar chart (signature plot, **Figure 3**).

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Figure 3. Example of a signature plot. Δ*G* blue, Δ*H* green, –TΔS red.

**Sample preparation and control experiments**

It is not only the interaction of the binding partners which can cause a change of heat. Different superimposed effects such as the dilution of the binding partners or other buffer components can also influence the release/absorption of heat during the titration. In particular, the widely utilized co-solvent dimethyl sulfoxide, which is used to dissolve many ligands that are insoluble in water, is characterized by a pronounced exothermic dilution. It should therefore be ensured that the binding partners are in identically composed solvents (buffer matching). In addition, control experiments, such as a "ligand versus buffer" titration, can be performed to account for the influence of the dilution heat.

During the association of the binding partners, changes in the protonation states can take place. The occurring proton transfers can lead to ionization of the buffer. The buffer specific ionization enthalpy Δ*H*ion thus released or absorbed, influences the enthalpy Δ*H*obs observed for the entire binding process. By repeating the measurement under identical pH in different buffers displaying different Δ*H*ion values, the number of protons *n*proton taken up or released and the intrinsic binding enthalpy Δ*H*bind can be determined (Equation 3).

(3)

**C-value**

An important parameter for planning and optimizing ITC experiments is the C-value, which reflects the shape of the binding isotherm. The C value is determined from the protein concentration [P] in the reaction cell using *n* and *K*D. (Equation 4).[4]

(4)

As a rule of thumb, for binding affinities in the nano- to low micromolar range, the ligand concentration chosen is 10 times the enzyme concentration. The enzyme concentration is usually between 10 µM and 50 µM. At higher *K*D values, the ligand concentration is often increased. Depending on the instrument, C should be between 1 and 1000 to determine binding affinities, with an optimum between 10 and 100 (**Figure 4**).

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Automatisch generierte BeschreibungFor ligands with low affinity, it is often very difficult to obtain a suitably high C-value. However, if the stoichiometric ratios are known, a titration with a C-value below 10 can be performed (this is called a low C titration). In this case, the bond isotherm loses its sigmoidal shape, but by pre-determination of the stoichiometry, *K*D and Δ*H* can still be determined with moderate accuracy. For ligands with very high affinities, C values are often above 500, which makes the binding isotherms too steep, and the inflection point cannot be reliably determined. The C value can be lowered by reducing the protein concentration, but if [P] is too small, the binding signals are no longer detectable by the ITC system.

Figure 4. Form of binding isotherms for different C values.

One possibility to reliably determine the affinities of particularly strong or weak ligands by ITC are displacement titrations. Here, a weaker ligand is pre-incubated in the cell with the protein and displaced by titration with a stronger binder. Thus, the observed affinity (*K*Dapp) of the high-affinity ligand is increased compared to the *K*D of the direct titration experiment. If the thermodynamic parameters of one of the ligands are known, the observed values for the displacement titration can be used to determine the *K*D value and the thermodynamic parameters for the unknown strong or weak binder (Equations 5 and 6)[5].

(5)

(6)

According to Zhang&Zhang in Equations 5 and 6, the index 1 denotes the values of the higher affinity ligand and index 2 the values of the lower affinity ligand.[5] L2tot is the concentration of the lower affinity ligand which is pre-incubated with the protein in the cell.

Execution

**Part 1: Direct titrations**

Determine the C-value of the titrations (use the given literature data for the KD-value), assuming a 1:1 stoichiometry. Evaluate whether this is suitable for each titration. Are changes to the experimental setup/data analysis necessary? Discuss this with your assistant.

**Benzamidine**

The weak inhibitor benzamidine (21.7 µM)6 is titrated against trypsin. The titration is performed in two different buffers, each as a duplicate. In addition, a control measurement of the ligand is performed against the respective buffer. The buffers are provided by the lab course assistant.

Table 1. Buffer composition.

|  |  |
| --- | --- |
| **Buffer** | **Composition** |
| **HEPES** | 50 mM HEPES pH 7.4, 100 mM NaCl |
| **TRIS** | 50 mM TRIS pH 7.4, 100 mM NaCl |

**Step 1: Prepare the required solutions**

**Important:** First calculate all needed volumes and discuss your results with the assistant before preparing the final solutions!

Stock solutions of trypsin (**150 µM**) and benzamidine (**50 mM**) are provided. The inhibitor stock is dissolved in water, the enzyme in buffer. You need to prepare the following solutions:

* Enzyme solution: **740 µL** with a concentration of **100** **µM** trypsin. (each for TRIS and HEPES buffer).
* Ligand solution: **540 µL** with a concentration of **2.5 mM** benzamidine (each in TRIS and in HEPES buffer).

Calculate the required volume of stock solution and complete the remainder with the respective buffer. Make sure to use the correct buffer with the correct stock solution for the enzyme solutions (e.g., diluting the TRIS stock solution of trypsin in TRIS buffer).

Further note that the ligand solution contains an excess of milli-Q®(MQ) water compared to the enzyme solution, since the stock solution consists of pure MQ water. Calculate the excess and determine the volume of MQ water that must be added to the enzyme solution to compensate for it (buffer matching). The volume of MQ water you calculated must be subtracted from the addition of buffer.

For control experiments and pre-rinsing of the sample cell, you additionally need 3 mL of each buffer with the same excess MQ water contained in the enzyme and inhibitor solutions.

**Step 2: Filling the microtiter plates**

* The previously prepared solutions are now pipetted into microtiter plates. 180 µL of the inhibitor solution and 370 µL of the enzyme solution are required per measurement.
* For the control measurement, one well is filled with 1 mL buffer, and an additional well is filled with 1 mL buffer to pre-rinse the sample cell.
* Each well must be labeled clearly and legibly!
* The programming of the experimental workflow using the MicroCal PEAQ-ITC Automated Control Software is then carried out under guidance of the assistant.

The titration is carried out fully automated by the ITC instrument (MicroCal PEAQ-ITC Automated, Malvern Panalytical, United Kingdom). The experiment temperature is 25 °C. The ligand is titrated into the sample cell with 19 injections of a volume of 2 µL, each at an interval of 150 s. The results of all experiments will be provided to you at the end of the week.

**Leupeptin**

The **strong** inhibitor leupeptin (40.4 nM)7 is titrated against trypsin. The titration is performed in HEPES buffer (see Table 1) as a duplicate. In addition, a control measurement of the ligand against the buffer is performed.

**Step 1: Prepare the required solutions**

**Important:** First calculate all needed volumes and discuss them with the assistant before preparing the final solutions!

Stock solutions of trypsin (**150 µM**) and leupetin (**20 mM**) are provided. The inhibitor is dissolved in MQ water and the enzyme in HEPES buffer. You will need to prepare the following solutions:

* Enzyme solution: **740 µL** with a concentration of **10 µM** trypsin.
* Inhibitor solution: **540 µL** with a concentration of **100 µM** leupeptin.

Further note that the ligand solution contains an excess of MQ water compared to the enzyme solution, since the stock solution consists of pure MQ water. Calculate the excess and determine the volume of MQ water that must be added to the enzyme solution to compensate for it. The volume of MQ water you calculated must be subtracted from the addition of buffer. For control experiments and pre-rinsing of the sample cell, you additionally need 3 mL of the buffer with the same excess MQ water contained in the enzyme and inhibitor solutions.

**Step 2: Filling the microtiter plates**

* The previously prepared solutions are now pipetted into microtiter plates. 180 µL of the inhibitor solution and 370 µL of the enzyme solution are required per measurement.
* For the control measurement, one well is filled with 1 mL buffer, and an additional well is filled with 1 mL buffer to pre-rinse the sample cell.
* Each well must be labeled clearly and legibly!
* The programming of the software is then carried out under guidance of the assistant.

The titration is carried out fully automated by the ITC instrument. The ligand is titrated into the sample cell with 19 injections of a volume of 2 µL, each at an interval of 300 s. The results of all experiments will be provided to you at the end of the week.

**Part 2: Displacement titration of benzamidine with leupeptin**

In this part of the experiment, a displacement titration of the low-affinity trypsin ligand benzamidine by the high-affinity ligand leupeptin is performed.

**Step 1: Prepare the required solutions**

**Important:** First calculate all needed volumes and discuss them with the assistant before preparing the final solutions!

Stock solutions of the enzyme (**150 µM**), benzamidine (**50 mM**) and leupeptin (**20 mM**) are provided. The inhibitor is dissolved in MQ water and the enzyme in HEPES buffer. You will need the following solutions:

* Enzyme solution: **740 µL** with a concentration of **10 µM** trypsin and **250 µM** benzamidine.
* Inhibitor solution: **540 µL** with a concentration of **100 µM** leupeptin.
* Control solution: **370 µL** with a concentration of **250 µM** benzamidine.

Calculate the volume of stock solution required and fill up the remainder with buffer.

For pre-rinsing the sample cell you additionally need 2 mL of the buffer with the same MQ water excess which is contained in the enzyme and inhibitor solutions.

**Step 2: Filling the microtiter plates**

* The previously prepared solutions are now pipetted into microtiter plates. 180 µL of the inhibitor solution and 370 µL of the enzyme solution are required per measurement.
* For the control measurement, one well is filled with 370 µL control solution, and an additional well is filled with 1 mL buffer to pre-rinse the sample cell.
* Each well must be labeled clearly and legibly!
* The programming of the software is then carried out under guidance of the assistant.

The titration is carried out fully automated by the ITC instrument. The ligand is titrated into the sample cell with 19 injections of a volume of 2 µL, each at an interval of 300 s. The results of all experiments will be provided to you at the end of the week.

Protocols

Write a short theoretical introduction. For the execution, reference can be made to the script. Deviations from the execution must be mentioned in the protocol!

The data determined on the experiment day are provided by the assistant. Determine the mean value for the duplicates and enter the values in Table 2. In addition, calculate the error values in each case. Thermograms, isotherms and signature plots are to be attached to the protocol in the appendix.

Table 2. Template for the result table of the experiments.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Titration | ITC-Buffer | *K*D | *n* | ∆*G* (kJ/mol) | ∆*Hobs* (kJ/mol) | –*T*∆*S* (kJ/mol) |
| benzamidine  vs. trypsin | TRIS |  |  |  |  |  |
| HEPES |  |  |  |  |  |
| leupeptin  vs. trypsin | HEPES |  |  |  |  |  |
| Leupeptin vs. trypsin/benzamidine  (Displacement titration) | HEPES |  |  |  |  |  |

For *K*D, *n* and ∆*H*obs the errors can be calculated by the Gaussian error propagation (Equation 7).

(7)

For ∆*G* and –*T*∆*S*, enter the standard deviation of the mean as the error.

The combination of experiments also allows the determination of proton transfers in the binding of benzamidine to trypsin. Furthermore, the affinity and the thermodynamic binding profile from the displacement titrations are to be calculated.

Determine the number of protons *n*proton taken up or released upon binding, the intrinsic binding enthalpy Δ*H*bind, and the resulting entropy term –*T*Δ*S*bind based on the benzamidine vs. trypsin titrations in different buffers. Present the results in a tabularized form. For this purpose, use the "Buffer ionization" tool of the *ITCcalc* calculator (available under: [ITC Calculator (uni-mainz.de)](https://itccalc.uni-mainz.de/)). **Important:** Use "." as decimal separator.For ∆*G*, use the average of the measurements in different buffers.

To evaluate the displacement titration, the *ITCcalc* website can be used as well (if you are unsure how to enter the values, you can use the "Tour function" for explanations). Compare your results with the direct titrations.

In the discussion, the measured values of all titrations should then be compared and discussed by each group. The following points are to be covered in the protocol:

* Discuss the determined binding isotherms. Which quantities can be read directly, which are calculated by the software?
* Create a bar graph (signature plot) in which Δ*H*, Δ*G* and -*T*Δ*S* are shown for the benzamidine vs trypsin titrations in TRIS buffer, HEPES buffer and after buffer correction.
* How do the following factors affect the **entropy** and/or **enthalpy** of complex formation: Desolvation of the ligands and trypsin, new formation of electrostatic interactions between ligands and enzyme, loss of degrees of freedom of the binding partners.
* How do the results of the different buffers differ? Discuss possible reasons.
* Consider the stoichiometry determined in the experiment. Does this seem reasonable to you? What are potential sources of error in the experiment that can influence it?
* Explain the necessity of the control experiment you performed. Which further control experiments would be useful to perform?
* What thermodynamic quantity must change during the association of the binding partners to obtain a signal during the measurement?
* What are the advantages of ITC over many other methods used to determine affinity? What are possible disadvantages?
* Discuss the C-values you determined. What solutions for too high/too low C-values did you use? Discuss whether these were successful for the titrations of benzamidine and leupeptin in HEPES buffer and the displacement titration.
* Calculate LE and LLE for benzamidine and leupeptin based on determined values. What do these parameters express?

References and further reading

[1] Pierce, M. M.; Raman, C. S.; Nall, B. T. Isothermal titration calorimetry of protein-protein interactions. *Methods (San Diego, Calif.)* **1999**, *19*, 213–221.

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[3] Williams, M. A.; Daviter, T. *Protein-Ligand Interactions* 1008; Humana Press: Totowa, NJ, 2013.

[4] Wiseman, T.; Williston, S.; Brandts, J. F.; Lin, L. N. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Analytical biochemistry* **1989**, *179*, 131–137.

[5] Zhang, Y. L.; Zhang, Z. Y. Low-affinity binding determined by titration calorimetry using a high-affinity coupling ligand: a thermodynamic study of ligand binding to protein tyrosine phosphatase 1B. *Analytical biochemistry* **1998**, *261*, 139–148.

[6] Talhout, R.; Engberts, J. B. F. N. Thermodynamic analysis of binding of p -substituted benzamidines to trypsin. *European Journal of Biochemistry* **2001**, *268*, 1554–1560.

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[Principles of isothermal titration calorimetry (ITC) - YouTube](https://www.youtube.com/watch?v=o_IpWcWKNXI)