S2004
Methods for characterization of biomolecular interactions
- classical versus modern

Isothermal Titration Calorimetry (ITC)

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Outline

- Calorimetry - history + a bit theory
- Isothermal titration calorimetry
  - Applications
  - Instrumentation
  - The Raw ITC Data
  - Evaluation of ITC Data

- Receptor - Ligand Interactions („thermodynamics behind“)
- ITC data – examples
  - „c values“

- Experimental set-up
- Sample preparation
- Troubleshooting

Calorimetry

Calorimetry (Latin *calor* - heat, Greek *metry* - to measure) is the termodynamic technique based on the **measurement of heat** that may be generated (exothermic process), consumed (endothermic process) or simply dissipated by a sample.

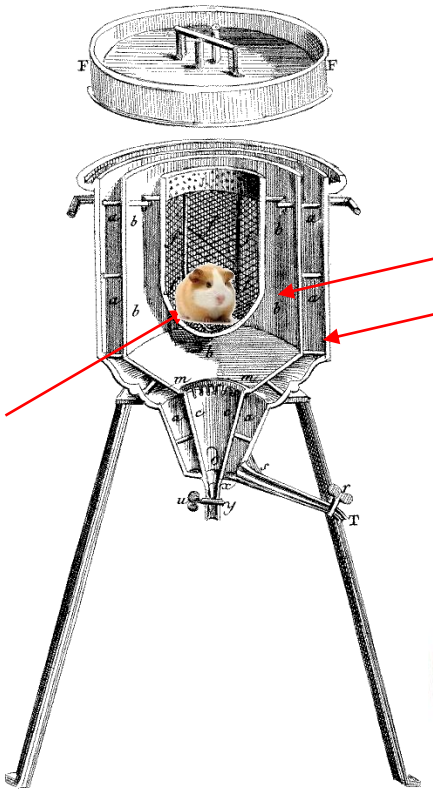
A **calorimeter** is an instrument used for measuring the quantity of heat absorbed or released in process of a **chemical reaction**.

1 calorie - express quantity of heat necessary to raise the temperature of 1 g of water by 1°C.

Heat is generated by almost all processes (physical, chemical or biological)

Calorimetry - heat changes detection

Antoine-Laurent de Lavoisier (1743-1794)

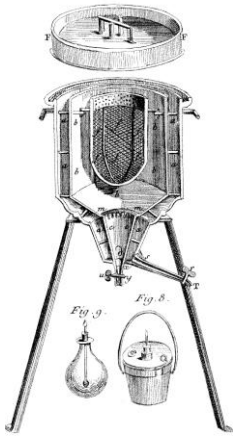


Lavoisier medal

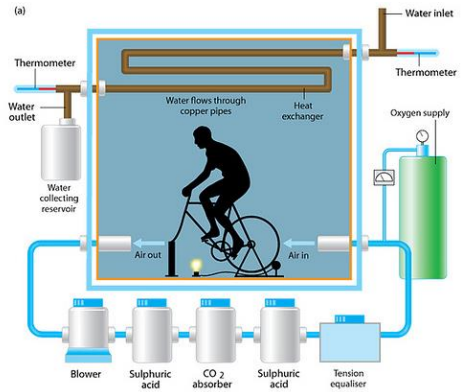
Lavoisier-Laplace calorimeter

The ice calorimeter was developed in the period **1782 to 1784** by the French scientists **Antoine Lavoisier** and **Pierre-Simon Laplace**. The central space of inner chamber contained burning oil, or an animal such as a guinea pig. The surrounding chamber contained ice. **Heat produced by the animal can be measured indirectly**, by assessing the amount of water that elutes from the bottom of the chamber, which is the impact of the animal's heat on the ice in the outer chamber.

Calorimetry



- **INDIRECT CALORIMETRY** - calculates the heat generated by living organisms when their metabolic processes yield waste carbon dioxide.



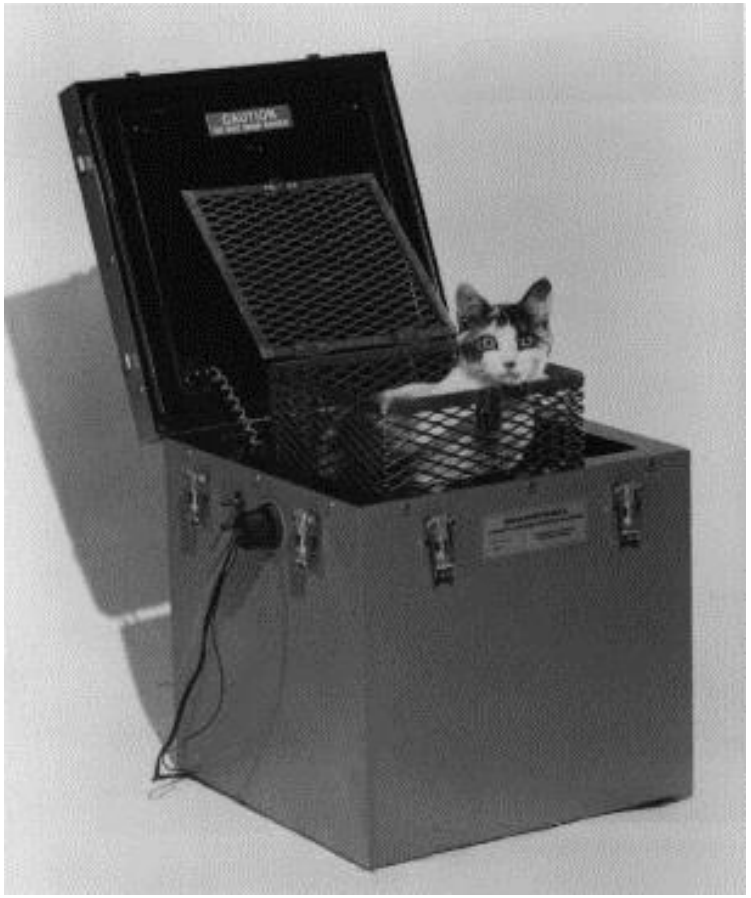
Energy expenditure from the O_2 consumption

- **DIRECT CALORIMETRY** - heat generated by living organisms may also be measured by direct calorimetry, in which the entire organism is placed inside the calorimeter for the measurement.
  - different types of direct calorimetry
  - sample is placed in the calorimetric cell

Calorimetry

Heat is generated by almost all processes (physical, chemical or biological)

- heat associated with biological reactions
- changes in animal metabolism resulting from nutrition, stress, etc.
- bacterial growth rates in fermenters
- interactions between molecules
- chemical reactions



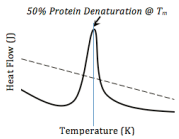
Microcalorimetry

Differential Scanning Calorimetry

- constant temperature **rate**
- thermal analysis („titrations“)
- *what happend when we heat/cool down the system?*

- During a change in temperature, DSC measures a heat quantity, which is released or absorbed excessively by the sample on the basis of a temperature difference between the sample and the reference material.

VP-DSC



Isothermal Titration Calorimetry

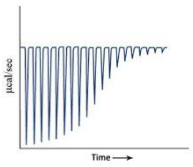
- constant temperature
- ligand titration
- *what happend when two (bio)molecules interact? (constant temperature)*

- Heat is released or absorbed as a result of the redistribution and formation of non-covalent bonds when the interacting molecules go from the free to the bound state.

VP-ITC

Auto-iTC200

iTC200



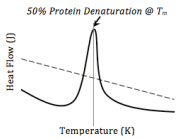
Microcalorimetry

Differential Scanning Calorimetry

- Biomolecular stability in solution
- provides insights into mechanisms of unfolding and refolding
- Midpoint **(T_m)** determination
- Enthalpy **(ΔH)**, heat capacity **(ΔCp)** of denaturation
- Characterisation of membranes, lipids, nucleic acids and micellar systeme

- **Equipment:** **VP-DSC (Malvern)**
- Solution heated/cooled from 10-130 °C

VP-DSC



Isothermal Titration Calorimetry

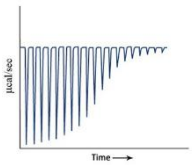
- Enzyme kinetics, biological activity or the effect of molecular structure changes on binding mechanism
- Complete thermodynamic profile of the molecular interaction in a single experiment (**stoichiometry, K_a, enthalpy ΔH and entropy ΔS values**) or kinetics parameters **K_m** and **k_{cat}**
- characterization of biomolecular interactions of small molecules, proteins, antibodies, nucleic acids, lipids and others

- **Equipment:** **VP-iTC, iTC200, AutoiTC200 (Malvern Instrum.)**

VP-ITC

Auto-iTC200

iTC200



Isothermal Titration Calorimetry

Applications / Advantages

- **receptor-ligand interactions**
  - interaction of small molecules
  - protein-protein interactions
  - nucleid acid interactions
  - …..others
- **changes in protein ionisation on binding**
- **critical micelle concentrations for detergents**
- **enzyme kinetics**

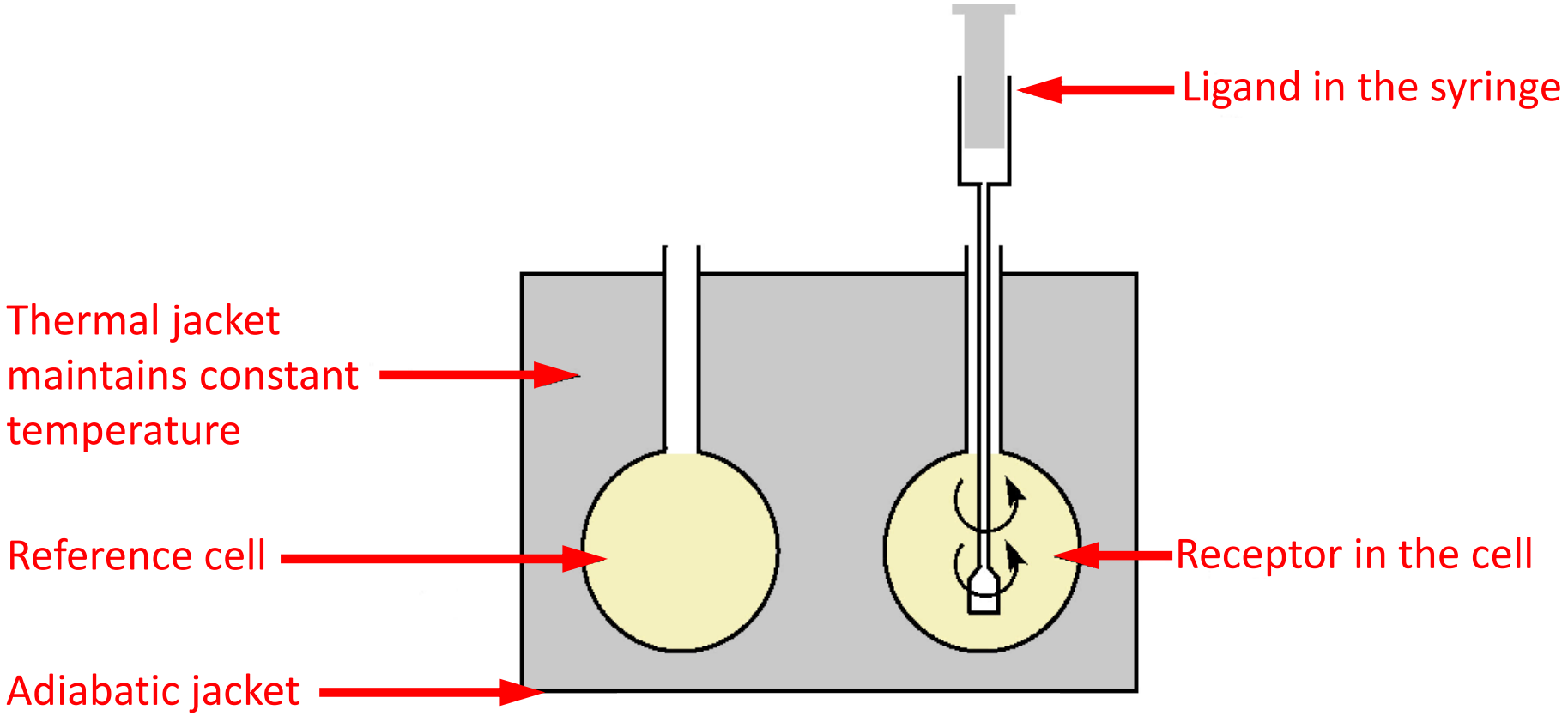
- **Experimental biological relevance**
- **Label-free**
- **In-solution**
- **No molecular weight limitations**
- **Optical clarity unimportant**
- **Minimal assay development**

- **Problematic low affinity interactions**
- **Sample consumption …**

Isothermal Titration Calorimeter

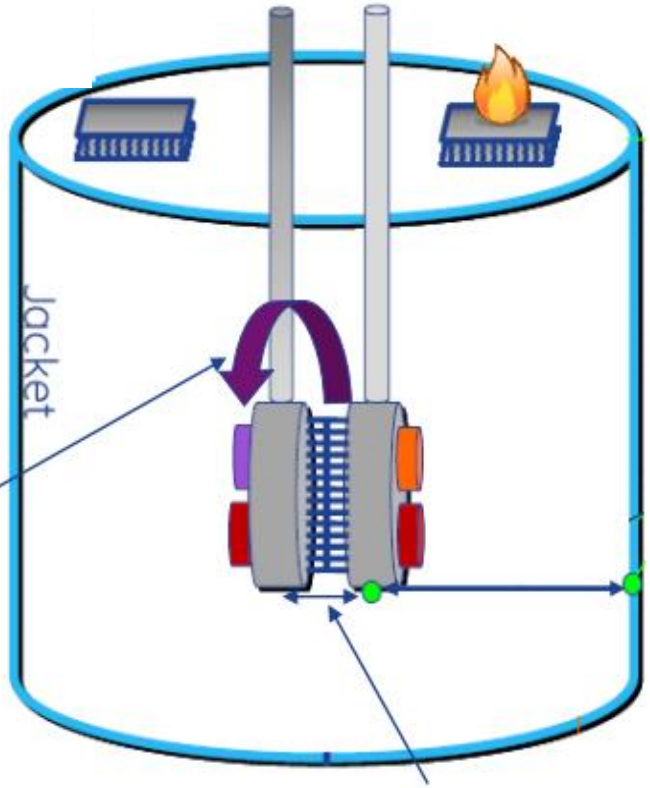
Microcal VP-ITC (Malvern Instruments)

Instrumentation



Both cells are from hastelloy alloy

Instrumentation



Feedback to the heaters which compenstate the temperature difference

Power feedback sensor which detects temperature difference between sample and reference cell and control the temperature maintenance

Goal ΔT~0

A constant temperature is controlled by two main heaters - one for each cell.
Each heater is controlled by a power feedback sensor.

In case of exothermic reaction - the sample cell gets warmer than reference cell - less power supplied to sample cell heater

ITC monitors these heat changes by measuring the differential power, applied to the cell heaters

Reference calibration heater

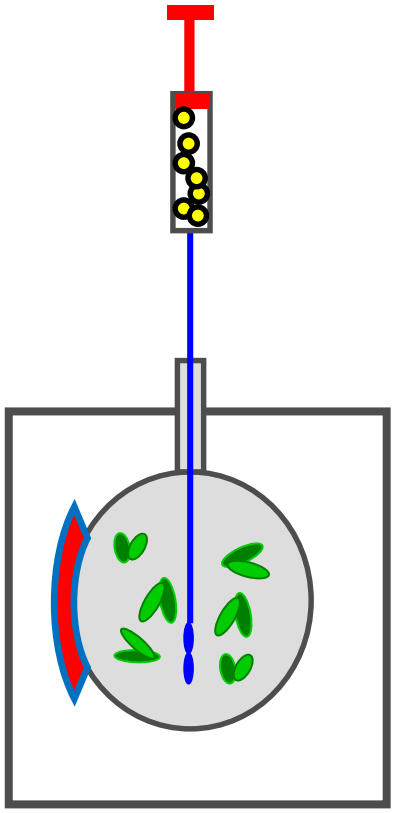
Sample calibration heater

Cell main heater

The Raw ITC Data

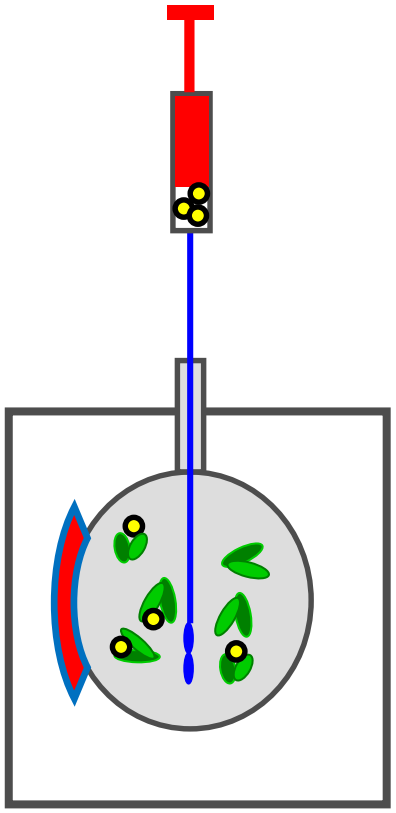
The Raw ITC Data

In the calorimetric experiment, ligand is titrated to the sample cell (receptor sample) in a number of small aliquots.



The Raw ITC Data

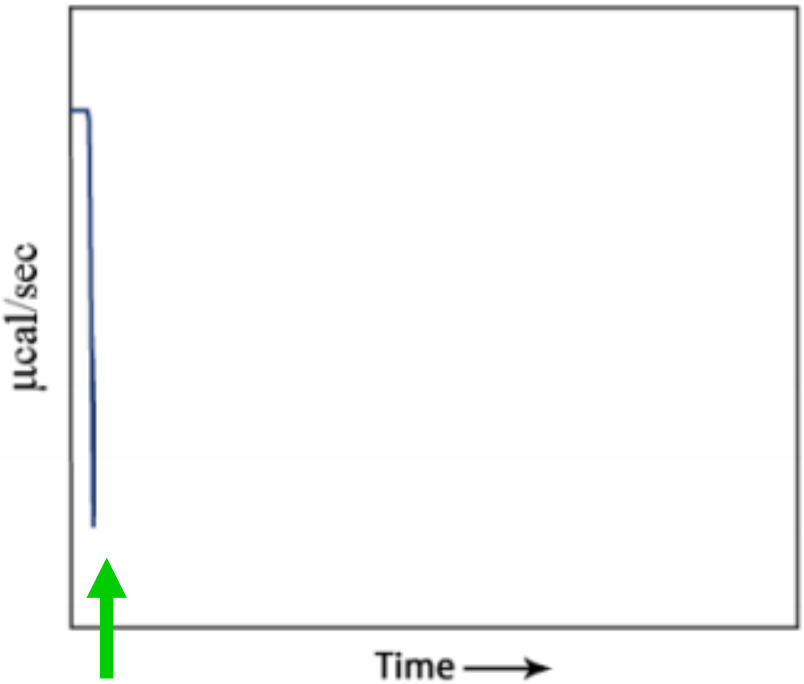
In the calorimetric experiment, ligand is titrated to the receptor in the sample cell in a number of small aliquots.



When substances bind, heat is either generated or absorbed.

The Raw ITC Data

Raw ITC data is a measure of the power difference supplied to each cell



The raw signal in the power compensation calorimeter is the power (µcal/sec) applied to the control heater that is required to keep the calorimeter cell from changing temperature as a function of time.

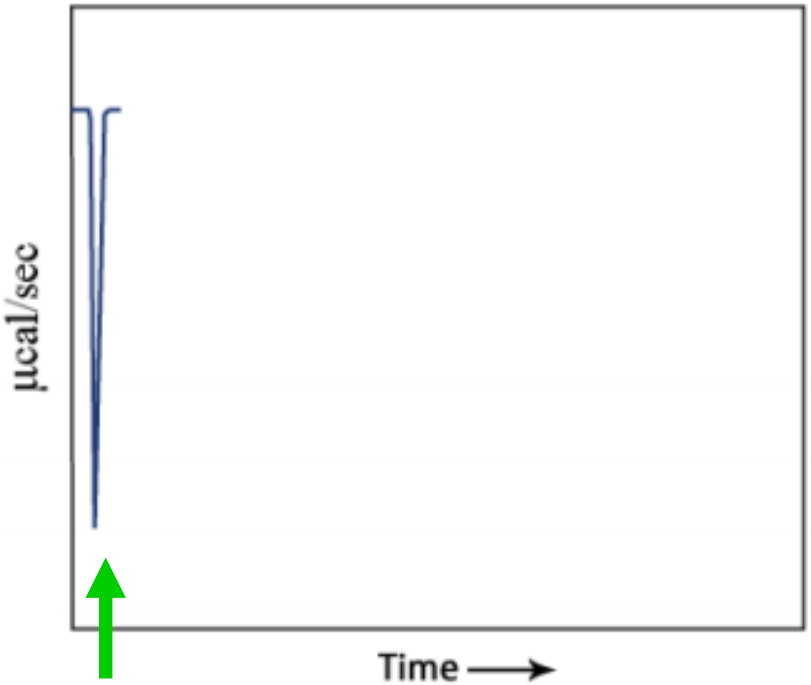
Start of titration

large peaks – lots of complex formed on each injection

equal height – virtually every ligand molecule becomes bound to receptor

The Raw ITC Data

Raw ITC data is a measure of the power difference supplied to each cell



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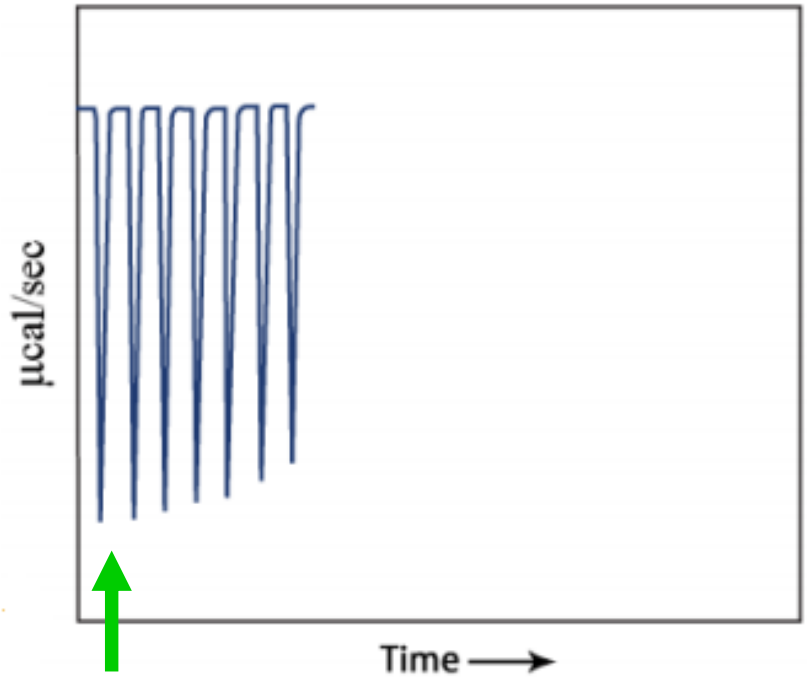

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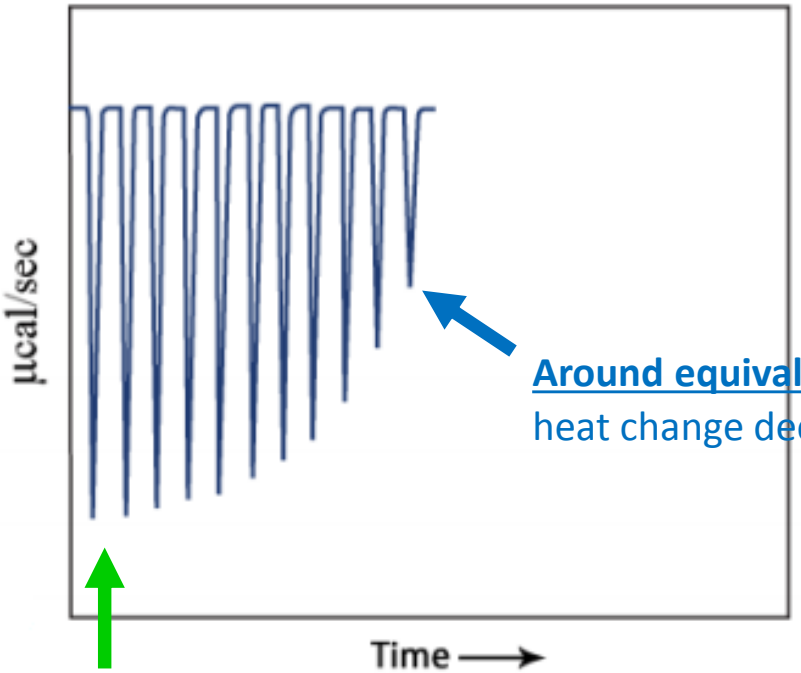

Around equivalence point
heat change decreases as binding sites fill up

Start of titration
large peaks – lots of complex formed on each injection
equal height – virtually every ligand molecule becomes bound to receptor

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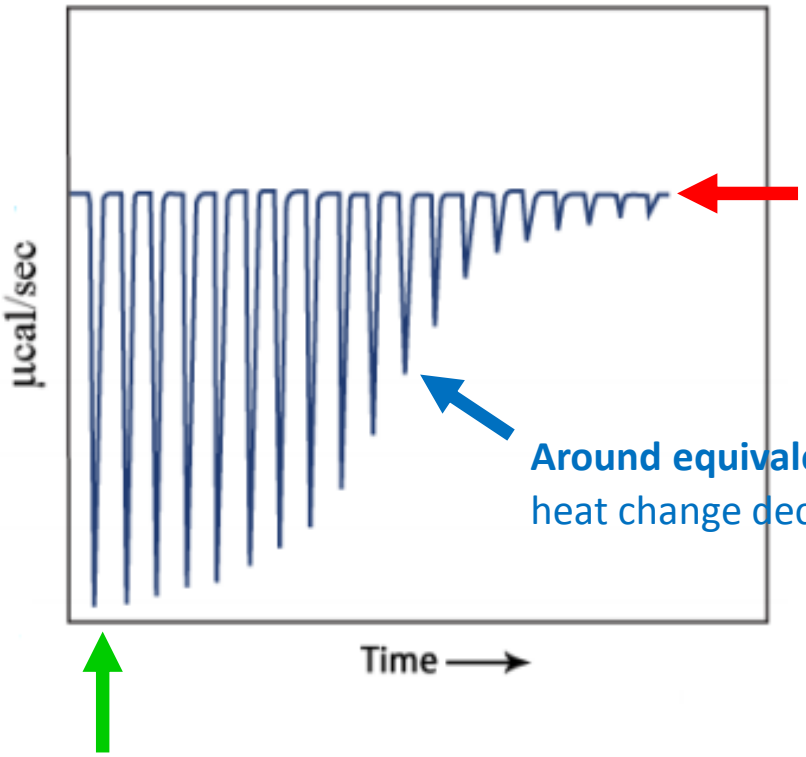
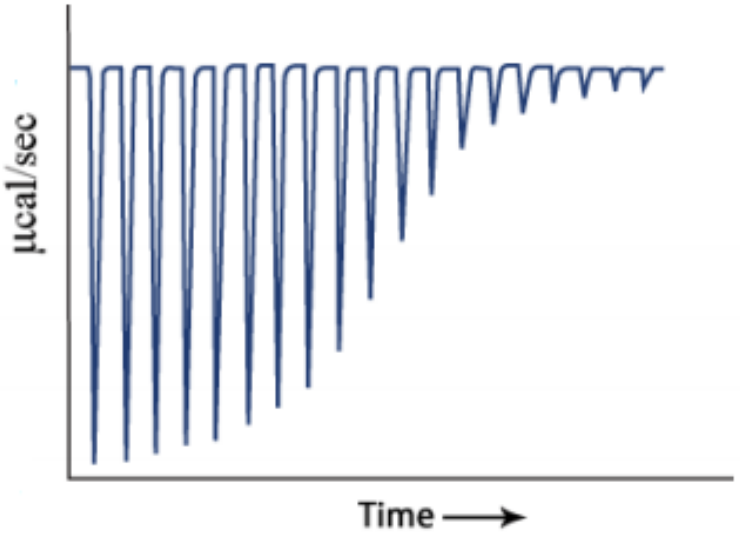


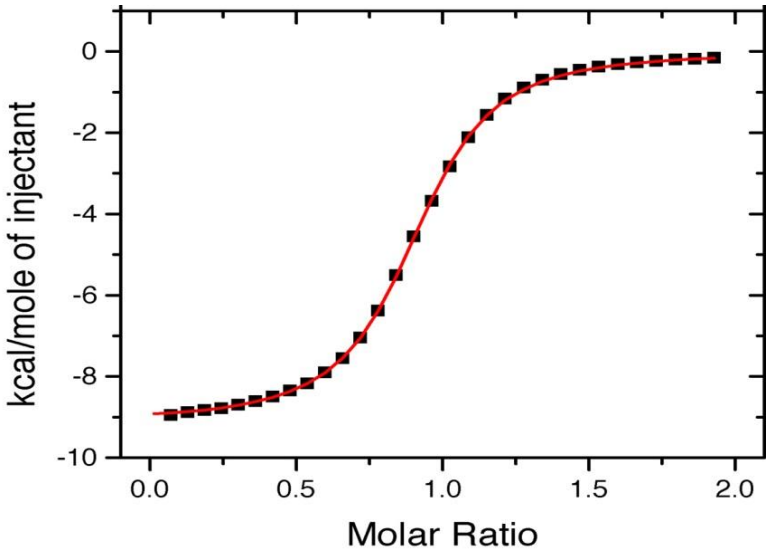
End of titration
all binding sites occupied – no further binding
only "dilution peaks" after addition of more ligand

Around equivalence point
heat change decreases as binding sites fill up

Start of titration
large peaks – lots of complex formed on each injection
equal height – virtually every ligand molecule becomes bound to receptor

Evaluation of ITC Data



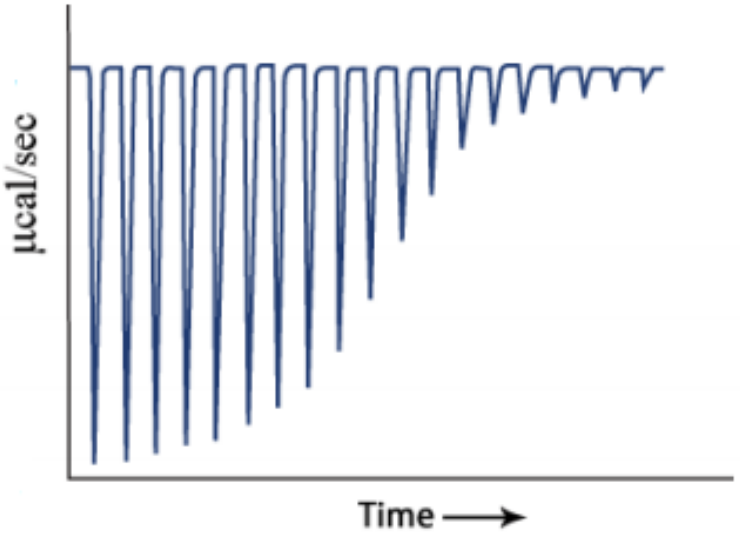


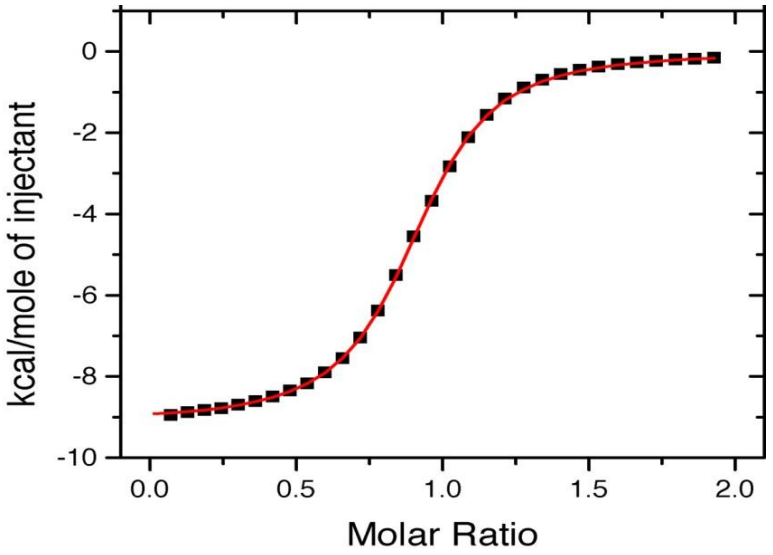
These heat flow peaks are integrated with respect to time, giving the total heat **released/absorbed after each injection point.**

The pattern of **the heat effects/mol of titrant as a function of the molar ratio [ligand]/[macromolecule]** can then be analysed to give the thermodynamic parameters of the interaction.

$\Delta H = \Delta Q$ / concentration of titrant

Evaluation of ITC Data





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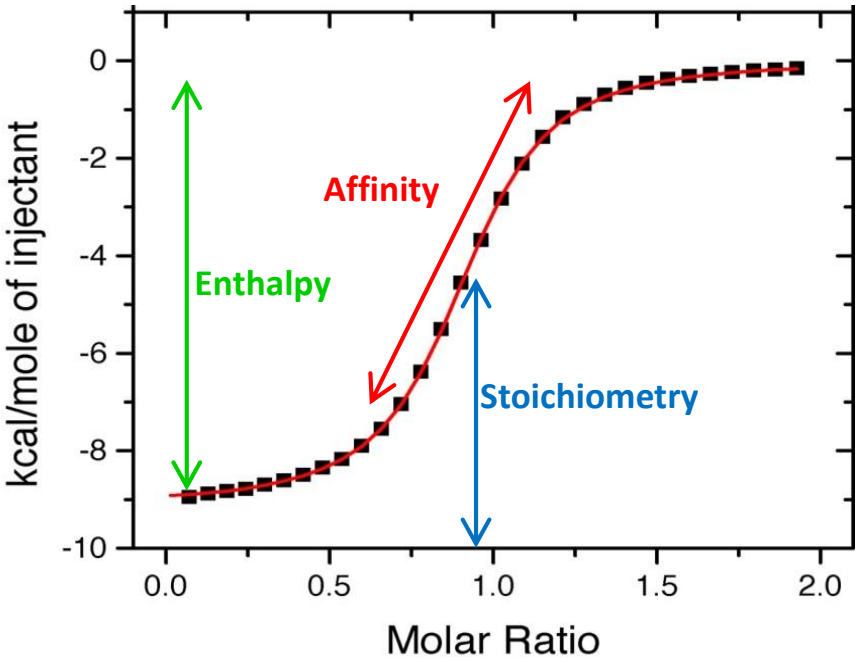


ligand / macromolecule

$$P + L \underset{K_d}{\overset{K_a}{\rightleftharpoons}} PL$$

In one ITC experiment:

- Enthalpy ΔH
- Equilibrium binding constant K_a
- Stoichiometry

…. calculate:

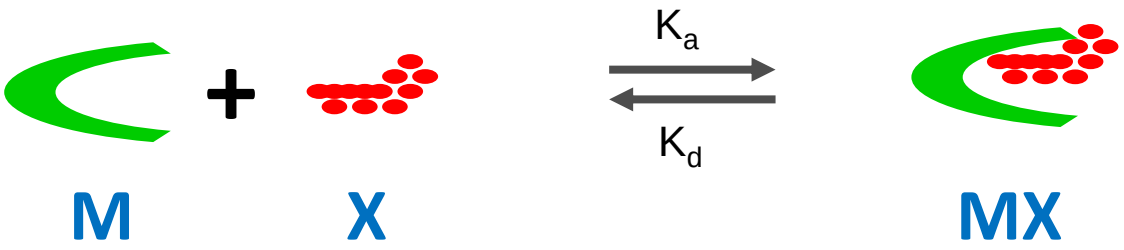
$$\Delta G = -RT \ln K_a$$

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

$$K_d = 1 / K_a$$

Receptor - ligand interactions

Receptor - ligand interactions



$$K_a = \frac{[MX]}{[M][X]}$$ [L / mol]

$$K_d = \frac{[M][X]}{[MX]}$$ [mol / L]

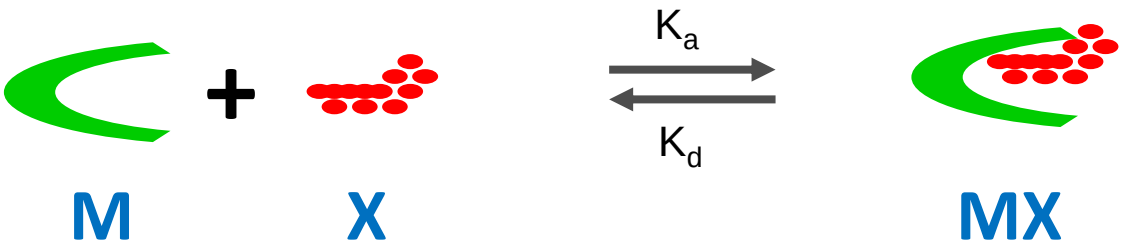
i.e. K_d is a concentration

High affinity = large K_a, small K_d

$K_d = 1 / K_a$

fast association, slow dissociation

Receptor - ligand interactions



$$\Delta G = -RT \ln K_a$$

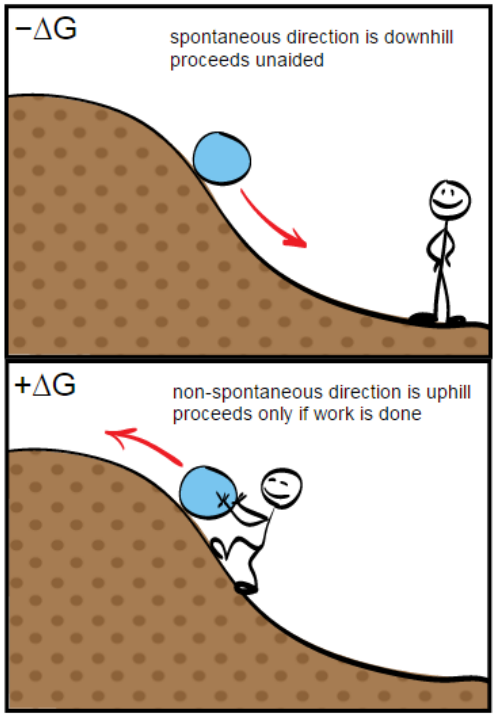
$$\Delta G = \Delta H - T\Delta S$$

Free energy — Enthalpy — Entropy

ΔG and spontaneous processes

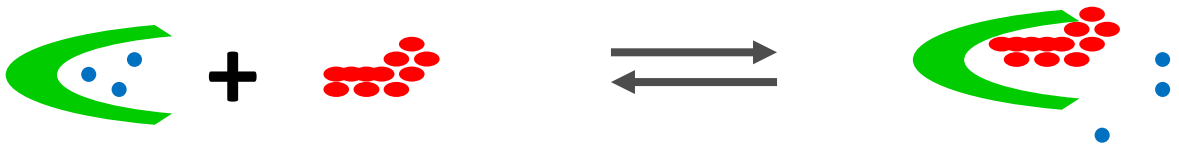
$$\Delta G = \Delta H - T\Delta S$$

$\Delta G \leq 0$ for spontaneous process



High affinity = high K_a, low K_d, high $-\Delta G$

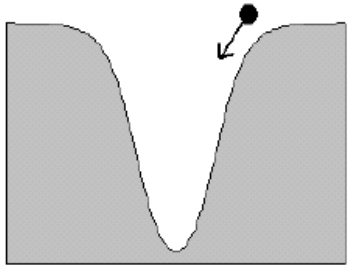
Receptor - ligand interactions



M X MX

- water molecules
- ionts
- protons

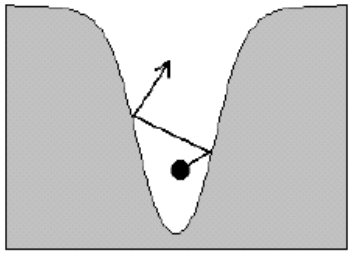
$$\Delta G = \Delta H - T\Delta S$$



Enthalpy : System has a tendency to reach the minimum energetic state.

ΔH has tendency to be negative.

….bonds are formated



Entropy : At the molecular level, Brown´s motion rises the entropy. Entropy rises with temperature.

TΔS has tendency to be positive.

Formation of bonds means that entropy is decreasing

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

Enthalpy

Changes in heat

Structure of the complex

- **Hydrogen bonding**
- **Van der Waals**

Structure of the solvent

- **i.e. water**

Enthalpy change - energy content of the bonds broken and created. The dominant contribution is from hydrogen bonds. Negative value indicates enthalpy change favoring the binding.

Entropy

Changes in disorder

Independent rotational and translational degrees of freedom

- **A complex is less disordered than two molecules**

Internal conformational dynamics

- **Flexible molecules lose entropy in the process binding**

Dynamics of the solvent

- **i.e. water**

Bonds formation means higher order of the system therefore entropy decreases

Negative is favourable

Positive favourable

Unfavorable enthalpy positive for entropically driven reactions:

Hydrophobic interactions

Solvation entropy due to release of water

Characterization of interaction

- ***Hydrophobic interaction:*** mostly characterized by less positive enthalpy, but positive entropy changes, due to the solvent reorganization around the nonpolar groups.

- ***Electrostatic interactions:*** entropically driven with less changes in enthalpy.

- ***Hydrogen bonds*:** enthalpically driven (estimated energy of one hydrogen bond is 5kcal/mol)

- ***Conformational changes*:** less changes in the enthalpy and entropy contributions.

- ***Water molecules*:** Structured water molecules / bulk water molecules

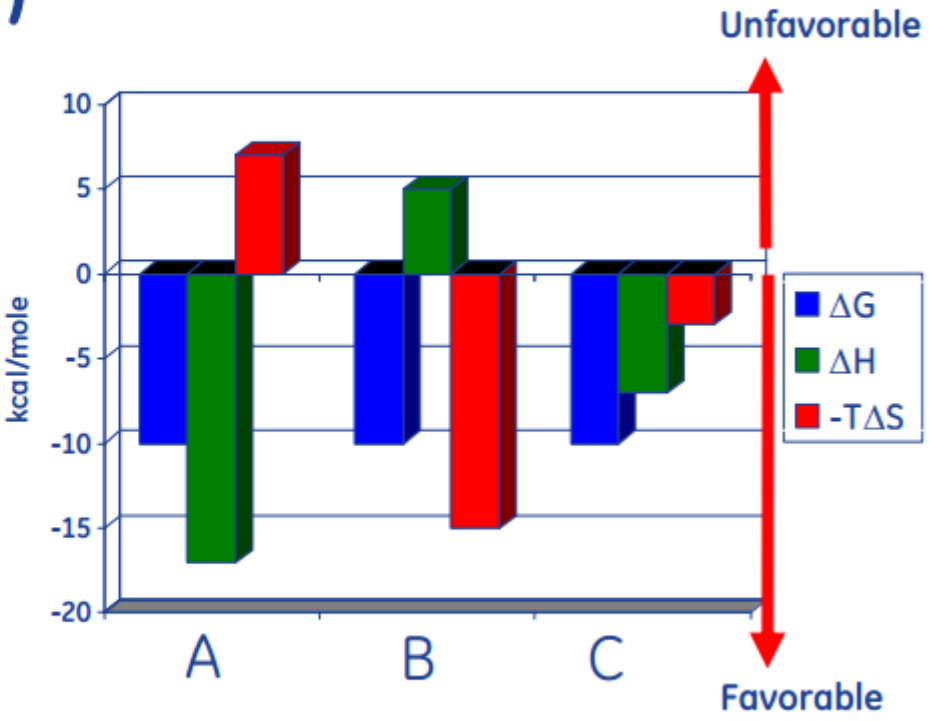
  Water molecules released from the protein binding site – this process is characterized by increasing of entropy, and the process is also enthalpically unfavourable.

Same affinity, different energetics! All three interactions have the same binding energy (ΔG)

A. Good hydrogen bonding with unfavorable conformational change

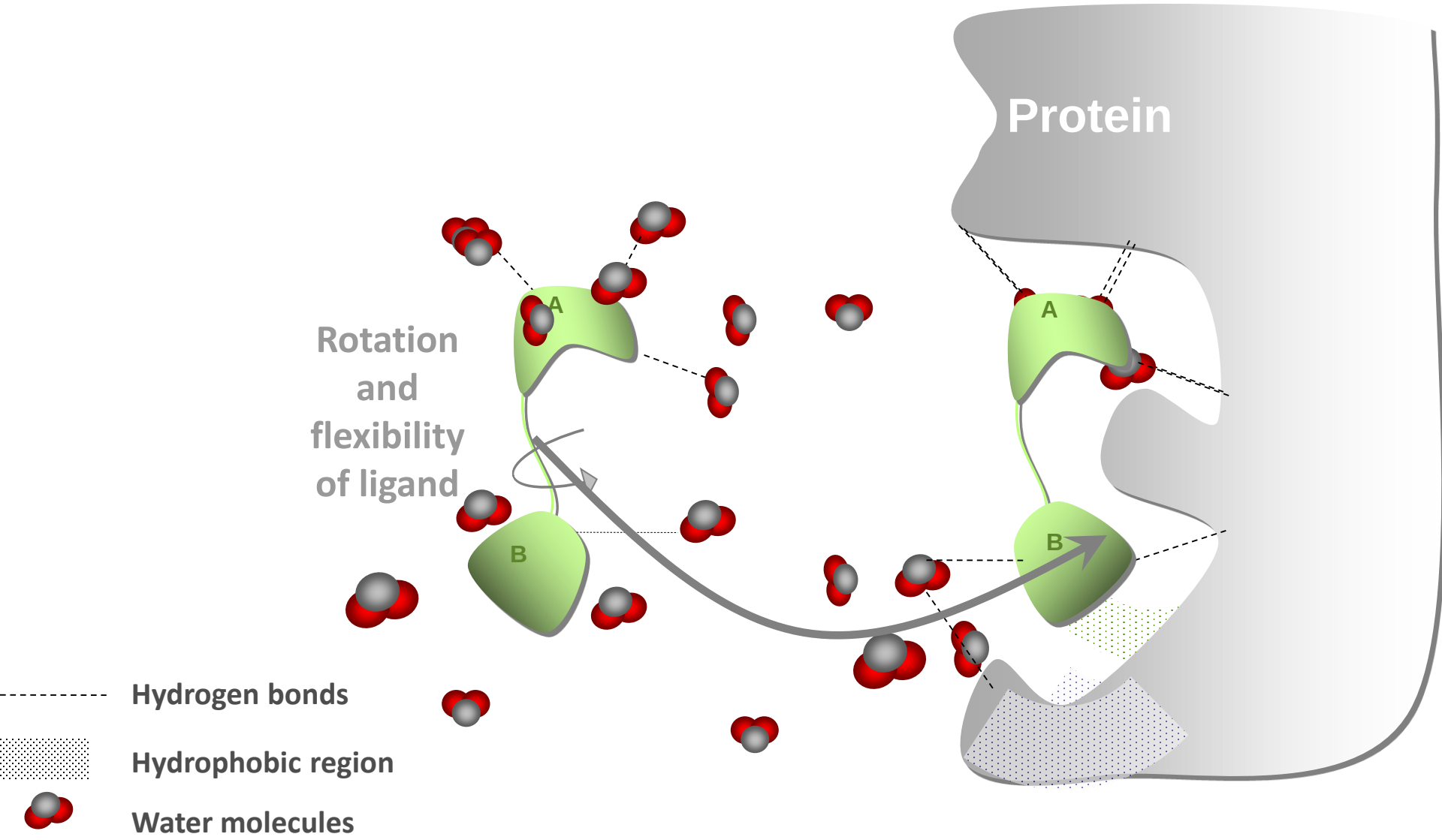
B. Binding dominated by hydrophobic interaction

C. Favorable hydrogen bonds and hydrophobic interaction



ITC results are used to get insights into mechanism of binding

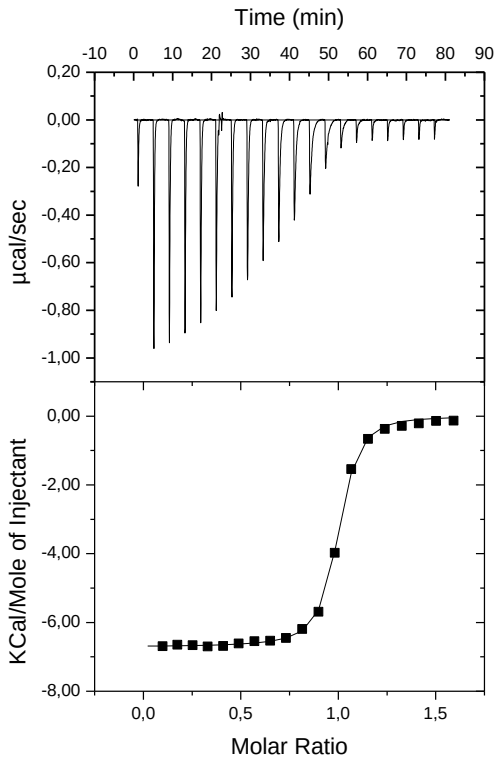
Receptor - ligand interactions

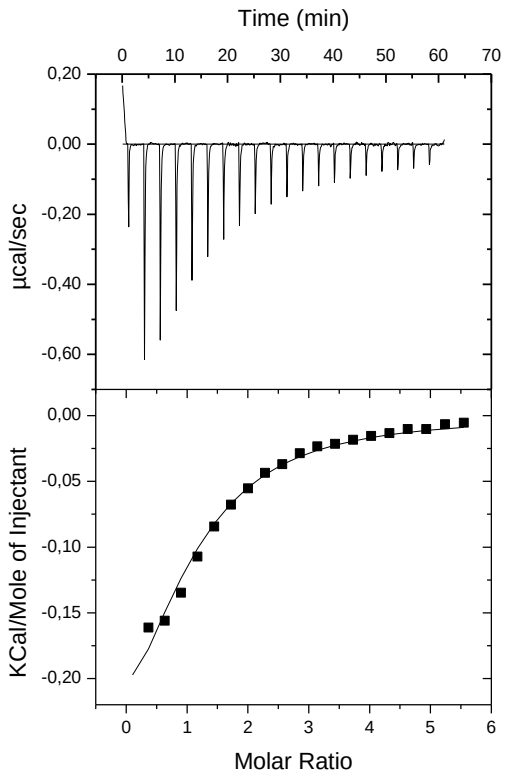


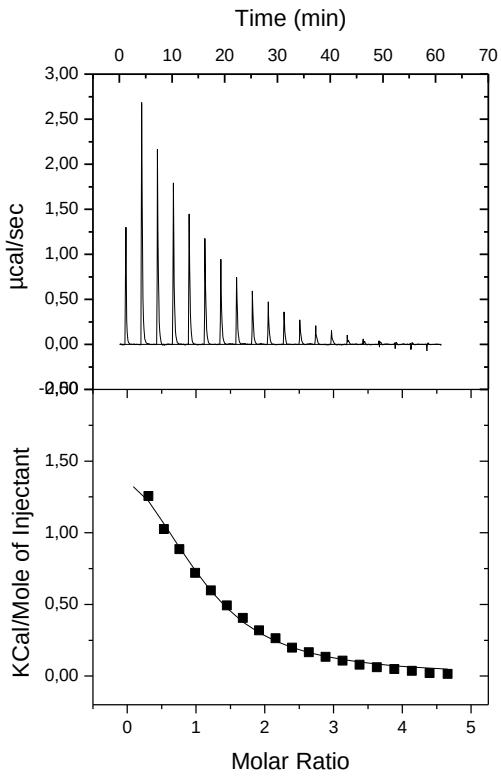
Back to the ITC Data

ITC Data

K_a 10^3 - 10^9 M^{-1} (K_d - 1 mM – 10 nM)





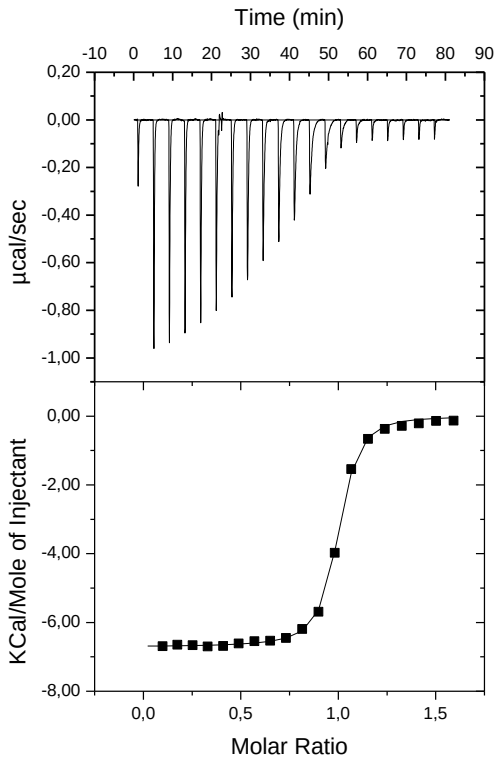


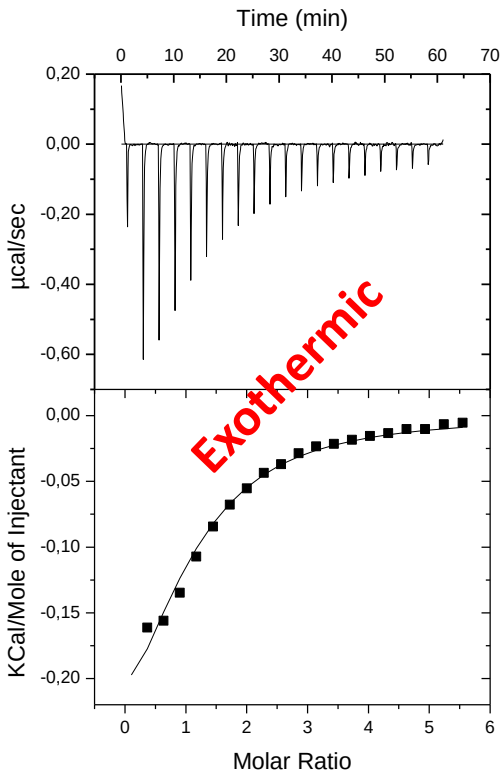
High affinity
K_a 10^5 M^{-1}

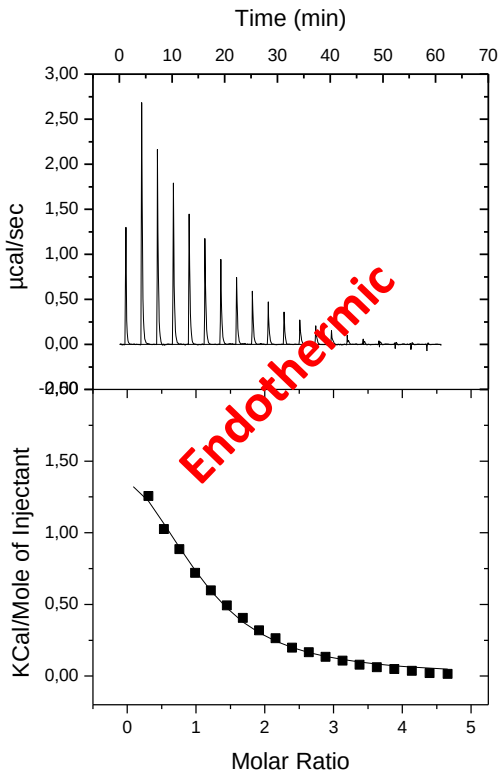
Low affinity
K_a 10^3 M^{-1}

ITC Data

K_a 10^3 - 10^9 M^{-1} (K_d - 1 mM – 10 nM)



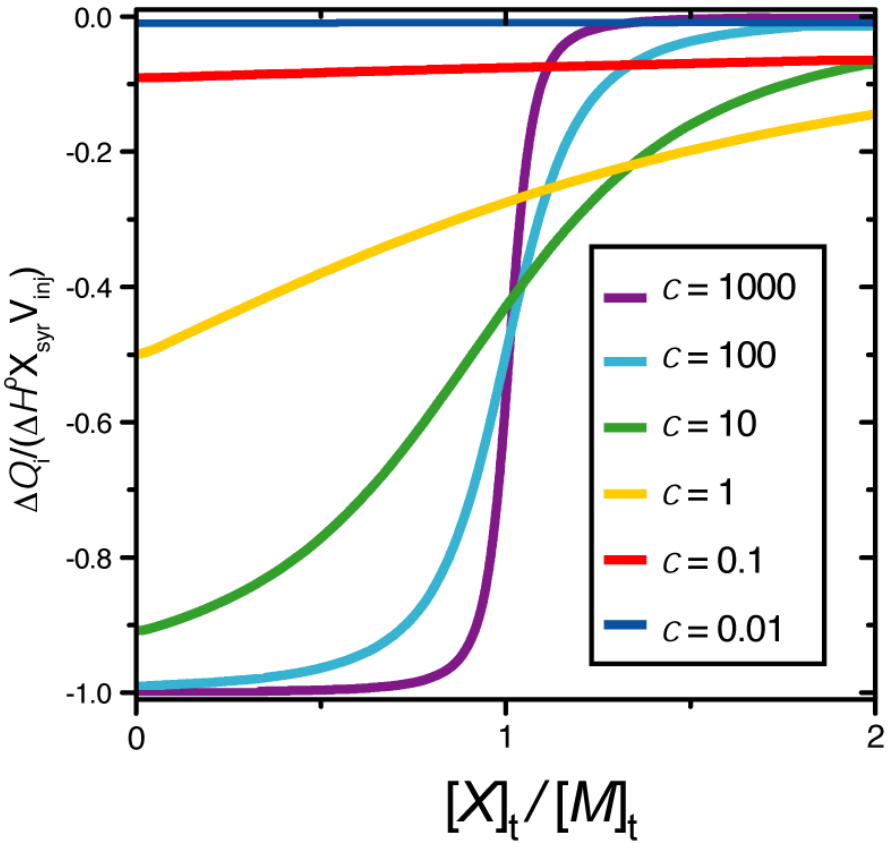




High affinity
K_a 10^5 M^{-1}

Low affinity
K_a 10^3 M^{-1}

The curve shape depends on the "*c*-value"



Generally….

c > 10

sigmoidal curve that becomes steeper as c increases

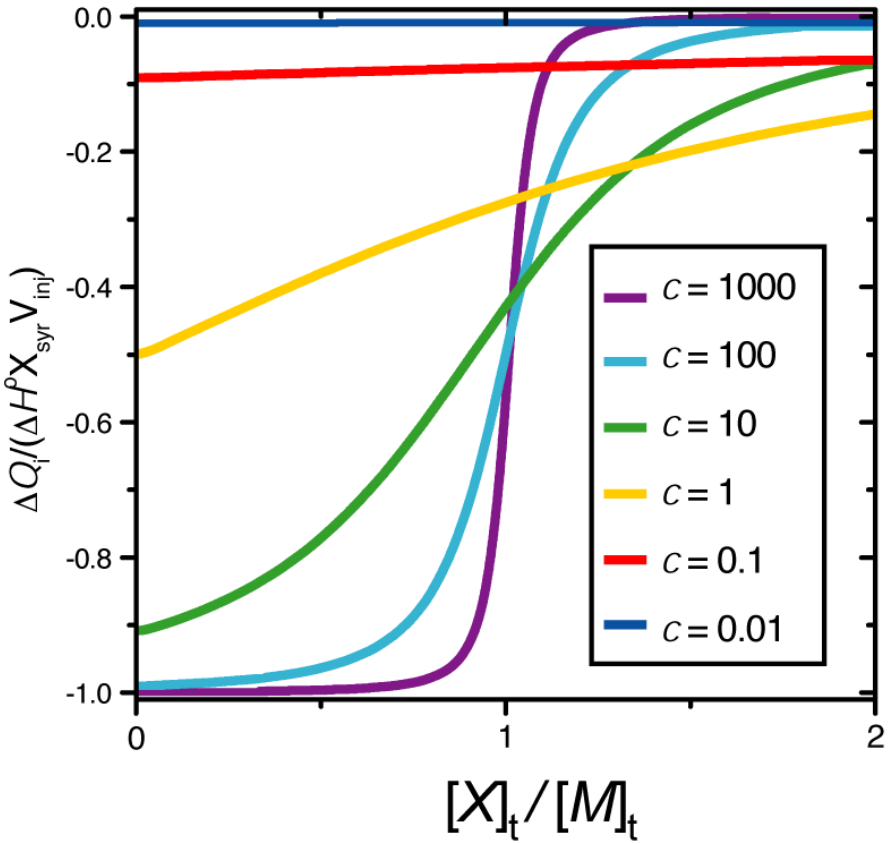
c < 10

curve becomes flatter

$$M = c / n \times K_a$$

$$c = n \times M / K_d$$

The curve shape depends on the "*c*-value"



$M = c / n \times K_a$

$c = n \times M / K_d$

For high affinity ligands
c > 500

$[M]_{total} >> K_d$

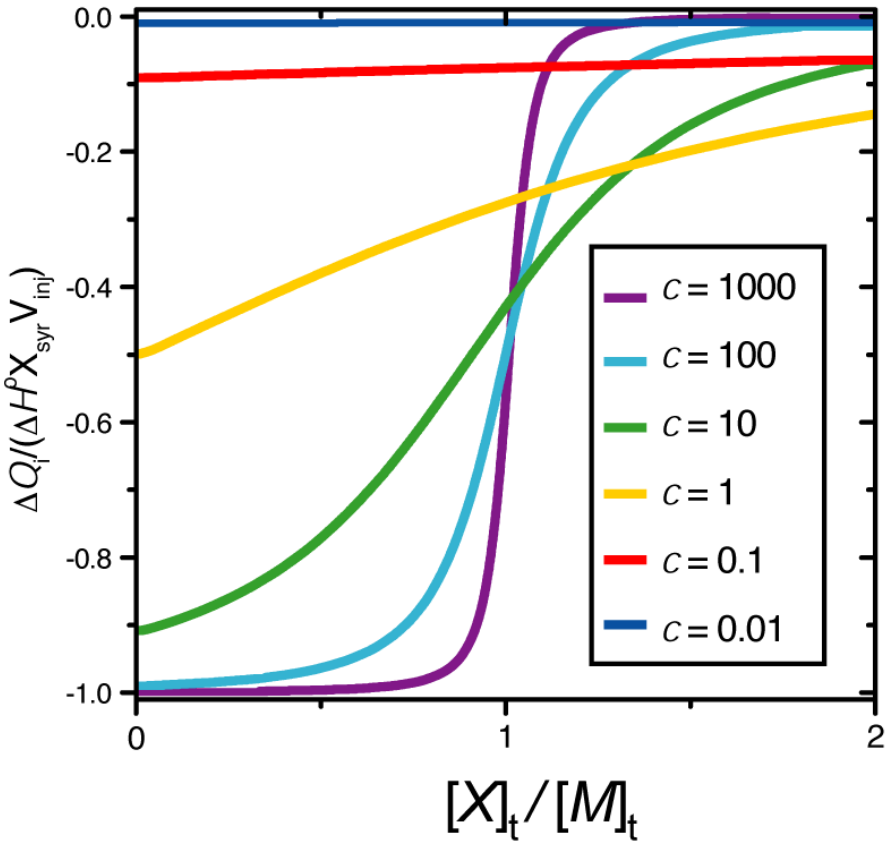
slope is too steep to determine K_d

only ΔH and n can be measured

For very high affinity ligands (low K_d) must use low macromolecule concentration

But low [M] gives very small signals…
Therefore K_d limit = 10 nM

The curve shape depends on the "*c*-value"



$$M = c / n \times K_a$$

$$c = n \times M / K_d$$

Low affinity ligands
c < 1

$[M]_{total} << K_d$

curve becomes very flat

For very low affinity ligands (high K_d) must use high macromolecule concentration
But proteins often soluble to only 1 mM…
Therefore K_d limit = 1 mM
(Reverse titration ???)

Or must add many equivalents of ligand…
K_d limit = 100 mM?

STOICHIOMETRY !!!

Very high and very low affinity systems can be studied using DISPLACEMENT TITRATIONS

Low affinity complex *High affinity complex*

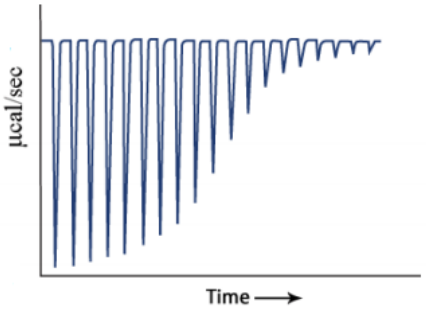
- High affinity ligand added to a solution of the low affinity complex
- **High affinity ligand displaces the low affinity ligand**
- Change in the apparent affinity and apparent enthalpy
- If parameters for one ligand are known, possible to calculate for the other ligand

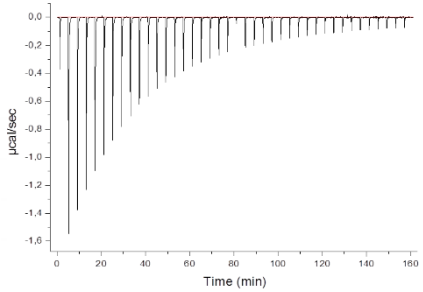
$$K_B = \left(\frac{K_A}{K_{Aapp}} - 1 \right) \frac{1}{[B]_t}$$

$$\Delta H°_B = (\Delta H°_A - \Delta H°_{Aapp}) \left(1 + \frac{1}{K_B[B]_t} \right)$$

ITC experimental set-up

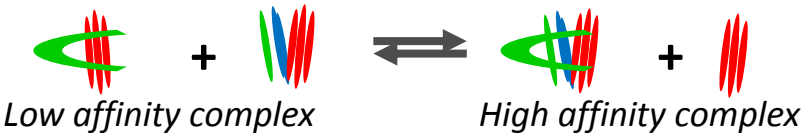
- **Set of titrations (continue injections)**
  - **Direct titration**
  - **Reverse Titration**
- **Competitive binding**
- **Single injection**



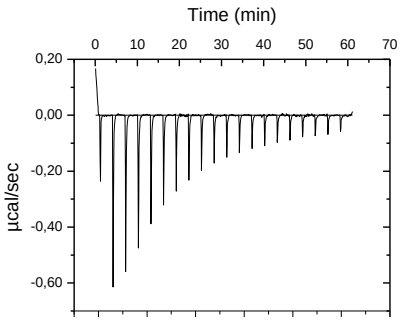


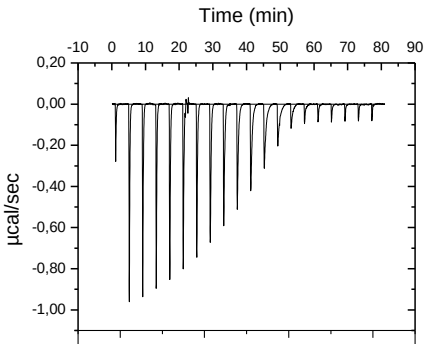
ITC experimental set-up

- Set of titrations (continue injections)
  - Direct titration
  - Reverse Titration
- **Competitive binding**
- Single injection



Low affinity complex *High affinity complex*





Low affinity complex *High affinity complex*

ITC experimental set-up

- Set of titrations (continue injections)
  - Direct titration
  - Reverse Titration
- Competitive binding
- **Single injection**

Sample preparation

- The **concentration of both samples must be determined preciselly**
- Samples must be in the **exactly same buffer** (heat of dillution)
- Dissolve your samples in lyophilisate form in the working buffer
  - Samples must be dialyzed exhaustively against working buffer
- For the first experiment (K_d is not known) **10 times higher concentration of ligand is recommended**
- Minimal concentration of macromolecule presenting in the calorimetric cell is **10 µM**
- **pH of the samples must be checked carefully**
- **Blank measurement**

- **Filtration and degassing of the samples**

Choice of buffer

Buffers have ionization enthalpies:

$$BH \xleftrightarrow{\Delta H_{ion}} B^- + H^+$$

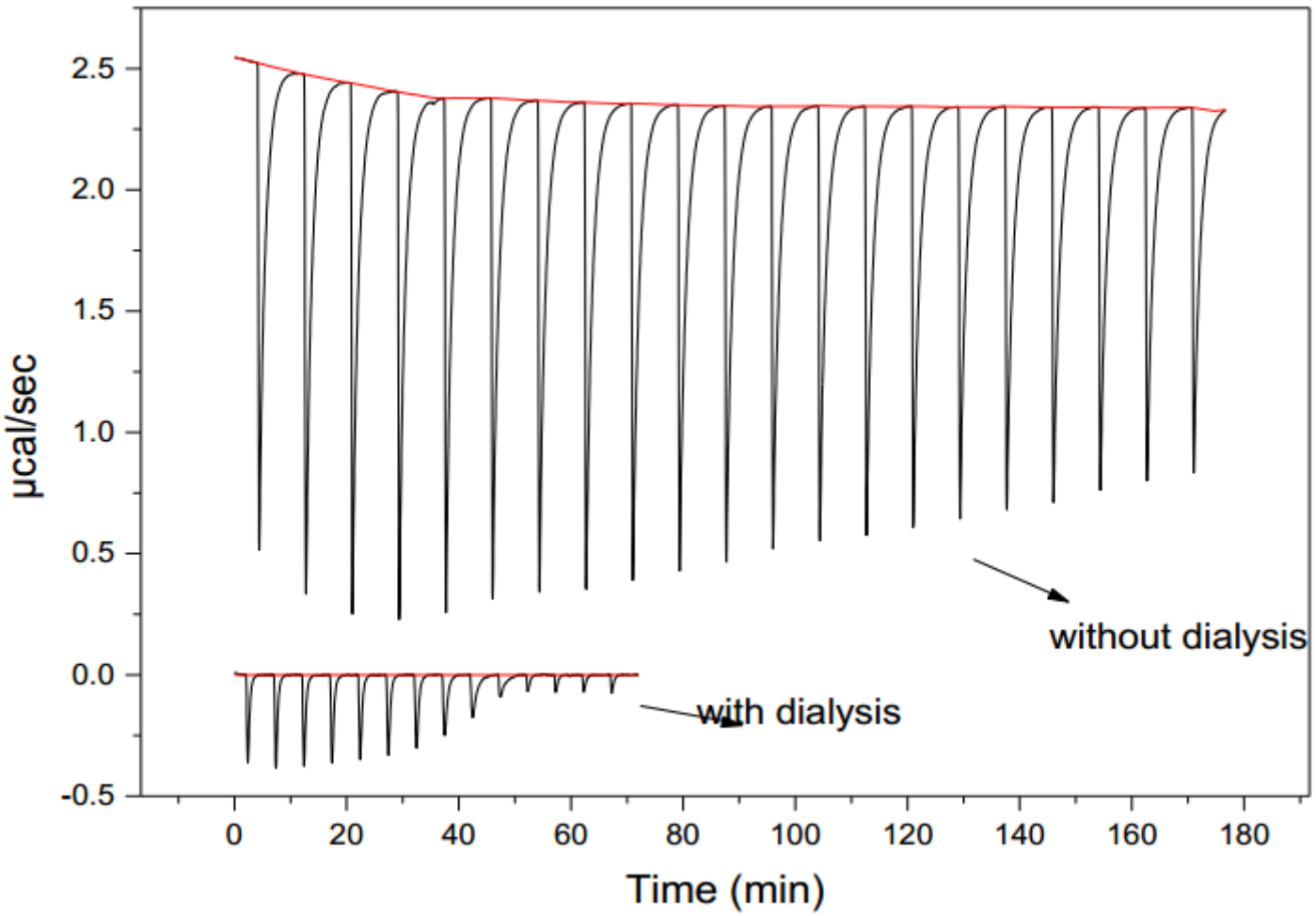
Use buffers with $\Delta H_{ion} \sim 0$

Including; phosphate, acetate, formate, citrate, sulfate, cacodylate, glycine

Quaternary amines (e.g. Tris) have high ΔH_{ion}

Troubleshooting

Buffer Mismatch

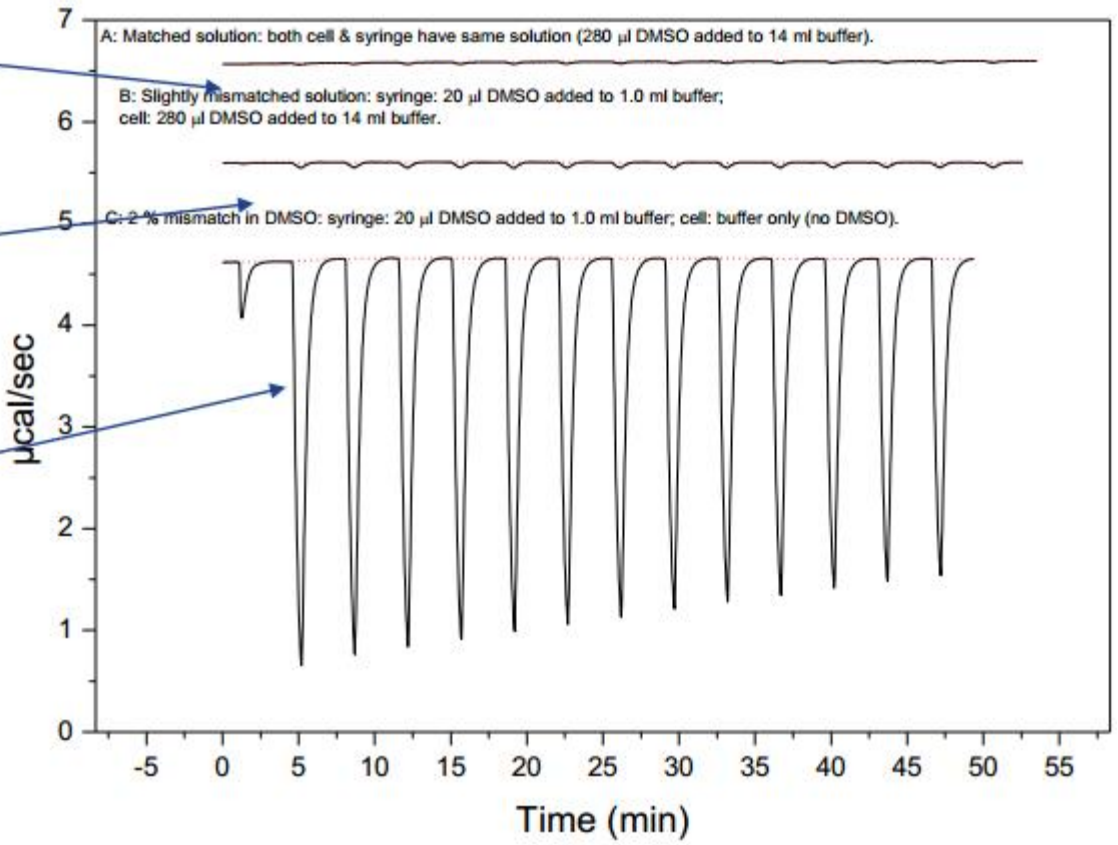


Buffer Mismatch

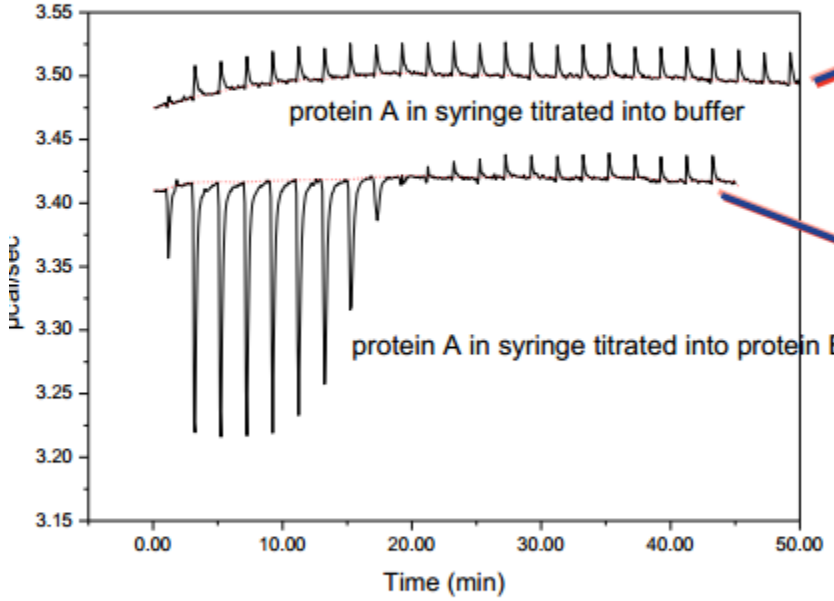
2% DMSO into
2% DMSO

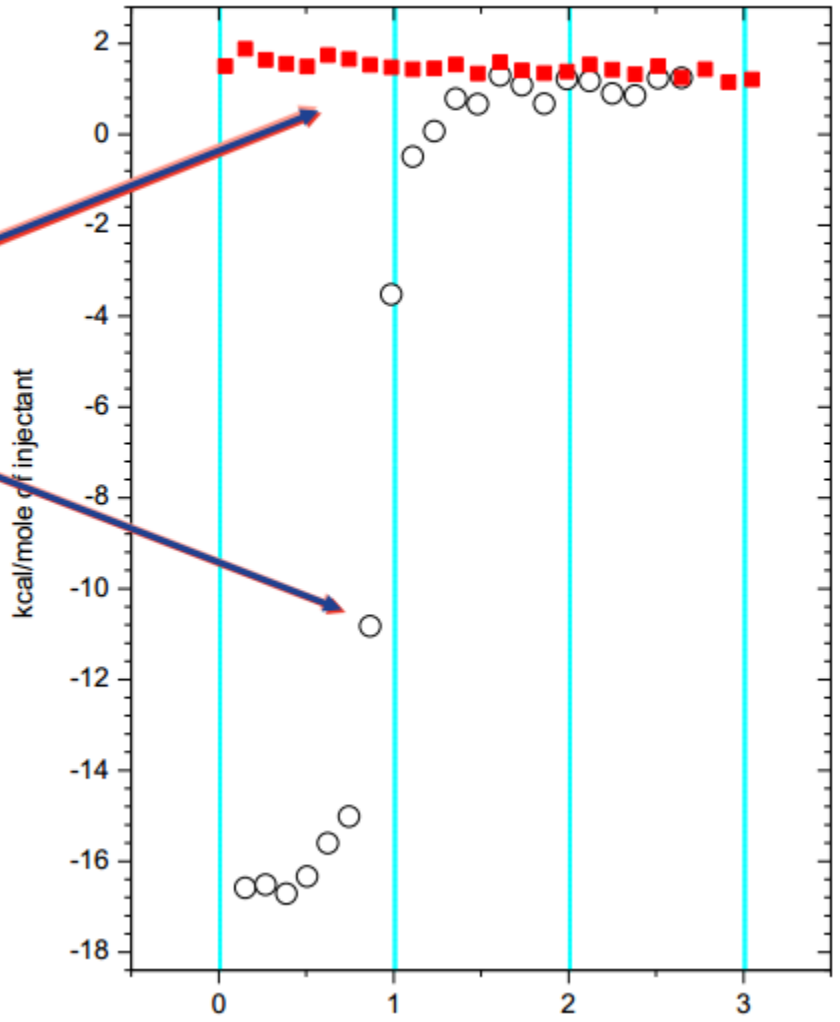
2% DMSO into
1.95% DMSO

2% DMSO into
0% DMSO

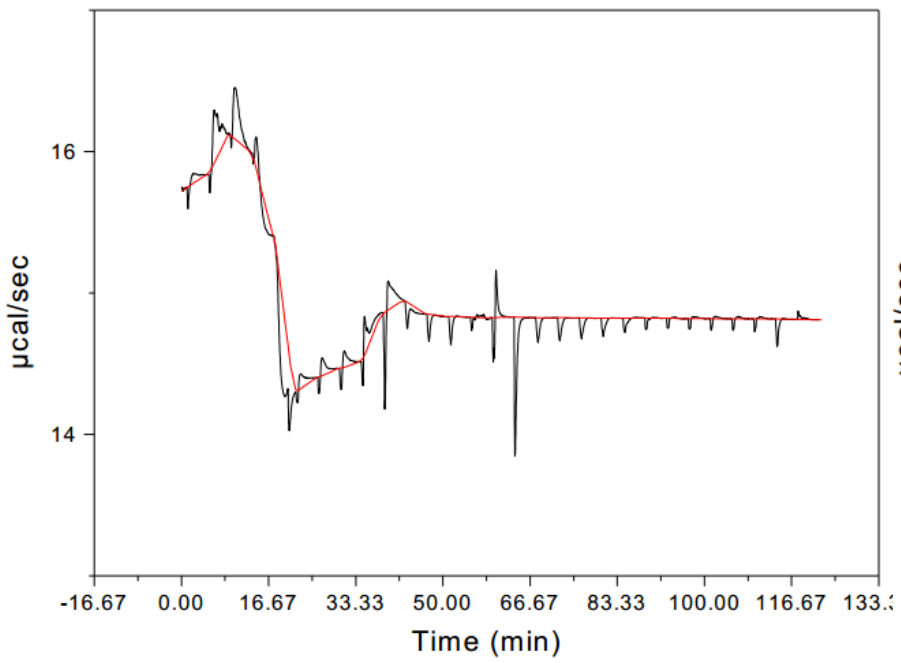


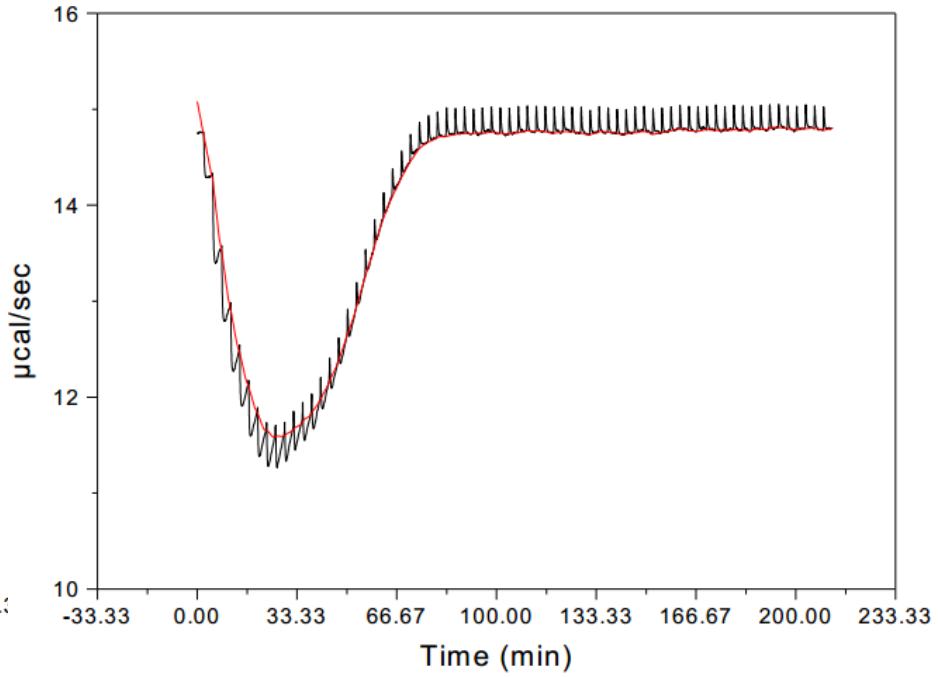
Blank experiment subtraction



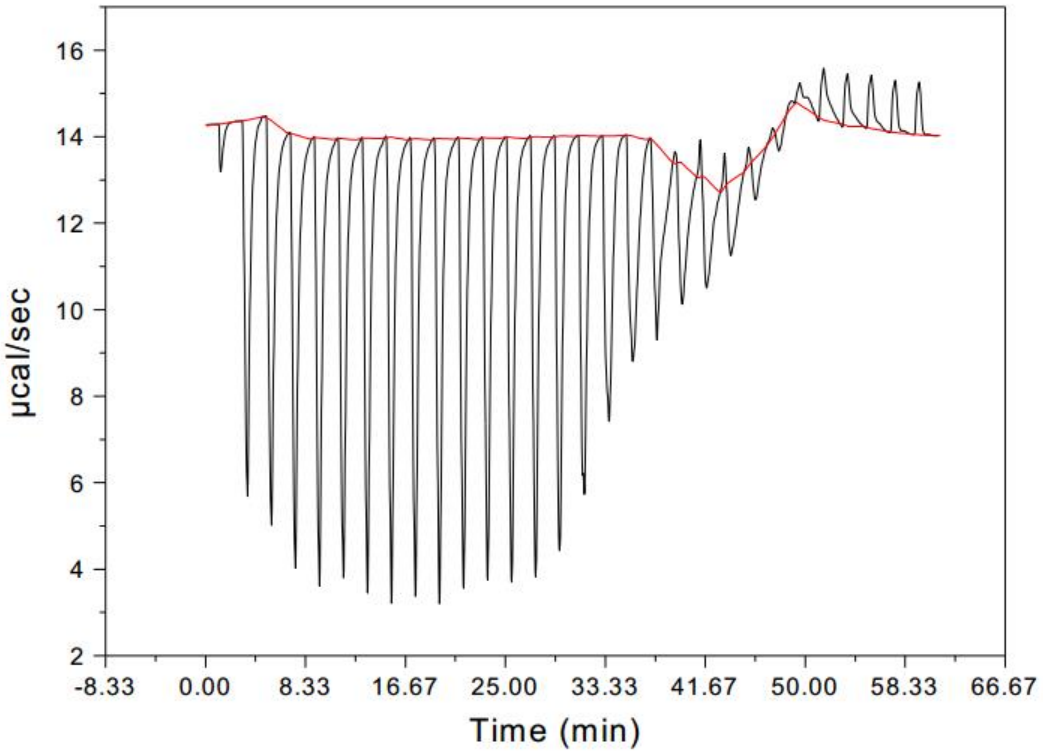


Air bubble / cleanless





Not enough time between injections




SOLUTION
SUPPORT
PROBLEM