LABORATORY OF ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies

Field: “Applications of physics in biology and medicine”

Specialization: “Molecular Biophysics”

Isothermal Titration Calorimetry as a tool for determining thermodynamic parameters of chemical reactions

PB4 v2

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1. Calorimetry

Calorimetry (from Latin *calor* = heat) is a science focused on measuring heat changes.

During almost every physical of chemical processes a heat effects are observed. They are related to change of internal energy of the system. Therefore release or absorption of heat is a universal parameter of processes, and calorimetry is a technique that can be used in studying almost every physic-chemical transformations, and molecules under study don't have to exhibit any particular features (like presence of chromophores for absorption or emission study, or atomic nuclei with special spin necessary for NMR experiments).

1.1. Two basic calorimetric methods

1.1.1. ITC – isothermal titration calorimetry

ITC is the only experimental technique which allows direct enthalpy change measurement occurring during chemical or physical process. Direct measurement of heat amount released or absorbed inside ITC calorimeter is performed while one reagent is added into another in particular volumes and particular time spacing.

Potential application varies from association and dissociation processes, determining mechanisms of molecular interactions(proteins with small molecules, DNA/RNA molecules, other proteins), influence of structural changes on molecules stability and binding mechanisms (protein mutants studies) to protein activity assays and chemical reaction kinetics. A priori, there is no limitations for molecular mass or reagents, their relative mass(i.e. small molecule interaction with large protein) or shapes, optical density of solution, as long, as both reagents are soluble.

For a simple complex formation, one titration is enough to obtain all important thermodynamic parameters of reaction: stoichiometry (N), association constant (K_a), enthalpy change (ΔH), entropy change (ΔS), free Gibbs energy change (ΔG)

Moreover ITC experiment performed at different temperatures yields heat capacity change ΔC_p [1].

1.1.2. DSC – differential scanning calorimetry

DSC is a technique designed for measuring heat capacity changes related to phase transitions. The system is heated and then cooled in controlled way.

Potential application varies from reversibility of thermodynamic transitions, stability of mutated proteins, folding and unfolding of proteins, DNA melting, dissociation of oligomeric proteins and large protein complexes, stability of protein-ligand complexes.

Single DSC experiment allows to obtain heat capacity change at constant pressure (Δc_p), phase transition temperature, phase transition enthalpy change (ΔH).

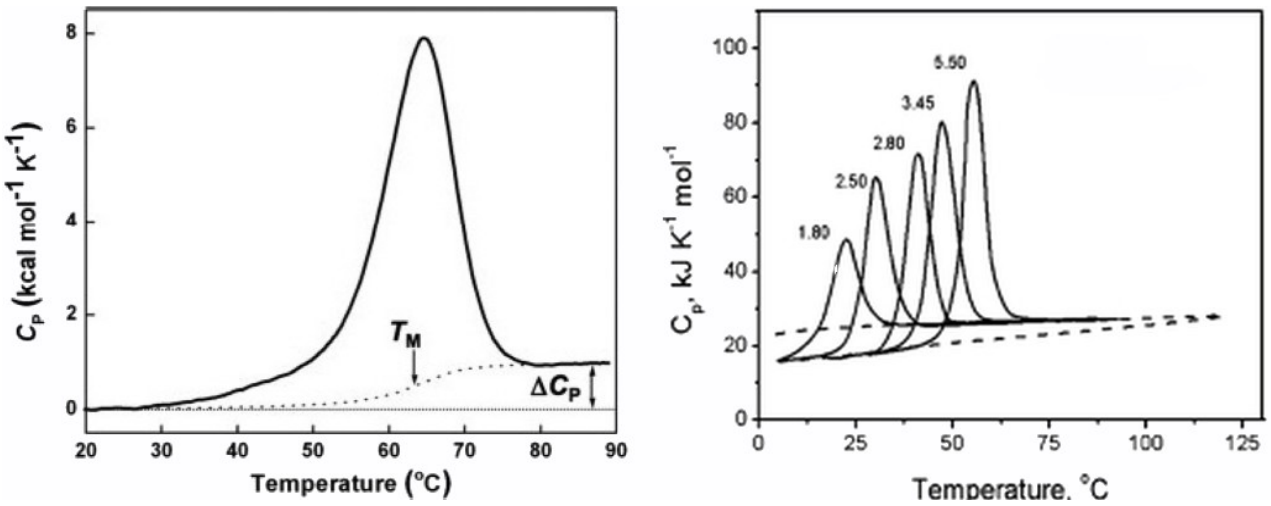


Figure 1. Typical results of DSC experiments. Melting of double DNA helix(left panel), unfolding of barnase (preiten) in pH gradient (right panel) [2]

2. Thermodynamics of ITC experiments

For simple association reaction $A+B \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} AB$ association constant is defined as follows: $K_a = \frac{[AB]}{[A][B]}$, where $[A]$, $[B]$, $[AB]$ are concetration of reagents and complex. Dissociation constant is reciprocal association constant.

In the equilibrium association/dissociation constant is related to Gibbs free energy with following relationship

$$\Delta G = -RT\,Ln\,K_a = RT\,Ln\,K_d \qquad (1)$$

where: R – universal gas constant, and T – temperature

Calorimetric titration are usually conducted under constant pressure (atmospheric pressure, as the cell is open to the air) and constant temperature (isothermal titration). In this conditions total heat effect (Q_{tot}), namely internal energy change of the system, related to studied process is equal enthalpy change (ΔH):

$$\Delta H = H_2 - H_1 = Q_{tot} \qquad (2)$$

Sign of enthalpy change indicates whether the reaction occurs with release of heat or require its delivery:

ΔH > 0 – exothermic reaction

ΔH < 0 – endothermic reaction

Enthalpy change is related mostly with formation and disruption of bonds.

Temperature (T) and pressure (p) are natural variables of thermodynamic function Gibbs free energy, which is defined as follows:

$$\Delta G = \Delta H - T\Delta S \qquad (3)$$

where ΔS is entropy change of the system.

Entropy is thermodynamic function describing conformational freedom of the system(is related to number of degrees of freedom of the system).

According to the second law of thermodynamics the entropy of an isolated system never decreases, such system will spontaneously proceed towards thermodynamic equilibrium, the configuration with maximum entropy, namely state with the highest possible number of degrees of freedom.

Entropy change is a measure whether the process is reversible or irreversible:

ΔS > 0 – irreversible reaction

ΔS = 0 – reversible reaction

Contributions to entropy may include: solvatation entropy, conformational entropy, dynamics of bonds, rotational and translational degrees of freedom of molecules

In ITC experiments entrpy is determined as a difference between Gibbs free energy change ΔG and enthalpy change ΔH .

Sign of Gibbs free energy change indicates whether the reaction is spontaneous or not:

ΔG < 0 – spontaneous reaction, system proceeds towards thormodynamic equilibrium, a state with minimal energy (negative enthalpy change ΔH < 0) and increase of "disorder" (positive entropy change ΔS > 0)

ΔG = 0 – thermodynamic equilibrium

ΔG > 0 – nonspontaneous reaction

3. ITC

3.1. Principle of operation of isothermal titration calorimeter

Isothermal titration calorimeter contains two experimental cell placed in adiabatic shield (called jacket) and a titration syringe. Both cells are identical, coin shape, placed symmetrically inside jacket. The solution of one of the reagents is placed in one of those cells (sample cell), the solution of the other in titration syringe. The other cell (reference cell) should be filled with buffer, the reagent are dissolved in, but in many cases water is good enough. The titration syringe is placed inside the sample cell (as shown on fig. 2), the tip of syringe is profiled with a shape of little blade, rotation of the syringe during experiment provides constant mixing of reagents.

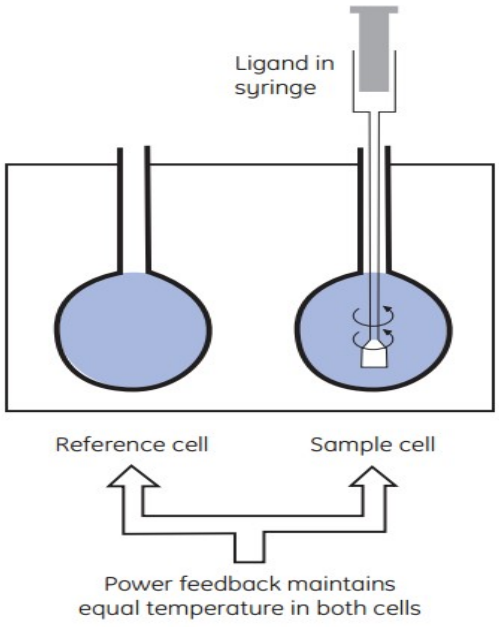


Figure 2. Simplified scheme of calorimeter [3]

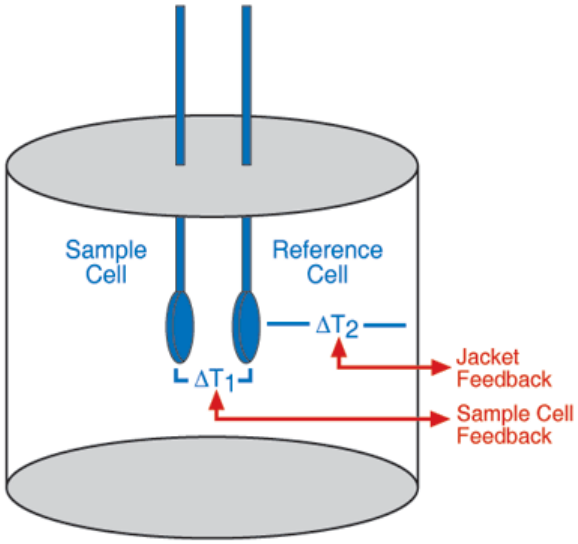


Figure 3. Simplified scheme of temperature control and measurement [3]

When experimental system is assembled, the important stage of equilibrating the system occurs. This is very important, to achieve equilibrium state before starting titration, as this is necessary for obtaining reliable data. Equilibrating is a multistep process. Firstly adiabatic jacket is equilibrated at desired temperature. Then both cell are driven to desired temperature and equilibrated there. Temperature difference between cells is monitored by Differential Power Sensor (DPS). The cell are considered to be at equilibrium, when the heat flow changes over time monitored by DSP are below certain value.

After addition of first volume of titrant into sample cell reaction starts, therefore heat is released or absorbed and the temperature of sample cell changes. The amount of heat delivered to or received from cell is equal enthalpy change (ΔH) of observed process. The real time measurement of heat flow is experimental signal. The result of ITC experiment is so called thermogram, namely plot of heat flow in time. If the experimental condition were well adjusted a typical thermogram is shown in left panel of figure 4.

If studied reaction is exothermic, temperature of sample cell rises and heat excess has to absorbed by calorimeter, the measuring system interprets this heat as negative (lost by experimental system) and picks in the thermogram are downward. Oppositely, if the reaction in study is endothermic, heat has to delivered to the sample cell (absorbed by the experimental system), thus the heat change is interpreted as positive and picks in the thermogram are upward.

Amount of heat released or absorbed in every step is calculated as an area of responding pick in the thermogram. When designing experiment, this is important, to set appropriate time spacing between consecutive injections of titrant, to ensure that the system manages to reequlibrate, and following every pick there will be a distinct section of baseline. By extrapolation of baseline sections between picks a baseline for whole thermogram is created, providing borders for pick area integration. Integrated heat changes plotted versus molar ratio of concentrations of ligand and binding sites gives a titration curve or binding isotherm.

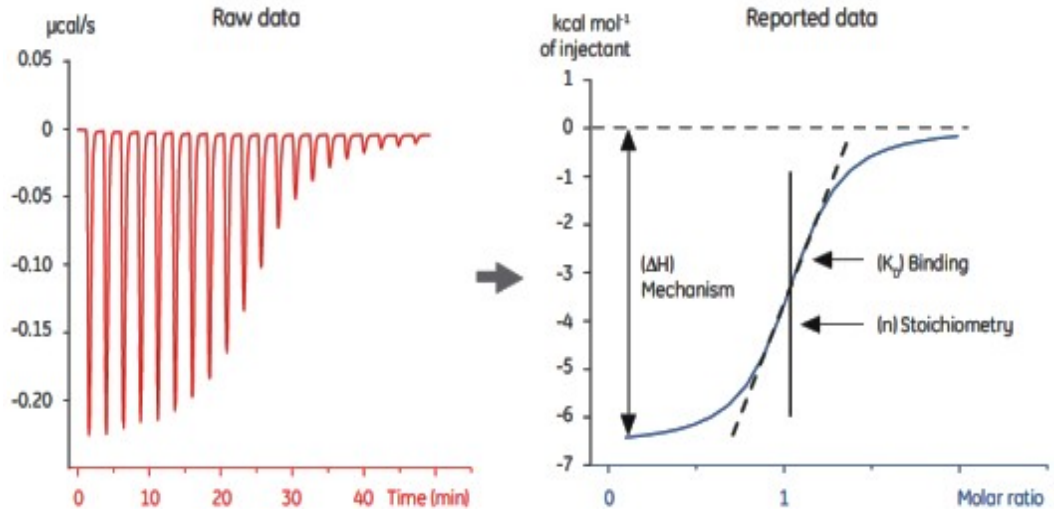


Figure 4. Typical thermogram and binding isotherm [3]

For the simple association reaction, when acceptor molecule has just one binding site for ligand, interpretation of titration curve is quite straightforward (right panel, fig 4). Distance between the upper and lower plateau determines enthalpy change (ΔH), slope of tangent to the titration curve slope determines the association constant (K_a), a point on the X axis corresponding to half of the enthalpy changes designate stoichiometry (N).

3.2. Limitations of method

Calorimeter measures total heat released or absorbed in the sample cell. Therefore it measures heat changes related to all the processes occurring in the cell. Thus this is up to the experimenter to design the experiment and prepare the samples to observe only reaction in study.

One of the basic, but important problem, is matching the buffers titrant and titrand are solved in. pH, ionic strength, concentration of salts and other additivities (like DMSO) have to be exactly the same in both solutions. Mixing of solutions of even slight concentration differences gives additional solvatation heat, and in case of buffer pH or concentration mismatch another problem occurs: protonation/deprotonation of buffer molecules. Ionisation energy for typical biological buffers vary from fraction to almost dozen kcal/mol, that is comparable with energy needed to from hydrogen bond.

In the table below heat of ionisation of typical biological buffers are given [4]

buffer	**ΔH_{ion} [kcal/mol]**
phosphate	1,22
HEPES	5,02
Tris	11,34

When the total heat change related to observed process is small, a very high protein concentration may be needed. This may be unachievable if a protein is poorly soluble and precipitates in higher concentrations or yield of protein isolated from natural sources or obtained with genetic engineering methods is low. On the other hand, if a total heat change is very high, the amount of heat released or absorbed may exceed

instrument response range.

In both cases, changing of samples concentration, whether this is diluting or concentrating may not be a solution, as one more aspect has to be taken into account. The shape of titration curve depends on product of binding sites concentration and association constant, so called Wiseman parameter, for which an optimal range exist (see below).

3.3. Samples preparation

The simplest solution of buffer mismatch is dialysis of macromolecule and ligand against the same buffer solution. In case of low molecular mass ligands dissolving it in the same buffer macromolecule was dialysed in is good course of action. That prepared samples has to yet filtrated and degassed, as it minimises noise and allows to avoid spikes caused by bubbles of air released from solution upon temperature changes.

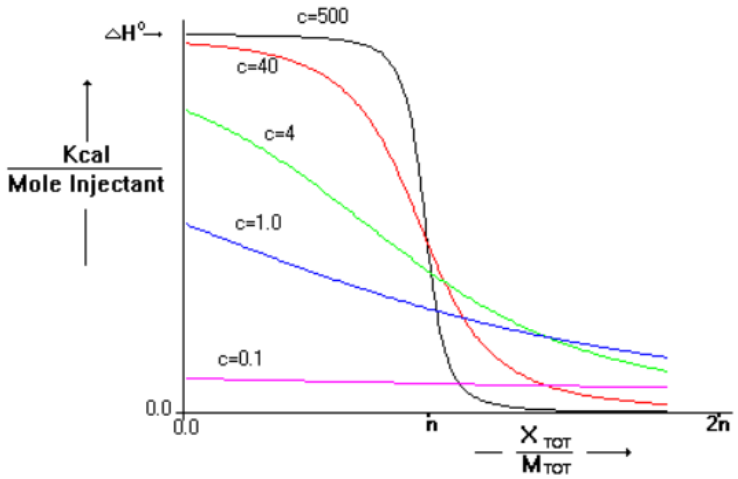
3.4. Shape of titration curve and Wiseman parameter

For typical system of ligand and receptor, titration curve has usually sigmoidal shape (fig. 4, right panel). For the first few steps, as the concentration of ligand is small, related to binding sites concentration, all added molecules are bound, and signal (ΔQ) is constant – initial plateau. With subsequent added volumes, subsequent active sites are saturated, the amount of acts of binding decreases in every step, and the measured signal changes – slope of titration curve. Finally all acceptor sites are saturated and the only observed signal is ligand dissolving heat – titration curve reaches upper plateau.

Wiseman parameter[5], also called sigmoidality factor, or simply c value, is defined as follows:

$$c = K_a \cdot [M]_{tot} \cdot N \qquad (4)$$

where K_a - association constant, $[M]_{tot}$ - total concentration of binding sites, N - stoichiometric coefficient.



Rys. 1 Kształt izotermy wiązania w funkcji parametru c [3]

In each binding experiment reagents concentrations should be in such a range, that both free reagents and complex concentrations are significant. If the concentration of binding sites is very much higher than $1/K_a = K_d$ all added ligand will be bound until saturation, and the binding isotherm is almost rectangular, with slope approaching infinity. In the opposite case, where the binding site concentration is much below K_d it is difficult

to approach full saturation, and the binding isotherm is very shallow with slope approachich zero. For accurate determination of K_d concetration of binding sites should not be much higher that K_d

Wiseman parameter facilitate adjusting ligand and macromolecule concentrations to obtain titration curve that is not to steep or to shallow. By the rule of thumbs, the optimal range of c value is 10-1000.

ΔH and K_a are obtained as parameters of function fitted to binding isotherm. But the most accurate method for obtaining these parameters is performing two titration, for different c values. ΔH is most precisely determined when titration curve has high c value, as the binding sites are well saturated and both plateaus are well defined, however the slope is two steep for determining K_a. Thus another titration has to be performed, with lower c value for K_a determination. A global analysis of these two curves would be the best option.

4. Models and equations

During this exercise formation of simple complex with stoichiometry of one will be observed, thus below, only single binding site model will be presented.

4.1. Model of identical, independent binding sites

This model is based on the assumption that all binding sites are identical(i.e. bind with the same association constant) and absolutely independent(no cooperation between sites is observed).

Used symbols:

Θ - fraction of liganded(saturated) binding sites

$1-\Theta$ - fraction of free binding sites

K_a – association constant

V_θ – active volume of sample cell (this is a volume of the coin shape part of the cell, without tube leading into it, moreover during experiment the volume of reaction mixture rises, the excess is just spilled above the reaction cell, and also not taken into account in calculations)

N – stoichiometry

$[M]_{tot}$ – total macromolecule(binding sites) concentration

$[L]_{tot}$, $[L]$ – total ligand concentration, free(unbounded) ligand concentration

Total heat Q released or absorbed by the macromolecule solution with Θ saturated binding sites:

$$Q = N\Theta[M]_{tot} V_\theta \Delta H \qquad (5)$$

This value can be calculated for every added ligand volume. But calorimeter measures difference in heat change between certain step (i) and previous step (i-1), thus signal measured by calorimeter is defined as follows:

$$\Delta Q_i = Q_i - Q_{i-1} + \frac{dV_i}{V_0}\left[\frac{Q_i + Q_{i-1}}{2}\right] \qquad (6)$$

where dV_i is volume change related to adding i-th ligand volume.

Equations (5) and (6) are general, they do not depend of reaction mechanism, until the fraction of saturated binding sites Θ is substituted, as this depends of number of active sites per molecule and reaction mechanism.

Calculating Θ within chosen model leads to obtaining equation of fitting function for data analysis.

Association constant expressed in terms of fraction of occupied binding sites is given as follows:

$$K_a = \frac{\Theta}{(1-\Theta)[L]} \qquad (7)$$

and total ligand concentration $[L]_{tot}$ in terms of free ligand $[L]$ and fraction of occupied binding sites:

$$[L]_{tot} = [L] + n[M]_{tot}\Theta \qquad (8)$$

Obtaining free ligand concentration $[L]$ from eq. 8 and substituting into eq. 7 a quadric equation for fraction of occupied binding sites Θ is derived:

$$\Theta^2 - \Theta\left[1 + \frac{[L]_{tot}}{N[M]_{tot}} + \frac{1}{N K_a [M]_{tot}}\right] + \frac{[L]_{tot}}{N[M]_{tot}} = 0 \qquad (9)$$

Solving above equation and substituting solution into eq. 5 the formula for total heat as a function total ligand concentration is derived:

$$Q = \frac{N[M]_{tot} V_0 \Delta H}{2}\left[1 + \frac{[L]_{tot}}{N[M]_{tot}} + \frac{1}{N K_a [M]_{tot}} - \sqrt{\left(1 + \frac{[L]_{tot}}{N[M]_{tot}} + \frac{1}{N K_a [M]_{tot}}\right)^2 - \frac{4[L]_{tot}}{N[M]_{tot}}}\right] \qquad (10)$$

Now above equation has to be rewriten in terms of total heat absorbed or released after i-th and i-1-th addition of ligand Q_i and Q_{i-1}. This two expressions(Q_i and Q_{i-1}) has to substituted into eq. 6 In order to obtain equation of fitting function for analysing experimental data.

This function is implemented in software, delivered with instrument, dedicated for ITC data analysis.

5. Bibliography

[1] Ilian Jelesarov, Hans Rudolf Bosshard: Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition, 1999
[2] Pooria Gill, Tahereh Tohidi Moghadam, Bijan Ranjbar: Differential Scanning Calorimetry Techniques: Applications in Biology and Nanoscience, 2010
[3] strona producenta MicroCal (part of GE Healthcare) http://www.gelifesciences.com/
[4] Alexander G. Kozlov, Timothy M. Lohman: Large Contributions of Coupled Protonation Equilibria to the Observed Enthalpy and Heat Capacity Changes for ssDNA Binding toEscherichia coli SSB Protein, 2000
[5] T. Wiseman, S. Williston, J.F. Brandts, L.N. Lin: Rapid measurement of binding constants and heats of binding using a new titration calorimeter, 1989

6. Requirement for preliminary test

The exercise begins with obligatory preliminary test. Student can start experimental part of the exercise only after passing this test. The form of the test, whether it is oral or written, the questions are closed or open, is chosen by lab assistant.

6.1. Recommended reading

1. Ilian Jelesarov, Hans Rudolf Bosshard: Isothermal titration calorimetry and differential scanning calorimetry as complementary tools to investigate the energetics of biomolecular recognition, 1999,12:3–18
2. John E. Ladbury (ed.) & Michael L. Doyle (ed.): biocalorimetry 2. Applications of calorimetry onthe biological sciences. Wiley. 2004

6.2. The preliminary test may include following subject:

The preliminary test will include issues covered in this instruction and during lecture on Methods of Molecular Biophysics.

1. What is calorimetry?
2. The basis of ITC & DSC techniques – principle of operation, measured physical quantity, obtained thermodynamic parameters.
3. Endo- and exo-thermic reactions. What processes, on the molecular level, occurs during association/dissociation reactions, which are related to heat release, which – heat absorption?
4. What processes, on the molecular level, occurs during association/dissociation reactions, what are their contributions to thermodynamic functions of state changes?
5. What is c value? How it can facilitate designing “good” ITC experiment.
6. How is build and works ITC calorimeter?

7. The exercise

7.1. Samples

Dependently of available chemicals and assistant decision one of the following association reaction will be studied:

1. complexing crown ether 18-crown-6 with barium ions
2. binding chitotriose by lysozyme
3. binding naproxen by bovine serum albumin (BSA)

Students will receive concentrated solutions of macromolecule and ligand, and corresponding buffer.

7.2. Performing exercise

1. Preparing and assembling the measuring system under the guidance of an assistant.

2. Determination the best conditions of the experiment with regard to Wiseman parameter and published data.
3. Preparation of solutions of protein and ligand at chosen concentrations, including filtering and desiccation.
4. Filling calorimeter cell and syringe with appropriate solution, assembly of the system, input selected measurement parameters into calorimeter control software and start measurement.
5. Analysis of results using dedicated software.

8. The report

The report shall contain

1. Description of studied phenomena
2. Description of materials and method, with brief discussion of limitations of the method
3. Analysis of obtained data, and interpretation of results, including calculation of Gibbs free energy for studied reaction
4. Comparison of own results with literature data.