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"

PPB4

The study of complexation of calcium ions with EDTA by isothermal titration calorimetry

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I. Introduction

All chemical and biochemical processes in which chemical bonds in molecules are created or broken, or hydration of polar and non-polar groups is altered, are accompanied by heat absorption or emission. The measurement of thermodynamic parameters characterizing this process allows to describe the occurring phenomenon. Not only does this have a cognitive, but also a practical meaning, for example in the studies describing the properties of drugs.

Calorimetry allows direct, quantitative measurement of heat exchange of the system with the environment, which is the result of ongoing chemical and physical processes. In contrast to indirect methods which require the specific properties of biomolecules, this technique allows for direct measurement of the parameters describing the changes of the system state. It can be used to study any molecular reactions in the case of transparent and opaque solutions or suspensions. There are typically no constraints on the mass of molecules and buffer composition. Due to the listed advantages, calorimetry is routinely used in investigations of interactions and conformational changes for a wide range of biopolymers, including proteins, nucleic acids and lipids.

The aim of the experiment is to:

- Learn the selected calorimetric measurement technique (ITC)
- Gain knowledge about the principles of operation and maintenance of the calorimeter
- Carry out a typical experiment involving the observation of calorimetric thermal changes occurring during the complexation of molecules
- Determine the thermodynamic parameters of the process
- Discuss the results.

II. Types of experimental calorimetric techniques

There are two types of calorimetric techniques:

1) Differential scanning calorimetry (DSC)
2) Isothermal titration calorimetry (ITC)

II.1 Differential scanning calorimetry (DSC)

DSC makes the measurement of phase changes, initiated by heat delivery, that occur in biopolymers, possible. Therefore, it has applications in research of phenomena induced by the temperature change (for example double - single helix DNA transition, protein unfolding,

oligomers dissociation into subunits, comparison of the mutants stability, interactions of proteins, polysaccharides, and non-biological polymers).

DSC allows to obtain the following thermodynamic parameters:

• unfolding enthalpy (ΔH), resulting from the emitted heat denaturation,

• phase transition temperature ($T_{p.f.}$) corresponding to the states in which 50% of the biomolecules are in the unfolded state - the higher the transition temperature the more stable biomolecules are,

• denaturation heat capacity change (ΔC_p), due to changes in the hydration of side chains, hidden, in the biologically active state in the interior of biomolecules and exposed in the denatured state.

In a typical DSC experiment one of the cells (reference) is filled with buffer, the other contains a solution of biomolecules in the same buffer. Cells are heated at a constant speed. The heat generated during thermal denaturation of the molecules is measured. A typical DSC thermogram is shown in the scheme (Fig. 1). From the plot of $C_p(T)$, we can determine: $T_{p.f.}$, ΔC_p, and next changes in enthalpy, entropy, Gibbs free energy and equilibrium constant of the process.

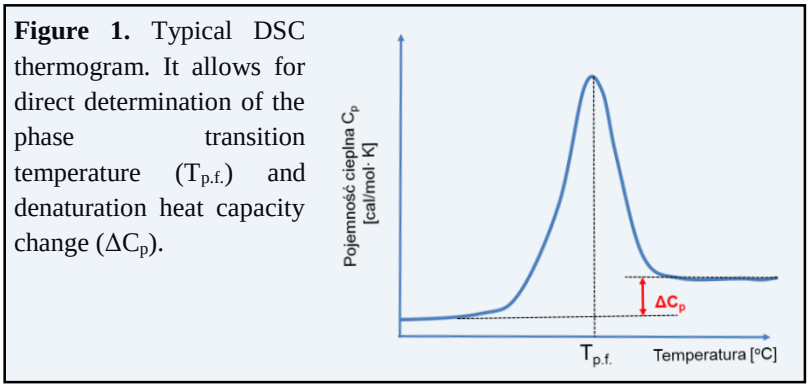


Figure 1. Typical DSC thermogram. It allows for direct determination of the phase transition temperature ($T_{p.f.}$) and denaturation heat capacity change (ΔC_p).

II.2. Isothermal titration calorimetry (ITC)

In a typical ITC experiment the heat generated during the biochemical reaction is measured. ITC technique is used in biophysical studies such as:

• associations and the mechanism of interaction of biomolecules (small molecules, proteins with ligands and other proteins, antibodies with antigens, nucleic acids, lipids, etc.),

• the impact of changes in the structure on molecules binding mechanisms (mutant protein),

• studies of biological activity and kinetics of enzymatic reactions.

Heat release measurements allow an accurate determination of the binding constant (K_{as}), reaction stoichiometry (N), changes in enthalpy (ΔH) and entropy (ΔS) and allow for a full description of the thermodynamic interactions.

Note:

1. Remember that the obtained parameters depend on the measurement conditions, for example temperature.

2. A limitation of the method may be too low concentrations of proteins and the associated low value of emitted or absorbed heat.

ITC advantages include: direct measurement of heat generated in the reaction, the possibility of determining the thermodynamic parameters in a broad range, the ability to study any molecular reaction and, usually, no restrictions on the mass of the particles.

ITC typical experiment involves the following steps:

1.Planning an experiment based on performed simulations. An important step is proper selection of the biopolymer and ligand concentration on the basis of the estimated value of the association constant (K_a) and sigmoidal parameter C defined as $C = [P]_t \cdot K_a$, where $[P]_t$ is the total planned concentration of macromolecules in a solution. The sigmoidal parameter (Fig. 2) which allows the most accurate thermodynamic parameters designation should be within the range of $10 < C < 1000$.

2. Preparation of the solution of the ligand and macromolecules in accordance with planned concentrations.

3. Collection of the data. ITC titration is carried out by repeated addition of small quantities of one reagent to the cell with second reagent with a fixed time interval, until the process of heat exchange will be finished.

4. Implementation of control measurements (eg. titration of the buffer by one of the reactants).

5. Analysis of the data, including amendments relating to the performance of control measurements and determination of thermodynamic parameters

6. Critical interpretation of the obtained results

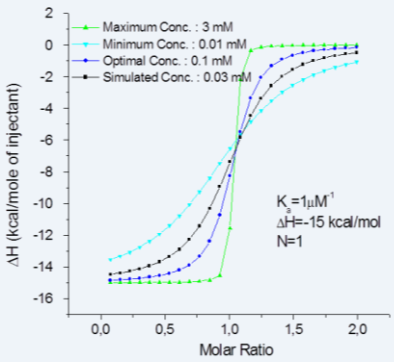


Figure 2. ITC titration curves obtained for association constant K_a=1μM^{-1} and four different, fixed concentration of macromolecules 0.01 mM, 0.03 mM, 0.1 mM and 3 mM. Sigmoidal parameter C is equal respectively: 10, 30, 100 and 3000.

III. ITC calorimeter construction

Modern ITC nanokalorymetr is shown in Fig. 3.

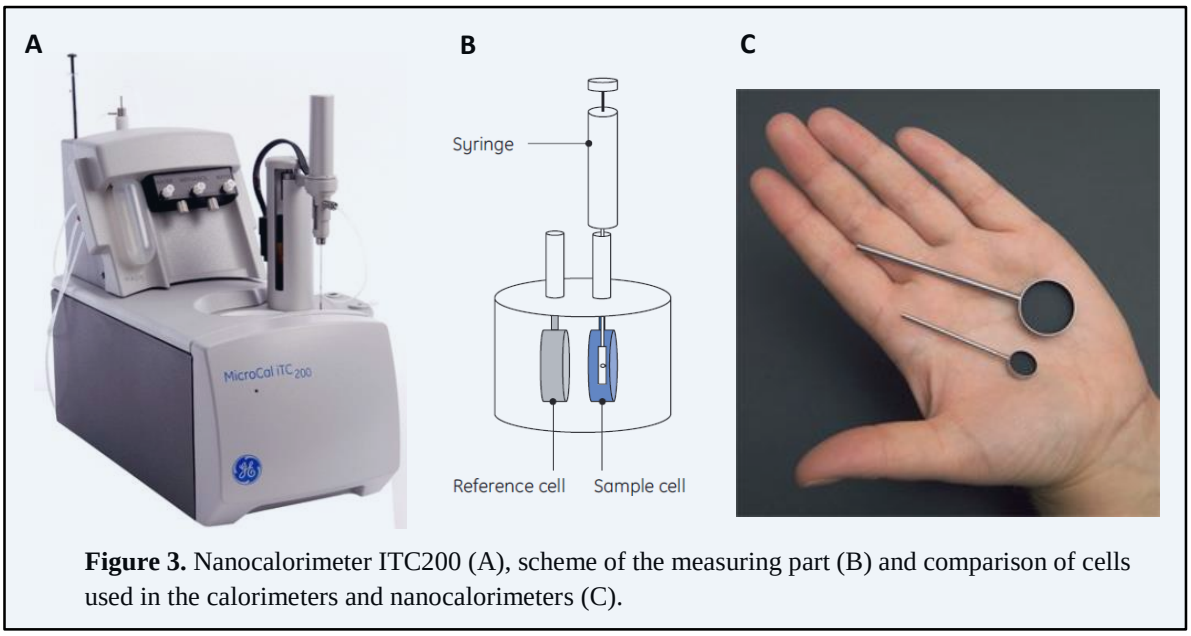


Figure 3. Nanocalorimeter ITC200 (A), scheme of the measuring part (B) and comparison of cells used in the calorimeters and nanocalorimeters (C).

The most important part of the ITC calorimeter are: the reference cell containing buffer and sample cell (Fig. 3B). Temperature sensor allows the thermal balance of cells before the experiment. Computer-controlled syringe allows you to add to the sample cell with a reagent solution the second reagent of a specific volume at specific time intervals. Measurement of the heat emitted or absorbed in the reaction is performed using the power compensation method. It consists of maintaining the sample cell at a constant temperature by a suitable controller, which cools or heats the cell during the reaction. A measured signal is power (μcal/s, μJ/s) needed to

keep the cell at a constant temperature (the same as the temperature of the reference cell), which is converted to heat emitted or absorbed during the reaction.

The result of measurements is a thermogram similar to the one in Fig. 4A. Analysis of the experimental data by dedicated software allows to determine thermodynamic parameters.

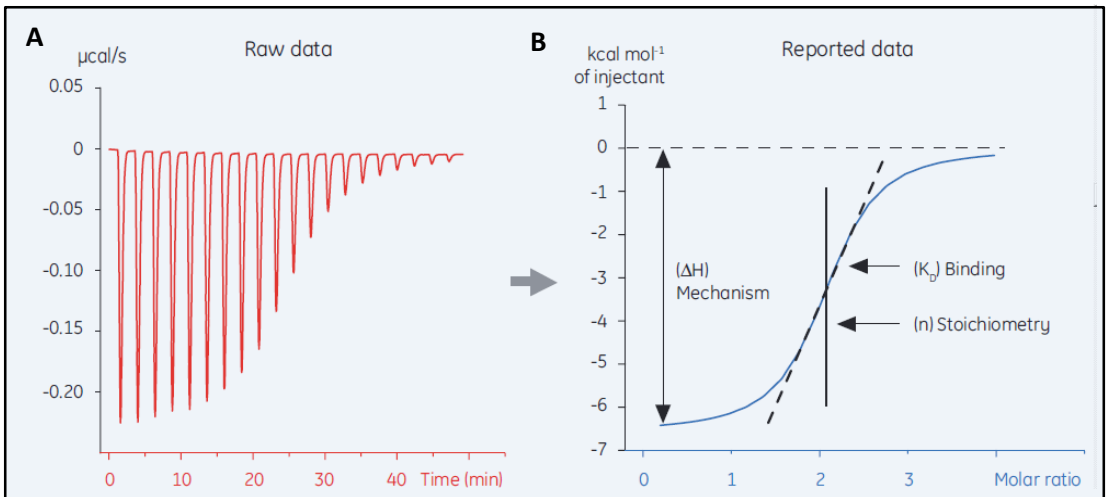


Figure 4. Typical ITC thermogram (A) and the graph obtained after the analysis of experimental data (B).

IV. The thermodynamic parameters obtained in the ITC measurement.

A detailed discussion of thermodynamic parameters can be found in the literature.

For the simplest association reaction

$$A + B \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} A \cdot B$$

dissociation constant is defined as:

$$K_d = \frac{[A][B]}{[AB]}$$

It is an inversion of the association constant.

The stoichiometry of N describes the quantitative ratio in which the chemicals react.

Standard change of reaction in Gibbs free energy is described as:

$$\Delta G^{\circ} = -RT \ln K_{eq} = RT \ln K_d$$

where: R - gas constant,

T - temperature

Thermodynamic parameters characterizing the interactions of macromolecules are:

enthalpy (changes of which result from the reorganization of intermolecular interactions, creation of new interactions, changes in the hydration of polar and non-polar groups), and

entropy (which describes the natural tendency of the system to achieve the highest degree of disorder, which is the sum of the changes in hydration entropy, conformational entropy and dynamics of binding associated with the translation and rotation of the particles after the formation of the complex). Changes in Gibbs free energy, enthalpy and entropy are linked in the following relationship:

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

For the spontaneous reaction ΔG is negative.

V. Entry requirements

Prior to the experimental part, students must pass an preliminary test. The teacher decides about the form of this test. The material on the calorimetry and method of the experiment performance is presented in this instruction and placed in the bibliography. Following topics may be discussed during the test:

1. Comparison of experimental techniques DSC and ITC. In which experimental problems are they used? Thermodynamic parameters, which we can obtain from DSC or ITC measurements.

2.Difference between endo- and exothermic reactions. The reasons of heat emission or absorption during the biochemical interaction macromolecule – ligand.

3. Definition of calorimetric parameters: enthalpy, entropy, Gibbs free energy, stoichiometry (N), dissociation constant (K_d), association constant (K_{as}).

4.Stages of ITC experiment. What is C parameter? What is its role during the experiment design.

5. Apparatus - Describe the most important part of the ITC calorimeter. What and how is measured during the experiment?

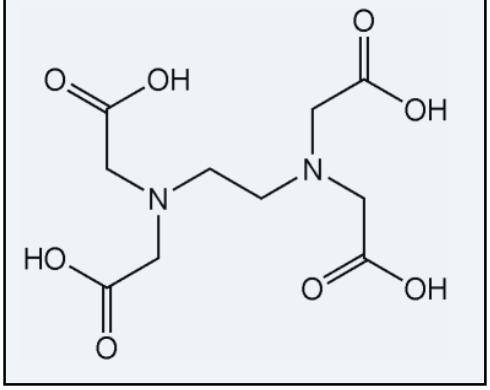
VI. The experiment

In the experiment, we will observe the complexation of EDTA (ethylenediaminetetraacetate, Fig.5) with calcium ions.

This is an exothermic reaction proceeding according to the scheme:

$$Ca^{2+} + EDTA(aq) \rightarrow [Ca^{2+}EDTA]$$

Figure 5. Structure of EDTA



We will determine the binding stoichiometry, enthalpy, entropy, association constant and Gibbs free energy.

There we have a 10 mM MES buffer, pH 5.6, EDTA at a concentration of 5 mM in 10 mM MES buffer pH 5.6, and the calcium chloride solution (conc. 50 mM),

The stages of the experiment:

1. Preparation of the ITC calorimeter is carried out under the supervision of an assistant (a part of the steps can be performed by the assistant).
2. Assuming the association constant equal to $1.24 \cdot 10^5$ M^{-1} determine the parameter C.
3. Prepare the solution of EDTA and calcium chloride in MES buffer with proper concentration by dilution of the stock solutions of EDTA (final concentration should be 0.4 mM, V = 500 μl) and calcium chloride (final concentration should be 5 mM, V = 100 μl).
4. Degass prepared solutions.
5. Discuss with the assistant and writing down the settings of the calorimeter.

Parameter	Settings	Parameter	Settings
Number of Injections		Volume 1st Injection	
Cell Temperature		Duration 1st Injection	
Reference Power		Volume after 1st Injection	
Initial Delay		Duration after 1st Injection	
Syringe Concentration of $BrCl_2$		Injection Spacing	
Cell Concentration of crown ether		Filter Period	
Stirrer Speed		Feedback Mode/Gain	
		ITC Equilibration Options	

6. Titrate the EDTA with calcium chloride.
7. If the clear initial and final plateau in the thermogram are not visible repeat the experiment for other concentrations of the reactants (optionally, if the titration curve looks correct experiment can be repeated at different temperatures).
8. Eventually, perform the control measurement (titration of MES buffer with calcium chloride).
9. Wash the ITC calorimeter after the experiment.
10. Describe the results, calculating the thermodynamic parameters, association constant and stoichiometry.
11. Compare the data obtained with literature data.
12. Interpret the results. Are the results consistent with literature data*? What could be the cause of the discrepancy? What is the impact of the parameter C value on the results? What is the impact of the temperature on the results?

Literature:

- John E. Ladbury (Editor), Michael L. Doyle (Editor), Biocalorimetry 2: Applications of Calorimetry in the Biological Sciences
- Matthew W. Freyer and Edwin A. Lewis, Methods in Cell Biology, 84 (2008), Isothermal Titration Calorimetry: Experimental Design, Data Analysis, and Probing Macromolecule/Ligand Binding and Kinetic Interactions
- Davis Sheeman – Physical Biochemistry: Principles and Applications, Ed. John Wiley and Sons, Inc., 2000
- Igor N. Serdyuk, Nathan R. Zaccai and Joseph Zaccai, Methods in Molecular Biophysics. Structure, Dynamics, Function, Ed. Cambridge University Press, 2007

*) Cited data comes from the MicroCal instruction
$K_a=1.24\cdot10^5$ M (±20%), N=0.966 (±5%), ΔH=-4120 cal/mol (±10%)