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"

PPB13d
Determination of the phase transition temperature (T_{pf}) for lysozyme by differential scanning calorimetry

KAPITAŁ LUDZKI
NARODOWA STRATEGIA SPÓJNOŚCI

UNIA EUROPEJSKA
EUROPEJSKI
FUNDUSZ SPOŁECZNY

Projekt *Fizyka wobec wyzwań XXI wieku* współfinansowany ze środków Unii Europejskiej w ramach Europejskiego Funduszu Społecznego

Introduction

Proteins are biologically active in a certain narrow range of temperatures. Rise of the temperature increases the rate of the reaction catalyzed by the enzymes, but above a certain temperature, protein denaturation takes place. This phenomenon exists for wild-type proteins and their mutants with altered stability. The numbers of mutated proteins used in biophysical studies is surging, for example in studies of enzymatic reaction mechanisms some amino acids are replaced in the active center of protein by other to confirm or exclude their role in catalysis. GFP mutants with altered emission spectrum or enhanced stability are also desirable as better biological markers than wild types proteins. In such studies, and many other the knowledge about the stability of the new mutants is especially important. If they would be proven unstable, they could not be used in measurements. An excellent method of checking protein stability is differential scanning calorimetry (DSC) relying on observations of the biomolecule behavior in the function of temperature.

Differential scanning calorimetry is an experimental technics allowing to measure the difference of the heat stream arising between the cell containing the sample and the reference cell, which are at any given time, under the same temperature conditions. The experiment can be performed at a constant temperature or the temperature can change gradually. In the second case, the change of the heat flux difference between cells can be seen.

In the simplest case, the DSC allows to determine the phase transition temperature (i.e. the melting point, T_m), while a further detailed analysis of the data allows for designation of the values describing the observed thermodynamic process (the heat capacity - C_p - as a function of temperature, ΔC_p between the native and denatured protein, the enthalpy change, $\Delta H(T)$, entropy, $\Delta S(T)$, the Gibbs free energy $\Delta G(T)$).

DSC allows for the study of various processes induced by temperature changes, such as: melting of DNA helix, unfolding and refolding of the proteins, decay of oligomers, the influence of pH and ionic strength on the stability of biomolecules, comparison of mutant stability, investigation of intermolecular interactions in proteins, polysaccharides, and non-biological polymers.

Typical DSC calorimeter is shown in Fig. 1

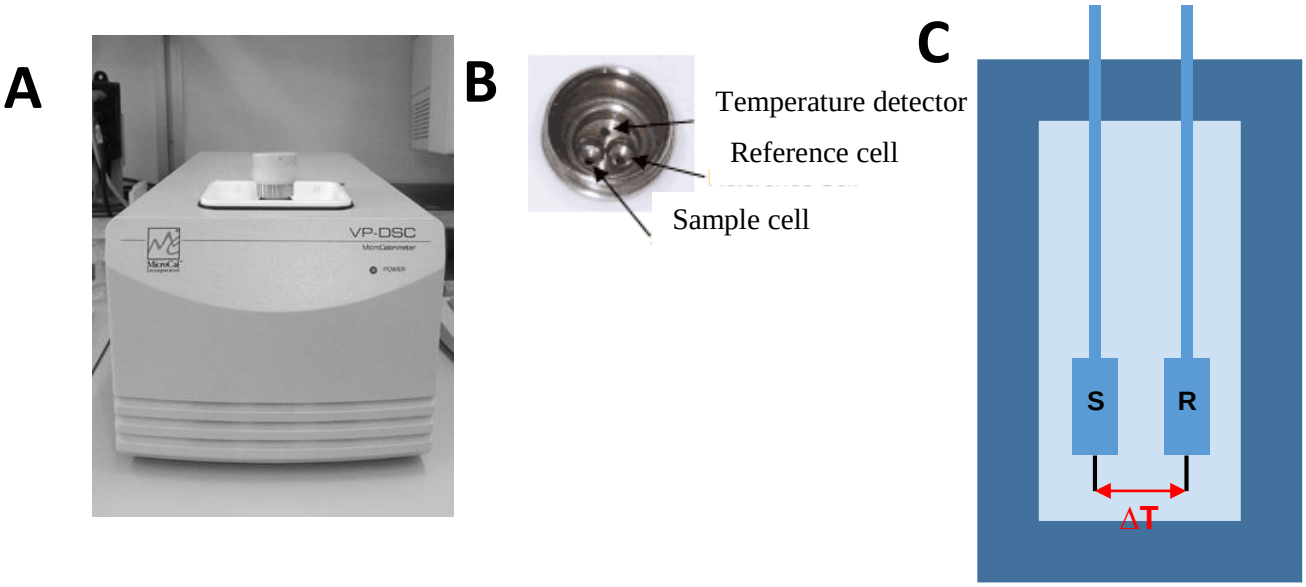

Figure 1. *DSC calorimeter (A) with the measuring chamber (B, top view) and a scheme of the measuring chamber (C).*

In a typical DSC experiment sample cell (S) contains approx. 2 ml of a protein at a concentration of 1-5 mg/ml. The reference cell (R) contains only buffer. Both cell are simultaneously heated or cooled. The maximum range of the temperature used is 10°C -130ºC. Special sensor measures the temperature difference appearing during the process and provides voltage to maintain the same temperature of both cells. A differential signal proportional to the provided voltage is recorded as a function of temperature.

The experiment is usually repeated several times and the measurements are averaged. This allows for determination of the thermodynamic parameters characterizing the observed process.

The thermodynamic parameters obtained from DSC measurements

In one-step model of transition from the native to a denatured structure

$$N \Rightarrow D$$

we can define a constant of equilibrium between native and denatured protein fraction in the temperature T as:

$$K_{den} = \frac{[D]}{[N]}$$

associated with the change of the Gibbs free energy (free enthalpy) of denaturation by relationship:

$$\Delta G_{den} = -RT \ln K_{den}$$

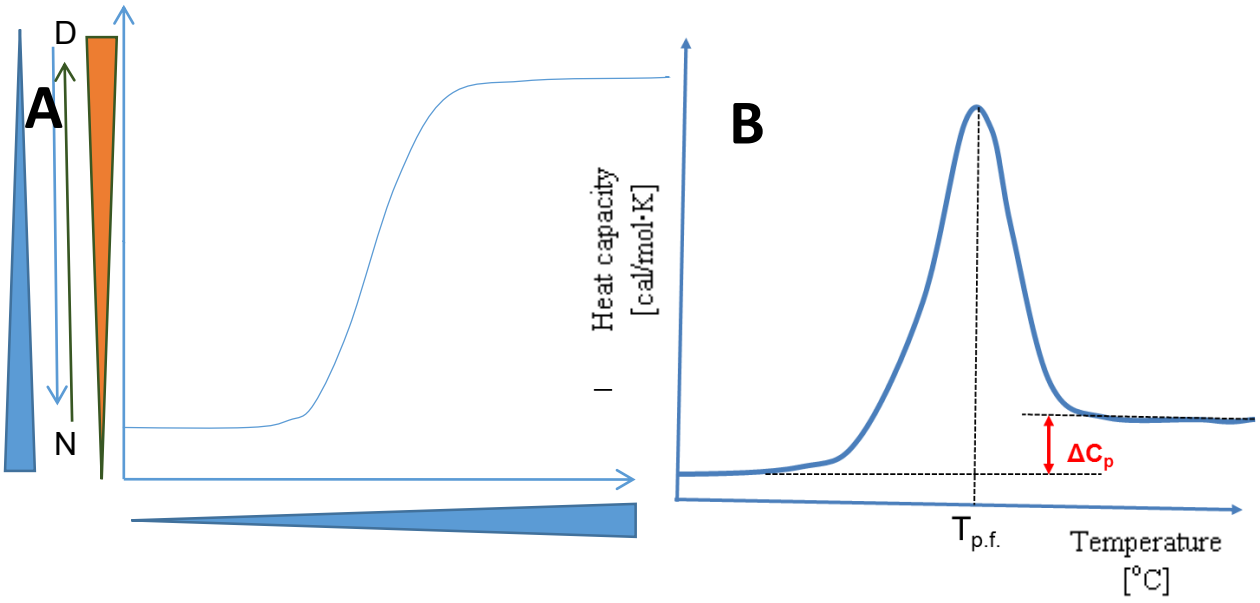

Figure 2. *Protein denaturation A) phase transition from native to denatured state. B) Changes in heat capacity as a function of temperature.*

In the DSC experiment we can measure heat capacity C_p in the function of temperature (T). Next we can determine from the $C_p(T)$ graph T_{pf}, ΔC_p and:

$$\Delta H_{D-N} = \int C_p(T)dT$$
$$\Delta S_{D-N} = \int \frac{C_p(T)}{T}dT$$
$$\Delta G_{D-N} = \Delta H_{D-N} - T\Delta S_{D-N}$$

Aim of the experiment

We will use the DSC method to investigate the unfolding of protein during the thermal denaturation. We will determine the temperature of phase transition (T_{pf}) from native to denatured state. We will investigate the lysozyme - a small protein consisted of 129 amino acids and a mass of 14.4 kDa. It is a hydrolytic enzyme that breaks down the peptidoglycan of the bacterial cell wall.

We will measure the power needed to equilibrate the temperature between sample cell, containing lysozyme, and the reference cell in a function of temperature.

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, object of the investigations, 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. ITC and DSC calorimetry - similarities and differences
2. Construction of the DSC calorimeter and calorimetric measurement technique.
3. Examples of the use of DSC calorimetry in a biophysical studies
4. The thermodynamic parameters, which can be determined by DSC calorimetry
5. Thermal denaturation of proteins

Realization of the experiment

Materials and instruments

We have:

1. 10 mM phosphate buffer pH 7.0 (10 ml)
2. Lysozyme at a concentration of 1 mg/ml (2 ml) in phosphate buffer identical to that above (one day before experiment protein was dialyzed against this buffer three times). Value of the extinction coefficient needed to determine the protein concentration in the sample cell is: $\varepsilon_{280nm}^{1cm}=38940\ M^{-1}cm^{-1}$, $\varepsilon^{1\%}_{280nm}=27.21\ cm^{-1}$
3. UV-VIS spectrophotometer
4. Calorimeter DSC (VP-DSC, Microcal or equivalent)
5. Vacuum pump with magnetic stirrer for degassing of solutions

- Directly prior the experiment, degas the protein sample and the buffer.

I. Measurement of the reference line of the buffer

1. Carefully load sample and reference cell by degassed phosphate buffer (do not enter the air bubbles into the cells)
2. In the control program select a range (30°C-90°C) and speed (60°C/hr) of temperature changes
3. Set Prescan Thermostat to 15 min, and Postscan Thermostat to 0 min
4. Close the cell gently tightening the metal screw, and then spin the white knob until the pressure monitored in the program will be equal to 30 psi.

5. Begin the measurement by pressing "Start DSC run"
6. During the measurement, determine the concentration of lysozyme by measuring the absorbance of the protein
7. After measuring allow the calorimeter to cool, and next remove the buffer from the sample cell.

II. Measuring the denaturation of lysozyme

1. Load the sample cell with degassed solution of lysozyme in phosphate buffer (do not enter the air bubbles into the cells)
2. Set the measurement parameters identically to those selected for the baseline measurement.
3. Close the sample cell carefully and begin the measurement by pressing "Start DSC run"
4. After measurements allow the calorimeter to cool. Next remove the buffer from the sample and reference cells
5. Write down the lysozyme condition after measurement (turbidity of the solution, aggregation and other visible changes)
6. Wash the calorimeter precisely by pouring approx. 0.5 l of deionized water through the sample and reference cells

III. Analysis of the experimental data

Perform an analysis of the experimental data using a dedicated program:

a) Open the sets of data for the protein and buffer
b) Subtract the reference line
c) Normalize results to the amount of pmol of lysozyme in the measuring cell (this is the reason why we had to determine the protein concentration accurately)
d) Determine the baseline,
e) Choose a two-state model for the analysis of data, and then determine the phase transition temperature T_{pf}.

Bibliography:

1. Igor N. Serdyuk, Nathan R. Zaccai and Joseph Zaccai – Methods in Molecular Biophysics. Structure, Dynamics, Function, Ed. Cambridge University Press, 2007
2. A. Cooper, M. A. Nutley, A. Wadood, "Differential scanning microcalorimetry" in "Protein-Ligand Interactions: hydrodynamics and calorimetry" S. E. Harding and B. Z. Chowdhry (Eds.), Oxford University Press, Oxford New York, (2000) p 287-318.