LABORATORY OF BIOPHYSICS FOR ADVANCED

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Folding and unfolding of proteins as a function of the denaturant concentration by emission spectroscopy

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Introduction

Each cell contains thousands of different proteins: enzymes, structural proteins, signaling, receptor proteins, proteins regulating genes expression and many other with specific functions, which help organisms to adapt to environmental conditions. (e.g. antifreeze protein in fish). In spite of such various functions, all proteins are composed of the same amino acids. That's what is different is the order of amino acids sequence in the protein chain, its length and the three-dimensional structure of biologically active macromolecules. The spatial structure of biomolecules plays fundamental role in the living cells. Only after the adoption of it the protein may properly work. However, if in the process of expression, the protein chain does not adopt the proper fold, it becomes useless and sometimes toxic to the cell. A good example are the prions, which are found in every organism and are harmless until they change conformation, becoming the infectious prion protein. Toxic and harmless prions have the same primary structure but differ in the secondary structure (PrPC protein which is not pathogenic possess three α-helices and two β -strands, and the pathogenic PrP Sc protein has mainly β-structures), which is reason of different physicochemical characteristics - PrPC is soluble in water, while PrPSc is insoluble.

Thus, if the proteins works properly, it must take the correct three-dimensional conformation. Despite the great diversity of protein conformation, you can find some common features. This is called secondary structure, among which the most common elements are β-sheet, α-helix and unstructured parts.

The process of adopting the correct three-dimensional conformation (folding) determines whether a protein will perform its functions. It is not surprising that protein folding is one of the key processes currently intensively studied by various scientific laboratories, using advanced biophysical techniques.

Three of the 20 amino acids which build proteins are fluorescent. These are: tryptophan, phenylalanine and tyrosine. However, only tryptophan changes emission intensity and shifts its maximum during a change of environment. It takes place during protein folding, associated with the shifting of tryptophan from the hydrophilic aqueous medium, into hydrophobic interior of the molecule. From the measurements of emission intensity changes, we obtain information on the stages of folding and determine whether it is a one step process, associated with the collapse into favourable energetically native structure, or if the process is multi-step - e.g the secondary structures form first, then it folds to form more complex systems.

Aim of the experiment

In this experiment we will use fluorescent spectroscopy to study the process of protein folding and unfolding. We will measure the natural emission of protein changes, associated with the change in the structure. As an additional marker we will use a label which emits fluorescence when bound to the hydrophobic region of protein.

The aim of the experiment is to:
Determine the relation between denaturant (guanidine hydrochloride) concentrations - and fluorescence of:
(1) the protein (we will observe the shifting the protein emission maximum and changes in emission intensity at the selected wavelength)
(2) a fluorescent label (BisANS), which is indicator of the appearance of intermediate structures during folding.

We will observe:

1. Changes in natural emission of protein during the increased of concentration of the denaturant (for biomolecules in a solution initially containing no denaturant) - unfolding of protein
2. The changes in emission of the protein during the reduction of the concentration of denaturant (for biomolecules in the solution initially containing a maximum concentration of denaturant) - protein folding
3. The change in emission of the fluorescent label, for which the emission intensity depends on the protein structure and has a maximum value for the molten globule state. These measurements will be taken for both, folding and unfolding of the protein.
4. On the basis of these measurements:
    a) we will specify the emission properties of the protein in the folded and unfolded state
    b) we will characterize processes of folding and unfolding of the protein
    c) we will determine whether the process of folding is reversible.

Materials and method

The protein can be denatured by increasing the temperature or by using detergents. However, such denaturation is often irreversible. Chaotropes, chemicals which, by changing the environment of the polypeptide chain, affect the unfolding of the protein are commonly used in this process. An exemplary member of this group is guanidine hydrochloride (CH_5N_3HCl), which in high concentrations destroys the three-dimensional structure of biomolecules. Decreasing of the GdnHCl concentration allows the protein to return to the biologically active structure.

4.4'dianilino 1, 1'-binaphthalene 5.5'-disulfonic acid (BisANS) can be connected to hydrophobic moiety in the proteins structures. Connected BisANS excited at 420 nm emits radiation with a maximum at 490 nm. This label is used to monitor the formation of intermediates (intermediate states), for which the hydrophobic surface is more accessible than in the case of the native protein. This state is named the molten globule structure - for this structure the main chain has a specific conformation as in the native protein, while the side chains are not yet well formed.

We will investigate the lysozyme - a small protein consisted of 129 amino acids with a mass of 14.4 kDa. It is a hydrolytic enzyme that breaks down the peptidoglycan of the bacterial cell wall.

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

$$N \Rightarrow D$$

we can determine the native protein fraction as a function of denaturant concentration. For this purpose we will measure the dependence of the fluorescence intensity at chosen wavelength on the denaturant concentration, with the assumption that the total fluorescence is the sum of fluorescence of the components:

$$y = y_N f_N + y_D f_D$$

where:

y - total fluorescence intensity

y_N - unit of native protein fluorescence

y_D - unit of denatured protein fluorescence

f_N - a fraction of the native form

f_D - a fraction of the denatured form

In result we obtained:

$$f_D = \frac{y - y_N}{y_D - y_N}, f_N = \frac{y_D - y}{y_D - y_N}$$

Hence, we can determine the percentage of native and denatured protein fraction as a function of denaturant concentration, and, next, the free energy ΔG

$$K = e^{\frac{-\Delta G}{RT}}$$

$$K = \frac{f_D}{f_N}$$

$$\Delta G = -RT \ln K = RT \ln \frac{f_D}{f_N}$$

where:

R - gas constant 8.31 J/(mol·K)

T - temperature [K]

Entry requirements

Prior to the experimental part, students must pass a preliminary test. The teacher decides about the form of this test. The material on the emission spectroscopy, 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. Emission spectroscopy. Jablonski diagram. The inner filter effect.
2. Construction of the spectrofluorimeter. Fluorescence measurement technique (excitation and emission spectra) in the UV-Vis range.
3. Properties of fluorescent proteins. Which amino acids decide about protein fluorescence? Excitation and observation wavelength range. The influence of the environment (polar, nonpolar) on emission intensity.

4. Protein folding. Models of the process. What happens if proteins do not fold properly?
5. The role of the reagents used in the study of folding (GdnHCl, urea, BisANS).

Realization of the experiment

There we have:

1 10 mM phosphate buffer pH 7.0 (buffer A)

2 10 mM phosphate buffer containing a 6 M GdnHCl pH 7.0 (Buffer B)

3 Bis ANS solution (conc. 2 mM, V=0,3 ml),

4 Protein solution at a concentration of 1 mg/ml in buffer A

5 Protein solution at a concentration of 1 mg/ml in buffer B

Steps of the experiment:

Day 1 and 2

1. Prepare of stock solutions with varying concentration of GdnHCl in the range of 0 - 4 M, and a volume of 5 ml according to the following table:

Concentration of GdnHCl [M]	Volume of buffer A [ml]	Volume of buffer B [ml]
0	5,00	0
0.2	4,83	0,17
0.4	4,67	0,33
0.6	4,50	0,50
0.8	4,33	0,67
1	4,17	0,83
1.5	3,75	1,25
2	3,33	1,67
2.5	2,92	2,08
3	2,50	2,50
4	1,67	3,33

2 Take the 1.3 ml of buffer with increasing concentrations of GdnHCl and measure the spectra in the range 300 - 450 nm with excitation at 280 nm and a slit width of 2.5 nm.

3 Take 1.3 ml of each solution and add 9.1 μl of protein in buffer A at a concentration of 1 mg/ml.

4 Measure the spectra of proteins initially folded during the unfolding in solutions with growing concentrations of GdnHCl

5 Take 1.3 ml of each solution and add 9.1 μl of protein in buffer B at a concentration of 1 mg/ml.

6 Measure the spectra of proteins initially unfolded during the folding in solutions with decreasing concentrations of GdnHCl

Day 3

Describe the measurement results

a) Prepare of the figures of fluorescence changes as a function of the concentration of GdnHCl in the selected wavelength for the protein folding and unfolding
b) Check the emission maximum shifts as a function of the GdnHCl concentration for the protein folding and unfolding

(remember to remove in the case of a) and b) background spectrum!)

Day 4 and 5

1. Prepare of stock solutions with BisANS (8 μM), varying concentration of GdnHCl in the range of 0 - 4 M, and a volume of 5 ml according to the following table:

The GdnHCl concentration [M]	Volume of buffer A [ml]	Volume of buffer B [ml]	Volume of 2mM BisANS solution [ml]
0	4,98	0	0,02
0.2	4,81	0,17	0,02
0.4	4,65	0,33	0,02
0.6	4,48	0,50	0,02
0.8	4,31	0,67	0,02
1	4,15	0,83	0,02
1.5	3,73	1,25	0,02
2	3,31	1,67	0,02
2.5	2,90	2,08	0,02
3	2,48	2,50	0,02
4	1,65	3,33	0,02

2. Take 1.3 ml of each of the prepared buffer solutions with increasing concentrations of GdnHCl and measure fluorescence of BisANS.
   (exc. wavelength 394 nm, obs. wavelength 478 nm, slit width 5.0 nm)
3. Take 1.3 ml of each solution with increasing concentrations of GdnHCl and BisANS and add 9.1 µl of protein in buffer A at a concentration of 1 mg/ml.
4. Measure the fluorescence of BisANS in the solutions of protein initially folded during the unfolding (exc. wavelength 394 nm, obs. wavelength 478 nm, slit width 5.0 nm)
5. Take 1.3 ml of each solution with decreasing concentrations of GdnHCl and BisANS and add 9.1 µl of protein in buffer B at a concentration of 1 mg/ml.
6. Measure the fluorescence of BisANS in the solutions of proteins initially unfolded during the folding (exc. wavelength 394 nm, obs. wavelength 478 nm, slit width 5.0 nm)

Day 5 and 6

Preparation of report.

1. Perform the BisANS emissions graphs for increasing concentration of GdnHCl, in the selected wavelength, the observation the folding and unfolding of protein
2. Determine the native fraction content in the experiments of folding and unfolding of protein
3. Determine the free energy ΔG.
4. Determine the range in which the protein has the form of a molten globule.
5. Check if the folding process is reversible (if hysteresis occurs).
6. Interpret the results.

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