LABORATORY OF BIOPHYSICS FOR ADVANCED

Experimental exercises for III year of the First cycle studies
Field: "Applications of physics in biology and medicine"
Specialization: "Molecular Biophysics"

Comparative studies on Enhanced Green Fluorescent Protein unfolding using chaotropic agent or acidification (Ex. 32a)

HUMAN CAPITAL
NATIONAL COHESION STRATEGY

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Experiment purpose

The purpose of experiment is to observe unfolding of very stable example of protein – the Enhanced Green Fluorescent Protein (F64L/S65T-GFP). Denaturation process will be achieved using solution of guanidinium hydrochloride (GdnHCl), a chaotropic agent, or by acidification with phosphate solution pH 1. Kinetic measurements will be conducted using stopped-flow technique to observe and compare the time constants in the function of GdnHCl concentration and in the function of pH value. Also comparison between denaturants should be performed. Measurements for time constants exceeding application of stopped-flow technique will be conducted using spectrofluorometer. Also stationary fluorescence measurements will be conducted with the purpose of identifying unfolding intermediate.

Introduction

Absorption and emission properties of amino acids and proteins

Proteins and peptides are composed of 20 essential amino acids, which in addition to the structural role may also be cellular oxidative substrates, neurotransmitters or hormones. Only three amino acids exhibit absorption and emission properties in near ultraviolet range: phenylalanine, tyrosine and tryptophan (Fig. 1). The conjugated π-electron system in aromatic rings is responsible for spectral properties of these amino acids.

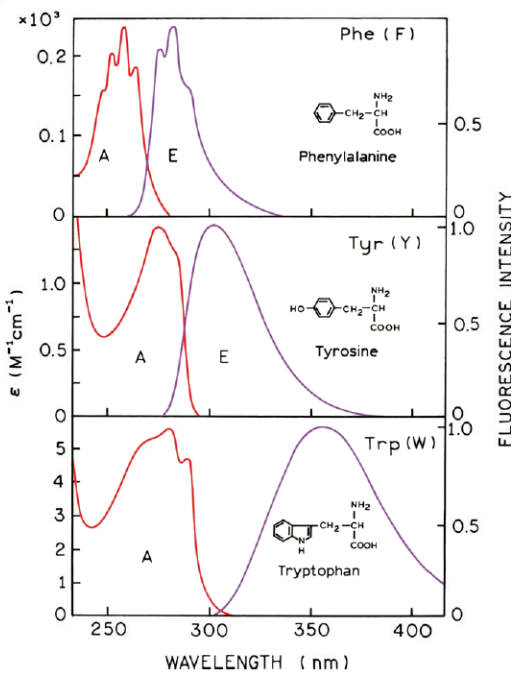

Fig. 1 Absorption (A) and emission (E) spectra of three aromatic amino acids in aqueous solution, pH 7, and their chemical structures [7]

Table 1. Spectral properties of aromatic amino acids in pH 7 (G. D. Fasman 1976 and J. R. Lakowicz 2006)

	max. λ_A [nm]	max. λ_E [nm]	ε [$M^{-1}cm^{-1}$]	Φ
Phe	257,5	282	15	0,03
Tyr	274	303	1 405	0,14
Trp	278	350	5 579	0,20

The most useful in protein studies is tryptophan, because of its unusual behaviour. It exhibits the most efficient quantum yield (Table 1) and fluorescence intensity and emission wavelength depends on hydrophobicity of its surroundings. It is a sensitive fluorescent probe of changes in protein conformation, denaturation process, in monitoring interactions of protein with ligand and subunit binding. Therefore, tryptophan is a useful tool in studies of protein function, folding and dynamics. One of its advantages is longer wavelength absorption spectrum in comparison with other aromatic amino acids. Shoulder at 295 nm makes possible to excite tryptophan independently from phenylalanine and tyrosine. Tryptophan is also very sensitive to fluorescence quenching, especially in contact with solution. Absorption and emission spectra of aromatic amino acids overlap each other, so resonance energy transfer is possible between phenylalanine and tyrosine or between tyrosine and tryptophan. Due to its properties tryptophan has a decisive influence on the

shape of the absorption and emission spectra of proteins, and therefore maximum of protein absorption is observed at 280 nm.

Green Fluorescent Protein

Green Fluorescent Protein (GFP) was discovered in the 60's of XX century by a Japanese scientist Osamu Shimomura during his research on fluorescent protein – aequorin. In 2008 Osamu Shimomura, Martin Chalfie and Roger Y. Tsien received Nobel Prize in chemistry "for the discovery and development of the green fluorescent protein, GFP". GFP occurs in *Aequorea victoria* jellyfish and with aequorin forms a system that makes upset jellyfish glows. Aequorin exists in a complex with coelenterazine (pigment occurring in some coelenterates) and when protein binds calcium ions it changes structure and as a result coelenterazine is oxidised to excited form of coelenteramide. Returning to the ground state coelenteramide emits blue light or through FRET (Förster resonance energy transfer) transfers its energy to GFP chromophore obtaining green fluorescence.

Green Fluorescent Protein is a small globular protein (about 27 kDa, 238 amino acids) with characteristic β-barrel structure (Fig. 3, left panel). β-barrel consists of 11 anti-parallel β-sheets and is closed with loops and distorted helical fragments. The only α-helice is coaxial and bears chromophore (p-hydroxybenzylideneimidazolidinone). Chromophore is shielded by β-barrel structure, which prevents its visible fluorescence from quenching by solvent molecules. The proper turn in α-helice and a very complicated network of hydrogen bonds keep chromophore's ring in appropriate arrangement. The distance between rings of chromophore and only tryptophan is about 11-15 Å, which makes possible resonance energy transfer (Fig. 3, right panel).GFP chain contains 11 tyrosine, 12 phenylalanine and 2 cysteine residues, which do not form a disulphide bond.

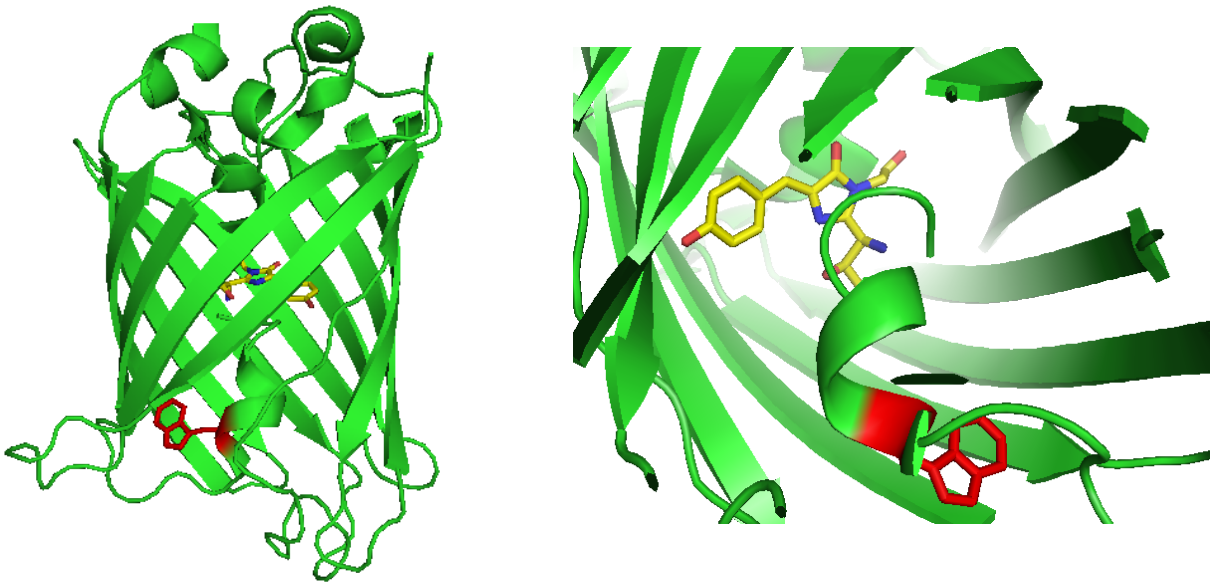

Fig. 3 Enhanced Green Fluorescent structure (left panel, PDB 2Y0G) and β-barrel interior with chromophore (yellow) and tryptophan (red).

In Enhanced Green Fluorescent Protein (EGFP) two point mutations were inserted: leucine instead of phenylalanine 64 and threonine instead of serine 65. These mutations entail more efficient protein folding, enhanced fluorescence intensity and reduction of absorption spectrum (Fig. 4). The latter is due to the change in the ionic form of chromophore, which in the wild-type protein (wtGFP) is present both in neutral (absorption at approx. 395 nm) and anionic form (absorption at approx. 475 nm), while almost the entire population of EGFP possess chromophore in anionic form. EGFP absorption spectrum reveals two peaks with maxima at 278 nm (originates from aromatic amino acids) and at 489 nm (originates from chromophore). Extinction coefficients for aromatic amino acids and chromophore were derived experimentally by Seifert et al. (2002) and in pH 7.3 are ε_{277} = 21 000 $M^{-1}cm^{-1}$ and ε_{488} = 38 000 $M^{-1}cm^{-1}$, respectively. When excited at 280 nm there are two peaks observed: at 336 nm (aromatic amino acids) and at 509 nm (chromophore), which the latter gives protein greenish-yellow glow.

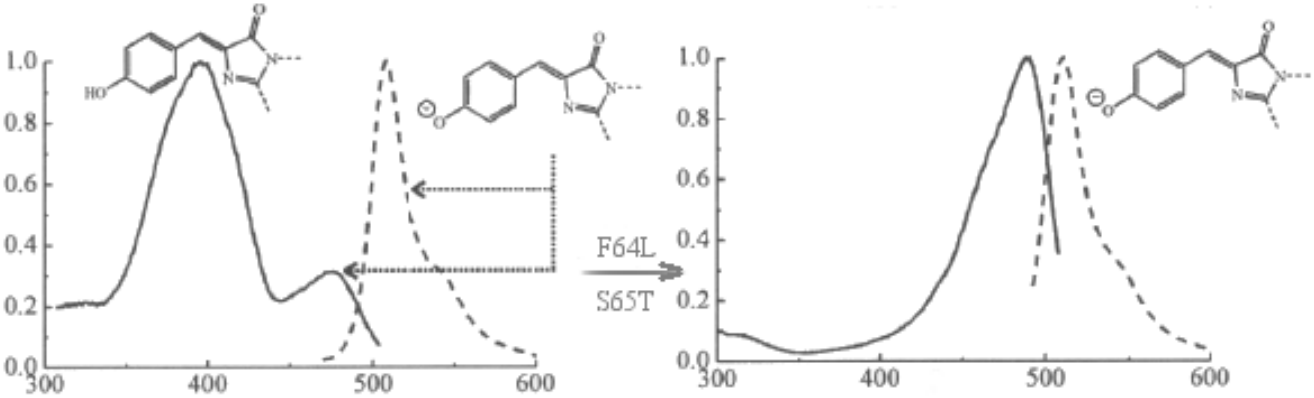

Fig. 4 Excitation (solid line) and emission (dashed line) spectrum of wtGFP (left panel) and the mutant F64L/S65T-GFP (right panel). R. Y. Tsien 1998, p. 520.

Green Fluorescent Protein unfolding

Reversible denaturation can be achieved by treating protein with chaotropic agent (urea, guanidinium hydrochloride) or by acidification. Guanidinium hydrochloride (GdnHCl) disrupts hydrogen bonding network in solution and affects protein interactions with surroundings what results in unfolding. On the other hand, acidification in most cases induces accumulation of positive charge on protein surface and changes association constant value of aspartic and glutamic acid what result in unfavourable electrostatic interactions causing unfolding. During GFP denaturation chromophore fluorescence is quenched, as disrupting compact β-barrel structure allows motions of chromophore ring and exposes it to interactions with solution. Though, loss of fluorescence is not the determinant of complete protein unfolding. Applied multiple biophysical techniques revealed that at pH 2.9, pH 2, pH 1.5 and pH 1 there was still residual protein structure, although no chromophore fluorescence was observed. Similarly, loss of the fluorescence during denaturation with high concentrated GdnHCl is not equal with reaching equilibrium state of protein. Depending on GFP mutant equilibration lasts even several days. In comparison with small and monomeric proteins GFP unfolding is relatively slow. Equilibrium unfolding revealed an unfolding intermediate both in denaturation by GdnHCl and by acidification. Its structure was similar in both cases and consisted of considerable portion of secondary and tertiary structure. Not all unfolding conditions are favourable for ob-

serving unfolding intermediate, probably because its structure is native-like. It is suggested that most of β-barrel structure remains intact except β-sheets 7-10, which are more susceptible to conformational changes. Kinetic measurements indicated that above 5 M GdnHCl unfolding time constant varies from 40 s to 76 s, but time constant value increases with decreasing GdnHCl concentration. Also depending on pH value and examined GFP mutant unfolding time constant varies from several dozen milliseconds to several dozen of seconds.

Stopped-flow technique

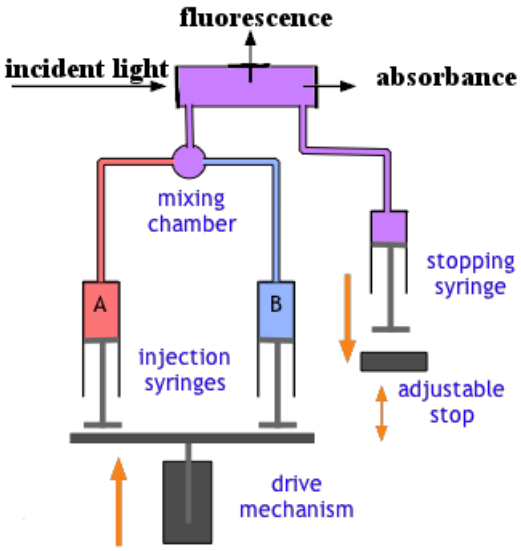

Fig. 6 Stopped-flow apparatus scheme, [1]

Stopped-flow technique is used to measure very fast kinetics, which half-lives are even millisecond order of magnitude. Stopped-flow apparatus scheme is presented on Fig. 6. Substrates of reaction are in injection syringes – dependent on kind of apparatus there are up to 4 injection syringes. Driving mechanism pushes injection syringes' pistons at the same time starting flow of substrates through mixing chamber to optical cuvette. Mixing element makes solution homogeneous by very fast mixing of substrates and initiates reaction. For solutions with high density difference a mixing element with special construction is needed. With the progressive movement of substrates the stopping syringe is filled and its piston is blocked after achieving desired volume. Stopping the flow starts observation of process and signal acquisition. Time between initiation of flow and beginning of measurement is called apparatus dead-time and usually lasts few milliseconds. The stopped-flow technique is coupled with absorption, fluorescence and circular dichroism spectrometers.

Data analysis

Unfolding kinetics might be describe by multiexponential function according to equation:

$$I(t)=\sum_i A_i \cdot \exp\left(\frac{-t}{\tau_i}\right)+A_{inf} \tag{1}$$

where
$I(t)$ – fluorescence intensity at time t
i – number of process phases
A_i – amplitude of phase number i
τ_i – time constant of phase number i
A_{inf} – value of fluorescence intensity in infinity

The best model with chosen number of phases should be selected on the basis of: a) analysis of fitting parameters with standard errors and b) analysis of residuals. Each time constant might be interpreted as a particular component of complex unfolding process.

Conduction of experiment

For time course measurements on spectrofluorometer students will receive 50 mM phosphate buffer with 300 mM NaCl without GdnHCl and with 6 M GdnHCl pH 8. Students should prepare a series of solutions with different concentration of GdnHCl. Protein unfolding will be induced by diluting concentrated EGFP sample in denaturing buffer directly in the cuvette. Solution will be mixed using magnetic stirrer. Students should select excitation and emission wavelength, slits width and time of measurement. Time course of background also should be recorded.

For stationary measurements on spectrofluorometer students will receive a series of 100 mM phosphate solutions in pH range from pH 1 to pH 8. Unfolding will be induced by diluting concentrated EGFP sample in relevant denaturing buffer directly in the cuvette. Solution will be mixed using magnetic stirrer. Perform fluorescence spectra with excitation wavelength λ_{ex} = 280 nm in range from 290 to 550 nm. EGFP concentration should not exceed 2 μM. Students should select appropriate values of remaining spectrofluorometer parameters. Background spectra also should be recorded.

Unfolding in function of GdnHCl concentration using stopped-flow technique will be performed in series of 50 mM phosphate buffers with 300 mM NaCl and different concentration of GdnHCl, pH 8. Substrates will be mixed in proportion 1:10 and GdnHCl concentration will be adjusted to receive final concentration 7.5 M, 7 M, 6.5 M, 6 M, 5.5 M, 5 M, 4.5 M, 4 M. Measurements in function of pH value will be performed in series of 100 mM phosphate buffers with addition of different amount of hydrochloric acid. After mixing in stopped-flow apparatus pH value should reach pH 1, pH 2, pH 3, pH 4, pH 5 and pH 6. In both types of experiments EGFP concentration should be 55 μM. Wavelengths of excitation and emission should be the same as in spectrofluorometric measurements. Students should select monochromators' slits width and adjust photomultiplier's voltage. When using stopped-flow technique it is necessary to repeat run in every measured condition. It is suggested to repeat each run 10 times and then average. Time course of appropriate buffers mixing also should be recorded.

Students should analyse data according to the following guidelines:

- for all types of time course measurements fit adequate form of equation (1)
- for unfolding time courses in the function of GdnHCl concentration measured on spectrofluorometer plot time constant versus GdnHCl concentration and determine presence of unfolding intermediate
- for stationary measurements in the function of pH value determine position of maximum and intensity of tryptophan peak in dependence on pH value and plot chromophore fluorescence intensity versus pH value and on the basis of these results determine presence of unfolding intermediate
- for stopped-flow technique measurements after determination of time constants, compare number of phases and time constants values between used denaturants

Student's report

Report should consist of:

- *Introduction* describing theory of experimental subject,
- *Materials and methods* with description of solutions and apparatus used in exercise and/or description of conducting experiment,
- *Results and discussion* presenting data, its analysis and interpretation. It can be divided in two distinct paragraphs if needed. Values of parameters should be rounded and presented with standard error. If calculation of experimental error was used, appropriate equations should be presented,
- *Bibliography*,
- *Appendix* if needed.

Preliminary test

Student is allowed to perform the exercise only after passing the preliminary test. The form of preliminary test is in decision of the assistant. Range of spectroscopy subjects were presented in this instruction, during lectures "Molecular spectroscopy" and "Molecular biology", during "Laboratory of elementary biophysics" and bibliography at the end of this instruction. Below there is a list of books that could be helpful in preparing to preliminary test:

- Gordon M. Barrow, *Introduction to molecular spectroscopy*
- Hermann Haken, Hans Christoph Wolf, *Molecular Physics and Elements of Quantum Chemistry: Introduction to Experiments and Theory*
- Joseph R. Lakowicz, *Principles of fluorescence spectroscopy*
- E. F. H. Brittain, W. O. George, C. H. J. Wells, *Introduction to molecular spectroscopy: theory and experiment*
- Raymond Chang, *Basic principles of spectroscopy*
- ed. D. W. Jones, *Introduction to the spectroscopy of biological polymers*
- Jeffrey I. Steinfeld, *Molecules and radiation: an introduction to modern molecular spectroscopy*
- Martin Quack and Frédéric Merkt, *Handbook of High-resolution Spectroscopy*

Before preliminary test read the following papers:

- ✔ S. Enoki, K. Saeki, K. Maki, K. Kuwajima, *Acid denaturation and refolding of green fluorescent protein*, Biochemistry 2004, 43, 14238-14248
- ✔ J-r. Huang, T. D. Craggs, J. Christodoulou, S. E. Jackson, *Stable intermediate states and high energy barriers in the unfolding of GFP*, J. Mol. Biol. 2007, 370, 356-371, particularly chapters „Chemical denaturation and fluorescence measurements" and „Kinetic studies on the unfolding of GFP
- ✔ S-T. D. Hsu, G. Blaser, S.E. Jackson, *The folding, stability and conformational dynamics of β-barrel fluorescent proteins*, Chem. Soc. Rev. 2009, 38, 2951-2965, chapters 3 and 4

You will receive them from the assistant.

The list of subjects that student might be asked about during preliminary test:

- Basic definitions related to electromagnetic radiation and spectroscopic measurements:
  - wavelength, frequency, wavenumber
  - radiant intensity, radiant energy density
  - molar absorptivity (molar extinction coefficient), integral absorption coefficient
  - ground state, excited state, oscillator strength
  - quantum yield
- Emission measurements:
  - schematic principles of emission measurements
  - what is the emission spectrum?
  - what is excitation spectrum?
  - quantitative description of emission (quantum yield, how to determine it?)
  - inner filter effect
- Electronic states of molecules:
  - molecular orbitals, what mean symbols: □, □*, □, □*, n
  - diagram of electronic states (S_0, S_1, S_2, ..., T_1, T_2,....), Jablonski diagram
  - possible absorption transitions and relative location of the bands corresponding to the frequency range of electromagnetic radiation
  - deactivation of excited states, radiative and nonradiative transitions, time constants characteristic for each process
- Absorption and emission properties of amino acids and proteins
  - which amino acids exhibit absorption and emission properties? why?
  - spectral characterisation of aromatic amino acids
  - particular tryptophan properties
  - how proteins can be examined spectrally?
- Green Fluorescent Protein:
  - structure and function
  - spectral properties
  - influence of F64L and S65T substitution on GFP
  - GFP unfolding

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(2) O. Shimomura, *Structure of the chromophore of Aequorea Green Fluorescent Protein*, FEBS Letters 1979, 104 (2), 220-222

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(4) I. Żak, *Chemia medyczna*, rozdział 13 „Aminokwasy i pochodne", Śląska Akademia Medyczna, Katowice 2001

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(6) S. Enoki, K. Saeki, K. Maki, K. Kuwajima, *Acid denaturation and refolding of green fluorescent protein*, Biochemistry 2004, 43, 14238-14248
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(13) J. S. Merkel, L. Regan, Modulating, *Protein Folding rates in vivo and in vitro by side-chain inteactions between the parallel strands of green fluorescent protein*, J. Biol. Chem. 2000, 275, 29200-29206