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"

Studies on green fluorescent protein refolding in dependence on pH using stopped-flow technique (Ex. 33)

HUMAN CAPITAL
NATIONAL COHESION STRATEGY

EUROPEAN UNION
EUROPEAN
SOCIAL FUND

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Experiment purpose

The purpose of experiment is to use stopped-flow technique in very fast kinetics using refolding of enhanced green fluorescent protein (EGFP) as an example study. The process should be studied in dependence on pH and characterised by a number of phases and relevant time constants in each of refolding conditions.

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.

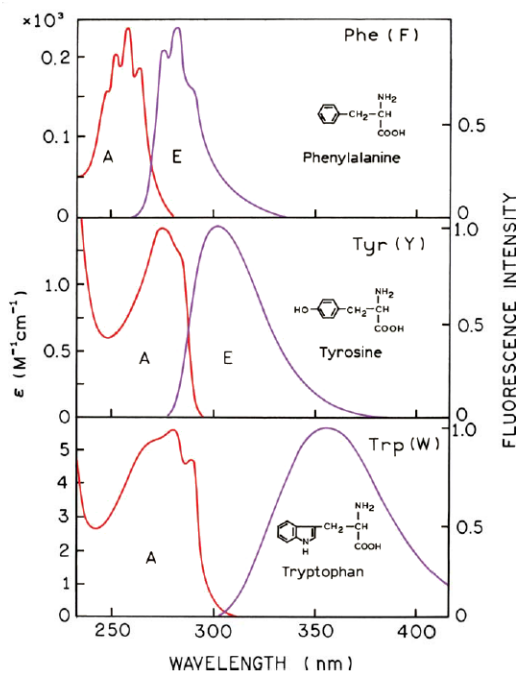

Fig. 1 Absorption (A) and emission (E) spectra of three aromatic amino acids in aqueous solution, pH 7, and their chemical structures [7]

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 or 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 and 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.

Protein folding

It is little known about protein folding and so there is still a lot to explore to understand better that very complex process. So far our knowledge about protein folding is to globular proteins or globular protein domains behaviour. Probably native state is preceded by defined folding pathways, which might be divided in different mechanisms. The two most extreme models of protein folding are: framework model and hydrophobic collapse model. In framework model the secondary structure forms rapidly and is used as a scaffold for formation of the tertiary structure. Two mechanisms were proposed to describe behaviour of proteins following the framework model:

- diffusion-collision mechanism with formation of the secondary structure, which then diffuses, collides and coalesces in the tertiary structure
- nucleation mechanism in which formation of protein core is very slow, but once is formed the acquisition of the native state is fast

At early stage of folding by hydrophobic collapse model a hydrophobic nucleus forms rapidly. At this stage water molecules and hydrophilic residues are ejected from protein core, then the secondary structure is being formed. Before protein achieves native state it adopts a molten globule state consisting of hydrophobic core, high percent of overall secondary structure and possible residual tertiary structure, which is very dynamic. To become native protein must acquire correct interactions and adopt rigid structure. Realistic pathways of protein folding are between these two mentioned models.

Protein folding might be two- or multi-state (Fig. 2). The simplest example of protein folding is two-state folding (D → N), in which protein forms native structure (N) directly from denatured state (D). In multi-state folding protein forms intermediate and transition states, what affects folding rate (e.g. D→ I→ N). It is possible that intermediates exist during all types of protein folding, but in two-state folding they are not observable, because they are unstable, high-energetic (exist in high-energy minima, slightly lower than denatured state) and do not have any influence on protein folding rate.

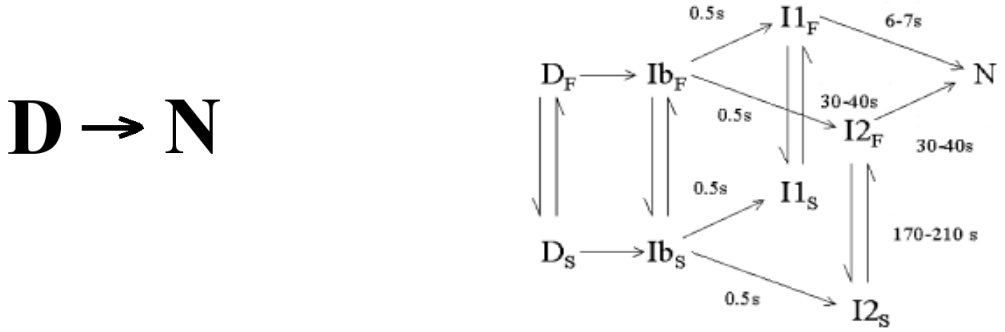

Fig. 2 Schemes of two-state folding (left panel) and multi-state folding on example of GFP mutant – Cycle3 (right panel, Enoki et al. 2004); D – denatured state, N – native state, IX_Y – different intermediates

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.

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 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.

Chromophore formation is auto-catalytic and origins from three amino acids: Ser65-Tyr66-Gly67. After proper protein folding tripeptide is in conformation which allows conducting internal chemical reaction resulting in cyclised ring. Maturation of this chromophore pre-form includes dehydration and oxidation. The time-limiting process is an oxidation and usually depends on molecular oxygen concentration.

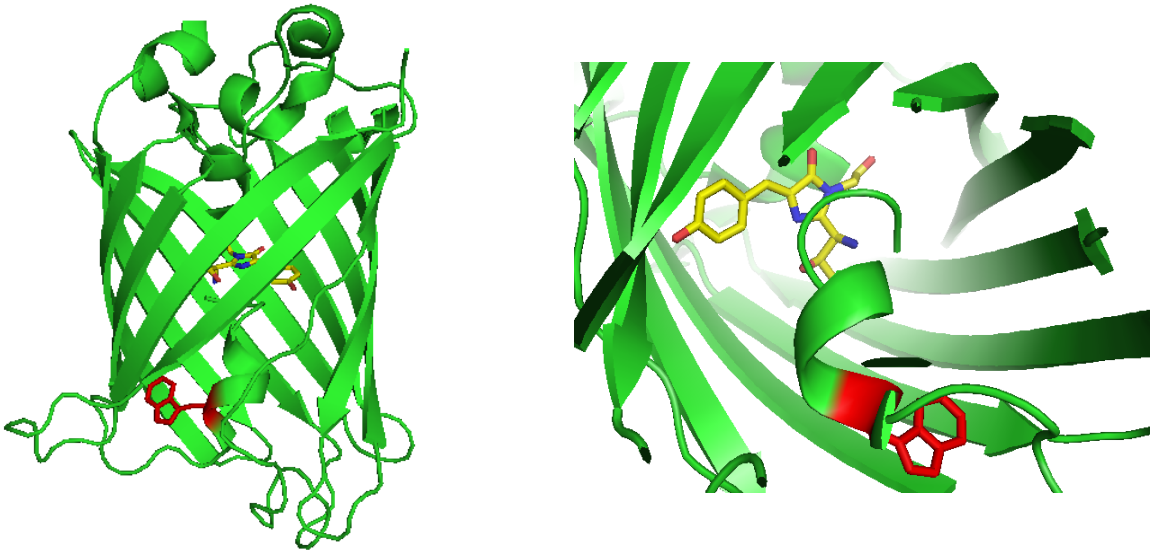

Fig. 3 Enhanced Green Fluorescent structure (left panel, PDB 2Y0G) and β-barrel interior with chromophore (yellow) and tryptophan (red).

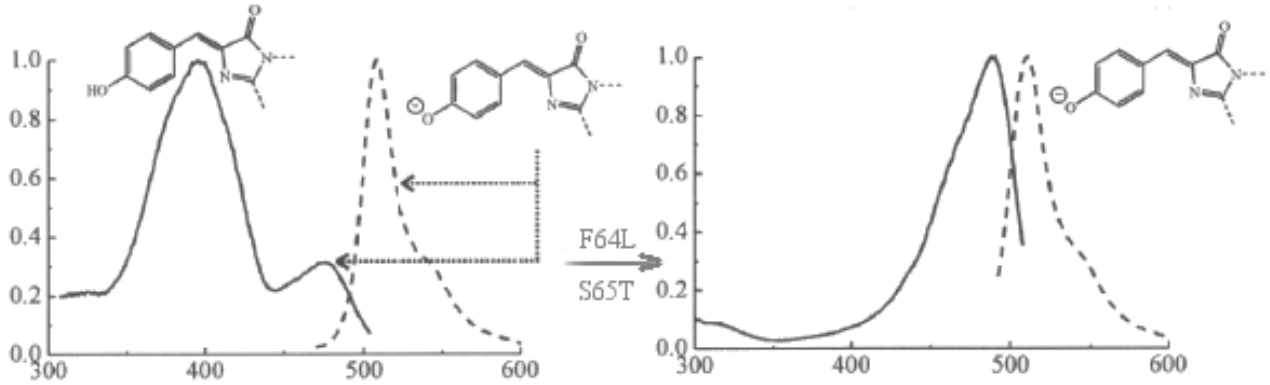

Fig. 4 Excitation (solid line) and emission (dashed line) spectrum of wtGFP (left panel) and the mutant F64L/S65T-GFP (right panel). R. Y. Tsien 1998, p. 520.

GFP folding

Refolding of GFP was examined by different kinds of experimental and computational techniques using a variety of GFP mutants, especially GFPuv (also known as Cycle3) and sfGFP (**s**uper**f**older GFP). Existence of chromophore affects protein refolding and makes folding landscape* more rough (more energetic barriers and local minima resulting in additional intermediates and transition states) relative to newly synthesized polypeptide chain (e.g. Fig 5). GFP refolds quite slowly in comparison with other small globular proteins. Equilibrium refolding in dependence on concentration of guanidinium chloride (GdmHCl -denaturating compound) revealed intermediate state with native-like structure consisting mainly of the secondary structure. Denatured protein rapidly adopts that flexible intermediate structure. However, transition from intermediate to native state is very slow. Research on refolding kinetics

*Folding landscape is a 3-dimensional representation of energy as a function of protein conformation and polypeptide chain topology; it has a shape of funnel, because it becomes narrow during folding as protein preserves native contact and possible conformations are reduced; the wide part of the funnel at the top represents all denatured states and the deepest narrowing is a global minimum with native state; local energetic minima make additional valleys and energetic barriers make piles of folding landscape.

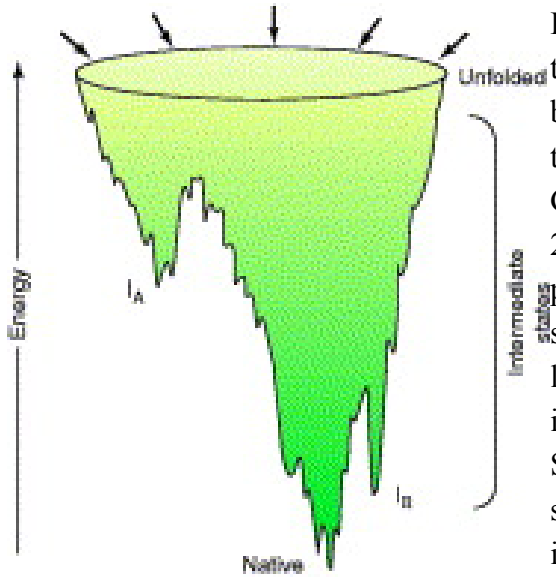

Fig. 5 Example of the folding landscape; denatured state at the top, native state at the bottom, IA, IB - intermediates (P. L. Clark, 2004)

in dependence on pH revealed multi-state process. It is related to prolines presence, which in denatured state are in any of *cis* or *trans* conformation, but in native state one of the ten is in *cis* conformation. Depending on initial proline arrangement GFP refolding follows fast or slow pathway. (Fig 2, right panel). First refolding phase, called burst phase, is not observable for stopped-flow apparatus so must be faster than 200 s^{-1}. Hydrophobic collapse occurs during this phase and probably packing the tryptophan into hydrophobic protein core. Subsequently appears intermediate with compact structure wherein tryptophan and chromophore are in distance enabling energy transfer. Among different intermediates states occurs molten globule intermediate wherein there is still no chromophore fluorescence because protein lacks its full rigid β-barrel structure. It is suggested that GFP refolding represents system of parallel folding pathways composed from at least 6 phases.

Stopped-flow technique

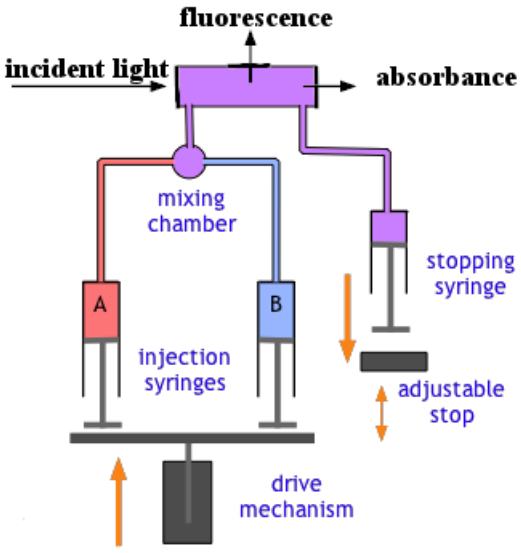

Fig. 6 Stopped-flow apparatus scheme, [1]

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

Green Fluorescent Protein folding is a multiexponential process (Enoki et al. 2004 and 2006) described by equation:

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

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

In the case when the process is not fully registered or photobleaching occurs, sometimes the phase fragment might be approximated by a linear function. Then equation (1) is modified to:

$$I(t)=\sum_i A_i \cdot \exp\left(\frac{-t}{\tau_i}\right)+a \cdot t+b \quad (2)$$

where a is a slope and b an intercept.

Data should be fitted with equations (1) or (2). 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.

Conduction of experiment

Step I

Students will receive:

- concentrated EGFP solution in 50 mM phosphate buffer with 300 mM NaCl, pH 8 (the concentration will be given by assistant)
- 100 mM phosphate buffer, pH 1
- 100 mM phosphate buffer, pH 8

At the beginning measure excitation and emission spectrum of EGFP in denatured (pH 1) and native state (pH 8) using spectrofluorometer. Analyse spectra and choose wavelengths of excitation and emission appropriate for stopped-flow measurements. Then plan an experiment for the next day and decide what volume of 20 μM EGFP should be prepared. Dilute protein in 100 mM phosphate buffer pH 1 to final concentration of 20 μM EGFP and prepare dialysis in the same buffer in dialysis tube.

Step II

Students will receive:

- set of 100 mM phosphate renaturation buffers of different pH value, which, when mixed in a ratio of 1: 1 with denaturing buffer pH 1, will give a final pH in the scale of pH 4 to 8
- β-mercaptoethanol (14,3 M)

Conduct a preliminary experiment of refolding using stopped-flow technique. Refolding will be initiated by pH-jump. Prior to folding β-mercaptoethanol must be added to concentration of approx. 28 mM in each renaturation buffer. β-mercaptoethanol is a reducing agent and prevents formation of disulphide bonds. Using denatured protein after dialysis conduct preliminary kinetic measurements. Depending on results choose appropriate time of measurement, widths of both excitation and emission slits and photomultiplier's voltage.

Prepare protein to dialyse for the next day of measurements.

Step III

Conduct experiment of refolding using stopped-flow technique and settled parameters of apparatus. For each pH value repeat at least 10 times each kinetic measurement. Measure background i.e. kinetics of mixing proper buffers. Always measure background the same day when refolding kinetics, because β-mercaptoethanol changes its spectrum during 24 hours. Remember to prepare adequate volume of renaturation buffer with β-mercaptoethanol to measure both refolding kinetics and background on the same sample (β-mercaptoethanol is very viscous and high concentrated, so preparing each sample may result in different final concentration).

Step IV

Analyse your kinetic data using equations given in this instruction. Remember to subtract background from your kinetic data. If kinetics were recurrent, average them before further analysis. If not, analyse each curve independently. It might happen, that mathematical analysis will be impossible, then try to interpret recorded phenomenon.

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" and bibliography at the end of this paper. 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
- ✓ S. Enoki, K. Maki, T. Inobe, K. Takahashi, K. Kamagat, T. Oroguchi, H. Nakatani, K. Tomoyori, K. Kuwajima, *The equilibrium unfolding intermediate observed at pH 4 and its realtionship with the kinetic folding intermediates in green fluorescent protein*, Journal of Molecular Biology 2006, 361, 969-982

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
  - spectrophotometer and spectrofluorometer construction

- 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:σ, σ*, π, π*, 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
- Electronic spectrum with vibrational structure:
  - in which region of electromagnetic spectrum electronic absorption and emission bands could be observed?
  - Jablonski diagram
  - scheme of electronic transitions allowed by selection rule
  - example of absorption and emission spectrum illustrating electronic-vibrational structure and possible transitions
- 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
  - refolding
  - chromophore formation
  - mutation influence on GFP
- Protein folding – models and mechanisms
- Stopped-flow technique – applications and principles of operation

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