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"

Green fluorescent protein refolding studies using stopped-flow technique (Ex. d12)

HUMAN CAPITAL
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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 characterised by a number of phases and relevant time constants.

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. The conjugated π-electron system in aromatic rings is responsible for spectral properties of these amino acids (Fig. 1). 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 for examplein studies 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 long wavelength absorption spectrum in comparison with other aromatic amino acids. Shoulder at 295 nm makes possible exciting tryptophan independently of phenylalanine and tyrosine. Tryptophan is also very sensitive to fluorescence quenching, especially in contact with solution. Due to its properties tryptophan has a decisive influence on the shape of the absorption and emission spectra of proteins, therefore maximum of protein absorption is observed at 280 nm.

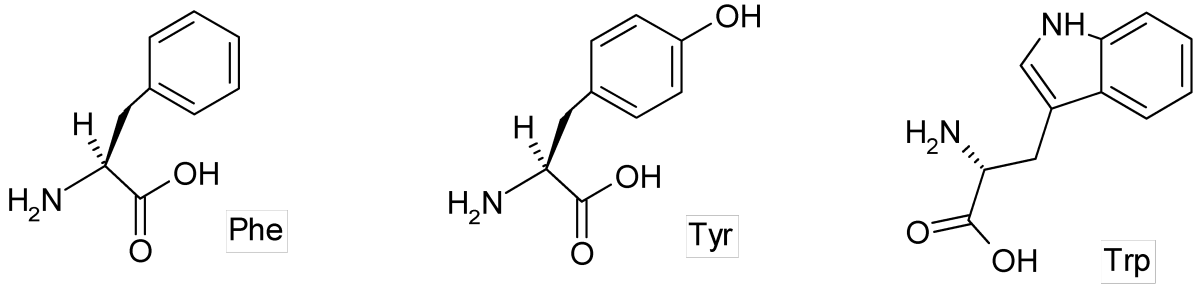

Fig. 1 Chemical structure of three aromatic amino acids, from the left: phenylalanine (Phe, P), tyrosine (Tyr, T), tryptophan (Trp, W)

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 limited to globular proteins or globular protein domains behaviour. Probably native state is preceded by defined folding pathways, which could 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. It includes ejection of water molecules and hydrophilic residues from protein core and is followed by formation of the secondary structure. Before protein achieves native state it forms 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 form 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. 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 adopts intermediate and transition states, what affects folding rate (e.g. D→ I→ N).

Green Fluorescent Protein

Green Fluorescent Protein is a small globular protein (about 27 kDa, 238 amino acids) with characteristic β-barrel structure (Fig. 2, left panel). β-barrel consists 11 anti-parallel β-sheets and is closed with loops and distorted helical fragments. The only α-helice is coaxial and bears chromophore. Chromophore is shielded by β-barrel structure, which prevents its visible fluorescence from quenching by solvent molecules. A very complicated network of hydrogen bonds and proper turn in α-helice keeps chromophore's ring in appropriate arrangement. The distance between rings of chromophore and only tryptophan is about 11-15 Å, which makes resonance energy transfer possible (Fig. 2, right panel).

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. Maturing of this chromophore pre-form includes dehydration and oxidation reactions. The time-limiting process is an oxidation and usually depends on molecular oxygen concentration. In wild-type GFP chromophore occurs in two ionic forms: neutral with absorption maximum at 395 nm and anionic with absorption maximum at 475 nm. In EGFP (F64L/S65T-GFP) only anionic form of chromophore occurs with absorption maximum at 489nm, which results in emission at 509 nm giving protein greenish-yellow glow. 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.

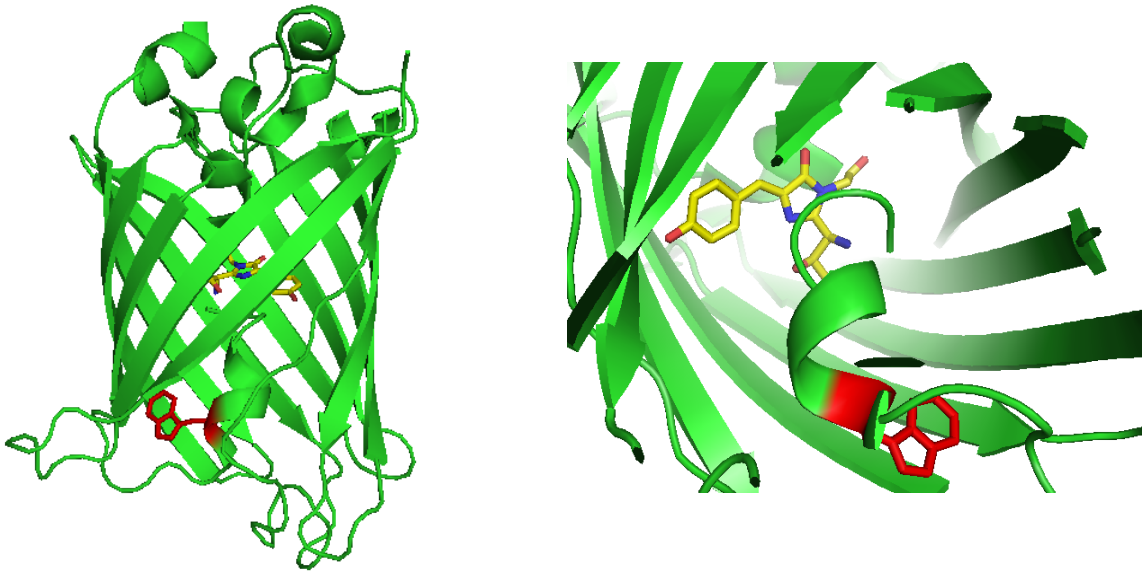

Fig. 2 Enhanced Green Fluorescent structure (left panel, PDB 2Y0G) and β-barrel interior with chromophore (yellow) and tryptophan (red).

Stopped-flow technique

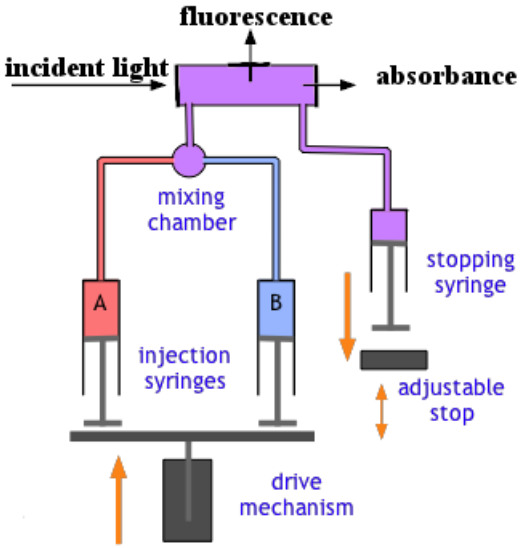

Fig. 3 Stopped-flow apparatus scheme, [1]

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. 3. Substrates of reaction are in injection syringes – dependent on kind of apparatus there are up to 4 injection syringes. Driving mechanism pushes pistons A and B in 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 observation 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:

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

where
$I(t)$ – fluorescence intensity at time t
i – number of phases of process
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, sometimes the phase fragment might be approximated by a linear function. Then equation (1) is modified to:

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

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/experimental errors and b) analysis of residuals.

Conduction of experiment

Student will receive the following solutions:

- 20 μM EGFP protein solution in 100 mM phosphate buffer pH 1
- 20 μM EGFP protein solution in 100 mM phosphate buffer pH 8
- 100 mM phosphate buffer pH 8
- 100 mM phosphate buffer pH 1
- 100 mM phosphate renaturation buffer, which will be mixed with solution pH 1 in ratio 1:1 giving final pH 8; renaturation buffer contains β-mercaptoethanol

and will use:

- fluorescence quartz cuvette with path lengths 10 mm and 4 mm
- Perkin Elmer LS 55 spectrofluorometer
- stopped-flow apparatus Applied Photophysics SX.18MV

1. Preliminary measurements: fluorescence steady-state measurements of EGFP in native state (pH 8) and denatured state (pH 1). On the basis of results student should select excitation and emission wavelengths for kinetic measurements.
2. Stopped-flow preliminary measurements: refolding kinetics measurement in the purpose of determining slit width for excitation and emission beams, time of experiment and voltage on photomultiplier.
3. Stopped-flow refolding kinetics measurements: experiment should consist of at least 10 repeats of a single refolding measurement.
4. Stopped-flow background measurements: experiment should consist of at least 10 repeats of a single measurement of mixing appropriate buffers.

Student can average data before further analysis if kinetics are recurrent

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/experimental 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. 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*

The list of subjects that student might be asked about:

- 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?)
- 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?
- Protein folding – models and mechanisms
- Stopped-flow technique – applications and principles of operation
- Green Fluorescent Protein – structure and chromophore formation

Bibliography

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3. M. Chalfie, *Green Fluorescent Protein*, Photochemistry and Photobiology 1995, 62 (4), 651-656
4. I. Żak, *Chemia medyczna*, chapter 13 „Aminokwasy i pochodne", Śląska Akademia Medyczna, Katowice 2001
5. V. Dagget, A. Fersht, *The present view of the mechanism of protein folding*, Nature Reviews, Molecular Cell Biology 2003, 4, 497-502
6. J. R. Lakowicz, *Principles of fluorescence spectroscopy*, Springer, 2006
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8. A. Bzowska, Handbook for exercise *„Spektroskopia absorpcyjna i emisyjna aminokwasów i białek, wykorzystanie w badaniach struktury i funkcji białek"*, Laboratory of biophysics for advanced, Warsaw 2012