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"

Application of absorption and emission spectroscopy of aminoacids and proteins in structural and functional protein studies

(PBdZ19)

HUMAN CAPITAL
NATIONAL COHESION STRATEGY

EUROPEAN UNION
EUROPEAN
SOCIAL FUND

Project co-financed by European Union within European Social Fund

1. The purpose of the exercise

The purpose of the exercise is determination (at pH 7.0 and temperature 25°C) of the following:

1. decimal molar extinction coefficient of enzyme PNP (Purine Nucleoside Phosphorylase) in the maximal absorption range of the protein (280nm),

2. PNP specific activity towards 7-methylguanosine using spectrophotometric initial velocity assay

3. dissociation constant of PNP-guanine complex using fluorescence titration.

2. The problem and methods

2.1. Absorption spectroscopy of aminoacids and proteins

Absorption and emission spectroscopy, the basics of which you know from Laboratory of Elementary Biophysics, is powerful tool in structural and functional study of proteins and their interactions. Of course structural information obtained with this kind of spectroscopy is not of atomic resolution, like for X-ray diffraction, but it gives important information about structure of molecules, and what is important structure in solution of known properties.

Unfortunately, many of molecules, essential for various biological processes, don't absorb radiation in near and middle ultraviolet(UV) range. Absorption in that spectral range comes from electronic transitions $\pi \rightarrow \pi^*$ i $n \rightarrow \pi$, thus from molecules having electron on π and n orbitals. Whereas in far UV nearly all substances absorb radiation, as it's related $\sigma \rightarrow \sigma^*$ transitions possible in almost all molecules, including buffers and other solvent used for protein solutions. Among essential biological molecules, in near and middle UV absorb aminoacids, purine and pyrimidine basis(so called nucleobases), as well as respective nucleosides and nucleotides, therefore proteins and nucleic acids absorb UV radiation. The proteins also exhibit absorption in far UV, in the region of 200nm, where is absorption band of peptide bond, which can be used for quite accurate determination of protein concentration, even if the exact sequence of the protein in unknown. Absorption in that region is quite similar for all proteins, and is approximately equal to $\varepsilon_{205nm}(1\ mg/ml) = 310\ cm^{-1}$ (Stoscheck, 1990).

Absorption spectroscopy can be used to determine concentrations of absorbing molecules, in studying enzymatic properties of proteins, such as inhibition by various ligands, natural or synthesised analogues. This method is rarely used in studying protein-ligand complex formation, as absorption doesn't depend significantly on the chemical environment of chromophore.

2.2. Emission spectroscopy of aminoacids and proteins

In contrast to absorption spectroscopy, the emission spectroscopy can be used to study interactions of molecules, like complex formation and conformational changes, like protein folding or denaturation. Unfortunately, among biologically important molecules, those which exhibit measurable fluorescence(their quantum yield is at least few percent) are even rarer that those which absorb near and middle UV.

In same handbooks on molecular spectroscopy, there is a simplified description of de-excitation processes as a straightforward releasing of oscillational and vibrational energy, concluded with a statement that electronic energy is too high to be released in a nonradiative processes, thus molecules fall back into ground state in

radiative way. If that was so, most of the molecules absorbing the UV radiation would have substantial emission quantum yield in that range, and they shall be suitable for studying with emission spectroscopy.

As this is not that simple, there are only three aminoacids and one nucleobase which are fluorescent under physiological conditions (water solutions, not extremal pH). These three aminoacids are tryptophan, tyrosine and phenylalanine. The nucleobase is 7-methylguanosine, and its nucleoside and nucleotide, occurring naturally in mRNA cap. Most snaturally occurring aminoacids and nucleobases demands modifications or quite exotic conditions regarding pH, to exhibit significant fluorescence.

Emission quantum yield and other spectral properties of fluorescent aminoacids are given in table 1.

Table 1. Spectral properties of aminoacids.
Data for aminoacids in 0,1M phosphate buffer at pH 7,0 from G.D. Fasman, Handbook of biochemistry and molecular biology, Proteins, I, 183-2-3, CRC Press, 3rd edition, 1976.

Compound	Maximum absorption wavelength [nm]	Absorption coefficient at maximum [1/M/cm]	Maximum emission wavelength [nm]	Fluorescence quantum yield
Tryptophan	278	5 579	348	0.20
Tyrosine	274	1 405	303	0.14
Phenylalanine	257.5	15	282	0.04

At this point, its worth mentioning a very unique and small group of proteins exhibiting fluorescence in visual range. One of those is Green Fluorescent Protein (GFP) from jellyfish, and its mutants emitting fluorescence of various colours, obtained with genetic engineering methods. These proteins serve as markers and are commonly used in biological and medical research.

Tryptophan, tyrosine and phenylalanine fluorescence quantum yield depends on chemical environment of particular residue and can be different from those given in table 1. Especially, tryptophan emission properties, regarding intensity and position of maximum, are sensitive to environment changes, particularly to polarity changes. Tryptophan emission spectrum is different, when in usually hydrophobic core of the protein and when exposed to solvent on the protein surface, thus different in folded and unfolded protein molecules. The position of spectrum maximum can change by as much as 20nm (red shift in polar environment). Tryptophan is good probe for monitoring polarity of its environment.

Overlap of aromatic aminoacids absorption and emission spectra frequently makes possible excitation energy transfer from phenylalanine to tyrosine and from tyrosine to tryptophan. This phenomenon is called fluorescence resonance energy transfer (FRET) and occurs only on very short distances, thus practically only in proteins in their native conformations and this is the reason why native proteins exhibit mostly fluorescence of tryptophan. Therefore FRET is another phenomenon allowing to study protein folding. FRET can also occur between fluorescent aminoacid and the ligand bound by the protein, thus can be used for following complex formation. In some cases binding the ligand may lead to changes in chemical environment of aromatic residue in protein, changing its quantum yield, giving another way to study complex formation with fluorescence emission spectroscopy. Beside changing natural protein fluorescence, ligand binding may lead to changes in fluorescence of ligand itself. For example a compound which generally don’t emit fluorescence, but its rare form(ionic, tautomeric, etc.) does, may emit in complex with

protein, as this rare form is the one, which binds to the protein. In such case protein-ligand complex may exhibit a band in its spectrum, not present in spectra of any of substrates. Such phenomena will be presented during this exercise.

2.3. Research object: purine nucleoside phosphorylases

Purine nucleoside phosphorylases (PNPs) is a family of proteins occurring in almost every living cell. These enzymes belong to, so called, salvage pathway and are absolutely essential for purine nucleoside metabolism(DNA and RNA building blocks) for those organisms which can't synthesize purine nucleosides from simpler chemicals. Phosphorylases isolated from various sources along with their potent and selective inhibitors have potential practical applications, mostly in medicine. Inhibitors of human PNP are potential immunosuppressive drugs or in therapy of autoimmune diseases. Inhibitors of PNP from various parasite organism are antiparasitic agents. Phosphorylases with lower specificity from some bacterias are used in enzymatic synthesis of purine nucleosides (Bzowska et al., 2000).

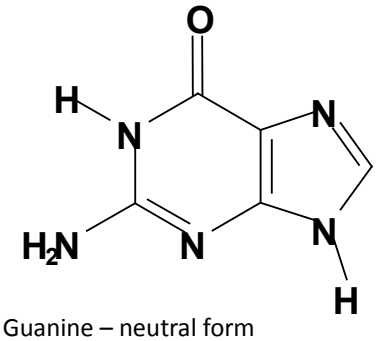
PNPs catalyse reversible phosphorolysis reaction of glycosidic bond in ribo- and deoxyribo- purine nucleosides:

β-purine nucleoside + Pi ↔ purine base + α-D-pentoso-1-phosphate

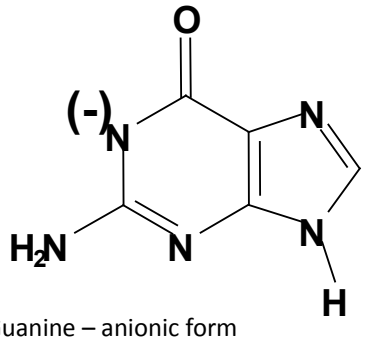
Phosphorolysis, other than more common hydrolysis, is a reaction of cleavage of a bond in presence of phosphate anion (HPO_4^{2-}, abbrev. Pi), not water molecule. In cells this reaction occurs mostly into direction of phosphorolysis(right in the above scheme), as products of reaction, purine bases and pentosophosphate, are substrates for other enzymatic reaction.

PNPs from mammalian and microorganism sources differ in structure and specificity. Mammalian PNPs are mainly trimers, they accept as substrates only 6-oxopurine nucleosides(guanosine and inosine), whereas PNPs of bacterial origin are mainly hexamers and they catalyse reaction with 6-oxo and 6-aminopurine nucleosides(also adenosine).

The purpose of the exercise is using absorption and emission UV-Vis spectroscopy to assign total protein concentration, active protein concentration (i.e. concentration of protein able to bind ligand and/or catalyse reaction), activity of enzyme along with dissociation constant and stoichiometry of interaction with one of the products of reaction: guanine.



Guanine – neutral form



Guanine – anionic form

Figure 1.
Structure of guanine, neutral and anionic (pK = 9.2) form. In both forms, tautomers with proton in position N(9) are shown (there are also form with proton in N(7) position).

Calf PNP is a homotrimer, namely is build from three subunits of identical aminoacid sequence and structure(Koellner et al., 1997; Bzowska et al., 2000) and is shown in figure 2. There are three tryptophan, several tyrosine and phenylalanine residues in protein sequence, therefore the natural fluorescence of this enzyme is significant. None of the tryptophans is located close to the active site, consequently ligand binding has little or no influence on protein fluorescence. However guanine binding (in absence of phosphate) leads to large emission increase of the ligand (Porter, 1992). The neutral form of guanine doesn't exhibit fluorescence, whereas naturally occurring anion with negative charge on nitrogen atom N(1) emits when guanine is dissolved in ethanol. Firstly it was suggested, that the reason for fluorescence increase of PNP-guanine complex was binding anionic form by the enzyme. This hypothesis was proved untrue (Stepniak et al., 2007). Later it was shown, that the enzyme preferably binds one of the tautomeric form of guanine, exhibiting fluorescence at decreased temperature(Breer, PhD thesis, 2011).

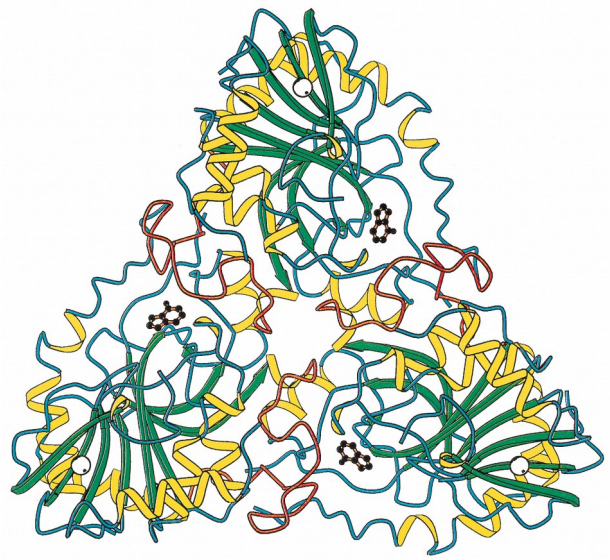


Figure 2.
Three dimensional crystallographic structure of calf PNP. In black shown ligand molecules (hypoxanthine) occupying active sites. Picture from Koellner et al., 1997, the first structure of calf PNP deposited in PDB.

Independently of the origin of fluorescence change upon complex formation, it can be used to determine parameters of PNP with guanine interaction, i.e. association/dissociation constant and stoichiometry.

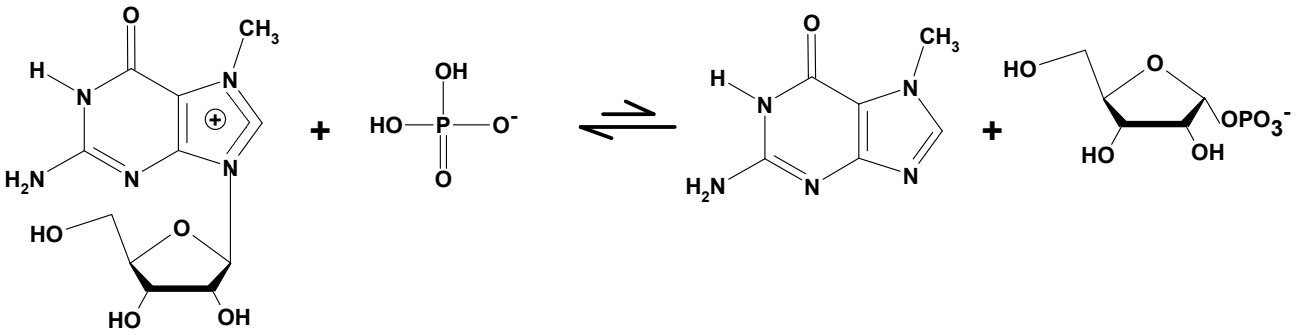


Figure 3.
Scheme of 7-methylguanosine phosphorolysis reaction. Nucleoside is shown in the cationic form (pK = 6.8). At pH 7.0, wherein the reaction is studied, neutral form is present. Precisely this is zwitterionic form, as after proton dissociation at N(1) position the ring has globally zero charge but the charge is delocalised and negative charge is localised mostly around N(1) and positive on imidazole ring.

For studying phosphorolysis reaction (figure 3), as a substrate 7-methylguanosine will be used, as its phosphorolysis is relatively easy to observe due to large differential absorbance of nucleoside and respective

base. The nucleoside is a substrate of varying concentrations, and the concentration of the second substrate, i.e. orthophosphate will be constant and very high – saturating (50 mM). As a source of phosphate ions the phosphate buffer 50mM pH 7.0 is used. Measurements are conducted at constant temperature 25°C.

2.4. Fluorescence intensity versus concentration relationship

Fluorescence intensity F is proportional to amount of radiation absorbed by the sample and its quantum yield. When observation of fluorescence is carried out in typical condition i.e. at an angle of 90 degrees to the excitation beam and there is no solvent absorption, fluorescence intensity is given by the following relationship:

$$F(\lambda_{exc}, \lambda_{ems}) = I_0 \Phi(\lambda_{exc})(1 - 10^{-\varepsilon(\lambda_{exc}) lc}) f(\lambda_{ems}) \qquad \textit{Eq. 1}$$

where: λ_{exc} – excitation wavelenght, λ_{ems} – emission wavelength, I_0 – intensity of exciting light, c – molar concentration of molecule, l – optical pathlenght, $\varepsilon(\lambda_{exc})$ – molar extinction coefficient, $\Phi(\lambda_{exc})$ - quantum yield of fluorescence emission, $f(\lambda_{ems})$ – normalised(area under curve equals one) emission fluorescence spectra.

When index $\varepsilon(\lambda_{exc}) lc$ is small enough(i.e. the concentration of absorbing molecules is small enough), the exponential function in above formula (Eq. 1) can be resolved into Taylor's series. After neglecting higher terms, formula simplified to the form:

$$F(\lambda_{exc}, \lambda_{ems}) = I_0 \Phi(\lambda_{exc}) \ ln(10 \varepsilon(\lambda_{exc}) lc) f(\lambda_{ems}) \qquad \textit{Eq. 2}$$

and observable fluorescence intensity is proportional to concentration of molecule in question.

In general the linear relationship is not observed, this is a phenomenon called inner filter effect. If fluorescence intensity is used to calculate a concentration of emitting molecule, in certain conditions, a correction has to be applied.

2.5. Correction for inner filter effect

Excitation and emission wavelength has to be chosen with regard to minimise total absorption of the sample at chosen optical pathlength. Absorbance must not exceed 0.3, as the correction has limited range of applicability due to neglecting higher terms in Taylor's series when obtaining formula given in equation 2. With the absorbance equals 0.05 error in fluorescence intensity is 5.5%, with absorbance 0.1 – 10.6%, with absorbance 0.2 – 20%.

All conducted emission measurement, especially all titrations, has to be corrected in regard to inner filter effect according to following formula:

$$F = F_{app} \cdot 10^{(A_{exc} + A_{ems})/2} \qquad \textit{Eq. 3}$$

Where F – corrected fluorescence intensity, F_{app} – observed(apparent) fluorescence intensity, A_{exc} – absorption of the sample at chosen excitation wavelength with regard to optical pathlength of excitation, A_{ems} – absorption of the sample at chosen emission wavelength with regard to optical pathlength of emission.

The correction for inner filter effect can be obtained experimentally. It involves performing titration of buffer with a ligand in exactly the same conditions(spectrofluorimeter configuration and ligand concentration and added volumes) as titration of protein solution with a ligand. Thus obtained titration curve has to be subtracted from protein-ligand titration curve.

In order to minimise inner filter effect the semi-micro cuvettes are used, i.e. cuvettes with optical pathlength of 0.4cm and 1cm, wherein shorter pathlength shall be placed as excitation path.

2.6. Correction for protein dilution

As the subsequent volumes of the ligand solution are added to the protein solution, concentration of the latter decreases. The fluorescence intensity versus ligand concentration relationship $F([L])$ has to be corrected for this dilution. Corrections shall be made by multiplying $F([L])$ by a factor V_L/V_0, where V_L – volume of solution after each added ligand volume, V_0 – initial volume of protein solution.

2.7. Protein-ligand complex

The simplest model of interaction between homooligomeric protein with a ligand (so called classical model) is based on two assumptions that (i) all subunits of the protein act independently, namely each of them binds ligand with the same dissociation constant Kd and (ii) binding subsequent ligand molecules adds equals contributions to total fluorescence of the system.

$$P + L \overset{K_d}{\Leftrightarrow} C \qquad \textit{Eq. 4}$$

where P – free protein (i.e. not liganded), L – free ligand, C – protein-ligand complex. All concentration refer to subunit concentration, not the whole trimer. In this model the oligomeric state of the protein is invalid, as the subunits act totally independent, namely like if it was solution of monomers.

Dissociation constant, Kd is given:

$$K_d = \frac{[L][P]}{[C]} \qquad \textit{Eq. 5}$$

Using relationships linking dissociation constant Kd, total protein concentration $[P_0]$ and complex concentration $[C]$: ($[P_0]=[P]+[C]$) along with ligand concentration $[L_0]$ ($[L_0]=[L]+[C]$), the analytical expression for total fluorescence intensity versus total ligand concentration is as follows (Eftink, 1997):

$$F([L_0]) = F_0 + \frac{1}{2} dF \left([L_0]+[P_0]+K_d - \sqrt{([L_0]-[P_0]+K_d)^2 + 4[P_0]K_d}\right) + [L_0]F_{lig} \qquad \textit{Eq. 6}$$

where F_0 – initial fluorescence of sample, therefore $F_0 = F_P$ – fluorescence of free protein, dF – total change of fluorescence during complex forming, then dF = $F_C - F_P$, where F_C is fluorescence of complex, F_{lig} – coefficient of fluorescence of ligand.

2.8. Fluorescence titration

Fluorescence(fluorimetric) titration is an experiment involving measuring of fluorescence intensity of mixture of protein and ligand at rising ligand concentration in conditions where excitation and emission

wavelengths are fixed. Obtained relationship between fluorescence intensity and ligand concentration is called titration curve. Fitting Eq. 6 to experimental titration curve allows to determine dissociation constant and coefficients of emission.

Total protein concentration $[P_\theta]$ also can be evaluated as a free parameter of fit. Then the obtained value of protein concentration shall be the concentration of active protein truly participating in reaction. If calculated protein concentration is close to determined active protein concentration, it strongly suggest, that classical model describes data well, and protein-ligand complex stoichiometry equals one. If fitted value deviates strongly from active protein concentration it may suggest more complicated binding mechanism.

In order to obtain both, dissociation constant and protein-ligand complex stoichiometry, from single titration curve, it is necessary to choose proper protein concentration, as the titration curve shape depends on protein concentration to dissociation constant ratio. Simulated titration curves for various protein concentration to dissociation constant ratios are shown on figure 4. If the protein concentration is small in comparison to dissociation constant, Kd can be determined, whereas stoichiometry can not. In this case negligible amount of ligand is bound to protein, therefore total ligand concentration and free ligand concentration are almost equal, and obtaining $[P_\theta]$ as a fit parameter is impossible (figure 4, panel C). Whereas, if protein concentration is significantly higher than dissociation constant, determining Kd is impossible, while stoichiometry can be obtain with high accuracy, as in that conditions so called stoichiometric binding occurs(figure 4, panel A).

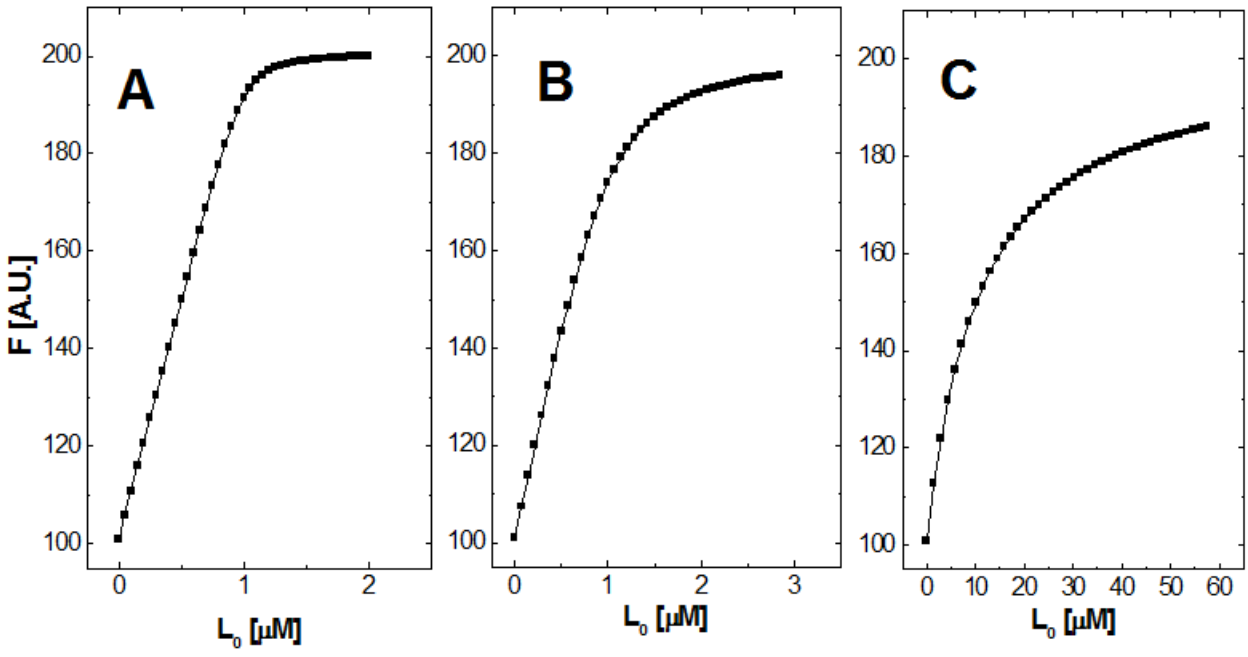


Figure 4.
Various shapes of titration curves due to different ratios of protein concentration to dissociation constant. All curves are simulated with classical model (Eq. 6). Panel A: stoichiometric curve, K_d = 0.01μM, P_{0act} = 1μM, panel B: optimal curve, K_d = 0.1μM , P_{0act} = 1μM, saturation 95%, panel C: not saturated curve(determining of stoichiometry impossible), K_d = 10μM, P_{0act} = 1μM, saturation 85%. For all simulated curves coefficients of emission are identical F_0 = 1, F_P = 300, F_{lig} = 0, dF = 100. Figure from PhD thesis of Dr. Karzyna Breer (doctoral thesis carried out at the Department of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw)

3. Requirement for preliminary test

The exercise begins with obligatory preliminary test. Student can start experimental part of the exercise only after passing this test. The form of the test, whether it is oral or written, the questions are closed or open, is chosen by assistant.

3.1. Recommended handbooks

G.M. Barrow: *Introduction to molecular spectroscopy*

C.A. Parker: *Photoluminescence of solutions*

J.R. Lakowicz: *Principles of fluorescence spectroscopy*

S. Underfriend *Fluorescence assay in Biology and Medicine*

Additionally, with this instruction, students receive:

1. Few initial pages of the paper: Stoscheck C.M. *Quantitation of proteins*, Meth. Enzymol. 182, 50-68 (1990

2. Respective fragment of the paper: A. Bzowska, E. Kulikowska, D. Shugar, *Purine nucleoside phosphorylase: properties, functions and clinical aspects*, Pharmacology and Therapeutics 88, 349-425 (2000), related to the scope of the exercise.

3.2. The preliminary test may include following subjects:

- Basic definitions related to electromagnetic radiation and spectroscopic measurements:
  - wavelength, frequency of vibration, wavenumber,
  - radiation intensity, radiation density,
  - molar extinction coefficient, integral extinction coefficient,
  - ground state, excited state, oscillator strength,
  - emission quantum yield, methods of determining the emission quantum yield.
- Radiation emission measurements:
  - schematic illustration of emission measurements geometry,
  - emission spectrum, excitation spectrum,
  - quantitative description of emission,
  - factors determining the shape and width of the outline of emission band,
  - inner filter effect and it's corrections.
- Electronic states of molecules:
  - definition of molecular orbital, what is the meaning of following symbols: σ , σ^*, π , π^*, n,
  - Jablonski diagram of electronic states: S0, S1, S2, ..., T1, T2,...., absorption and emission transitions and relative location of respective absorption and emission bands on the frequency radiation scale,
  - processes of de-excitation, radiative and nonradiative transitions, the characteristic time constants of these processes.
- Absorption and emission spectra with oscillatory structure:

- characteristic radiation range of absorption and emission electronic transitions,
- scheme of energetic levels – Jablonski diagram,
- scheme of allowed transitions and selection rules,
- exemplary shape of absorption and emission spectra illustrating the regularity arising from the structure of electron-vibrational levels and transitions between levels.

- Experimental methods of determining and calculating protein concentration, with regard to absorption spectroscopy in UV region:
  - methods based on aromatic aminoacids absorption,
  - methods based on peptide bond absorption,
  - methods based on the formation of coloured complexes (colorimetry),
  - computational methods based on absorption of aromatic aminoacids.
- Protein specific activity:
  - definition and units,
  - purposes of determining specific activity,
  - methods of specific activity determination .
- Dissociation and association constant of protein-ligand complex:
  - definition of dissociation constant, units, relation to substrates and complex concentrations,
  - relation between association and dissociation constant,
  - relation of dissociation constant with Gibbs free energy and temperature.
- Fluorescence titration:
  - fluorescence titration definition,
  - purpose of fluorescence titration, parameters obtained from this measurement,
  - aspects of planning successful titration experiment,
  - relationship describing shape of titration curve and parameters obtained from this model,
  - corrections necessary to interpret properly the results.

4. The exercise

4.1. Samples and methods

Students receive pre-prepared solutions necessary to comply with exercise:

- protein solution (calf PNP in 20 mM HEPES buffer pH 7.0 at a concentration of approx. 150μM (monomers concentration).

- guanine solution 1st grade in 0.1N NaOH at a concentration of approx. 1.8mM (guanine poorly dissolve in neutral pH)

- guanine solution 2nd grade in 20 mM HEPES buffer, at a concentration of approx. 25μM

- 7-methylguanine, solution in water at a concentration of approx. 1 mM

- 20mM buffer HEPES pH 7.0 (phosphate free)

- 200mM buffer phosphate pH 7.0

All measurements will be performed in quartz, semi-micro cuvettes, i.e. cuvettes with optical pathlengths 0.4 and 1cm. For fluorescence measurements cuvette with conical cavity at the bottom, for magnetic stirrer, will be used. Orientation of cuvettes relative to the incident beam, for both absorbance and fluorescence measurements are given in figure 5. For fluorescence measurement, in order to minimise inner filter effect shorter pathlength should be for excitation and longer for emission.

Figure 5.
Geometrical orientation of semi-micro cuvettes relative to incident beam and detector for absorbance and fluorescence measurements.

Minimal volumes of experimental samples are as follows: 1.2ml of solution in absorption cuvette , 1.3ml in emission cuvette when using Schimadzu spectrofluorimeter, 0.9ml when using Perkin Elmer spectrofluorimeter.

Initial concentration of PNP shall be approximately 1μM. Based on substrates and complex absorbance and fluorescence spectra with regard to minimising inner filter effect best excitation and emission wavelengths λ_{exc} and λ_{ems} for titration experiment should determined.

Best wavelength for studying 7-methylguanosine phosphorolysis is 260nm. Differential extinction coefficient of substrate (7-methylguanosine) and product (7-methylguanine) at pH 7.0 and 260nm is $\Delta\varepsilon$ = 4600 $M^{-1}cm^{-1}$, whereas extinction coefficient of 7-methylguanosine At 280nm is equal to ε = 8 500 $M^{-1}cm^{-1}$.

Abortion measurements are performed using spectrophotometers Cary 50, Cary 100 or Shimadzu 2401PC, whereas fluorescence – spectrofluorimeters Perkin-Elmer LS55 or Shimadzu RF501PC

4.2. Performing exercise

1. Protein concentration determination by measuring the absorbance in 20 mM HEPES buffer pH 7.0 at

    a) 205nm

    b) 280nm and 205nm

2. Protein extinction coefficient determination in maximum located in the region of 280nm based on results from section 1.

3. Extinction coefficient calculation in the region 280nm based on the data in table 1 and protein sequence (found in databases).

4. Comparison of extinction coefficient determined in section 2, calculated in section 3, calculated with

ProtParam software and obtained from literature (Table 3)

5. Spectrophotometric determination of specific activity towards 7-methylguanosine in 50mM phosphate buffer pH 7.0, at 25°C and saturating concentration of 7-methylguanosine. Assuming that the Michaelis constant is 13μM calculate saturating concentration. Concentration cannot be to high due to the possible deviations from Lambert-Beer rule.
6. Catalytically active protein concentration determination by comparing the measured specific activity with the highest specific activity found in the literature (find in received literature appropriate data and use information that the specific activity towards inosine is 34 U/mg).
7. increased fluorescence observation of the PNP-guanine complex: registration PNP, guanine and their complex spectra.
8. Performing fluorescence titration of PNP with guanine in 20 mM HEPES buffer pH 7.0, 25°C .
9. Performing control fluorescence titration (with only buffer in cuvette for inner filter effect correction)
10. Based on section 8 & 9 dissociation constant determination of guanine binding to calf PNP and binding stoichiometry assuming that catalytically active protein (a) does bind guanine, (b) does not bind guanine.

The student is expected to plan the experiment in details in order to perform task described above. Prior to commencing experiments the student have to consulted planned experiments with the assistant leading the laboratory class.

5. *The report*

The report should contain

Introduction – general, theoretical aspect related to your task

Materials & Methods – present used equipment, test compounds and measurement conditions

Results & Discussion - describe and interpret the results

Table 2. Exemplary scheme of titration

Step	Guanine solution	Added volume [μl]	Concentration of guanine [μM]	Sample volume V_L	Factor V_L/V_0	PNP concentration [μM] $[P_0]V_L/V_0$	Fluorescence
1	2nd grade	0		(=V_0)			
2		1					
3		1					
4		1					
5		1					
6		1					

7		1					
8		2					
9		2					
10		2					
11		2					
12		2					
13		2					
14		5					
15		5					
16		5					
17		5					
18		5					
19		5					
20		10					
21		10					
22		10					
23		10					
24		10					
25		10					
26	1st grade	1					
27		1					
28		1					
29		1					
30		1					
31		1					

6. *Literature*

C.A. Parker, *Photoluminescence of solutions*

J.R. Lakowicz, *Principles of fluorescence spectroscopy*

S. Underfriend *Fluorescence assay in Biology and Medicine*

7. Bibliography:

C.M.Stoscheck , Quantitation of proteins, Meth. Enzymol. 182, 50-68 (1990)

D.J.Porter, Purine nucleoside phosphorylase. Kinetic mechanism of the enzyme from calf spleen., J. Biol Chem. 267, 7342-7351 (1992).

G. Koellner, M. Luić, D. Shugar, W. Saenger, A. Bzowska, Crystal structure of calf spleen purine nucleoside phosphorylase in a complex with hypoxanthine at 2.15 Å resolution. J. Mol. Biol. 265, 202-216 (1997).

K. Stepniak, B. Žinic, J. Wierzchowski, A. Bzowska, Nucleosides, Nucleotides and Nucleic Acids 26, 841-847 (2007).

M.R. Eftink, Fluorescence methods for studying equilibrium macromolecule-ligand interactions, Methods Enzymol. 278 (1997) 221-257.

K. Breer, "Biophysycal basics of catalysis of purine nucleoside phosphorylases – study of wild type protein and its mutants "., PhD thesis made at Department of Biopysisc, IFD, WF, UW (2011)

E. Kulikowska, A. Bzowska, J. Wierzchowski, D. Shugar, Properties of two unusual and fluorescent substrates of purine nucleoside phophorylase: 7-methylguanosine and 7-methylinosine, Biochim. Biophys. Acta, 874, 355-363 (1986).

A. Bzowska, E. Kulikowska, D. Shugar, Purine nucleoside phosphorylase: properties, functions and clinical aspects, Pharmacology and Therapeutics 88, 349-425 (2000).

8. Tables of extinction coefficients and molecular masses of studied compounds

Table 3.

Compound	**Molar mass [g/mol]**	**pH**	**Wavelength at maximum [nm]**	**Extinction coefficient [$M^{-1}cm^{-1}$]**	**Extinction coefficient of 1% protein solution (10 mg/ml) in 1cm cuvette**
PNP	32 093	7	279	30 840	9.6

Table 4.

Compound	**pH**	**Wavelength at maximum [nm]**	**Extinction coefficient [$M^{-1}cm^{-1}$]**	**Observation wavelength [nm]**	**Differential extinction coefficient Δε [$M^{-1}cm^{-1}$]**	**pK**
Guanine	Neutral form	246 275.5	10 700 8 200	-	-	9.2
Guanine	Anion	273	8 000	-	-	9.2
7-methylguanosine	pH 7.0 (mixture of ionic forms)	258	8 500	260	4 600	6.8
	Neutral form pH 8.8	242 283	4 500 8 100			

	Cation pH 2.0	258	10 000			

Coefficients from:

1. R.M.C. Dawson, D. Elliott, W.H. Elliott, K.M. Jones (Eds.), Constituents of RNA and related compounds, and spectral data and pK values for purines, pyrimidines, nucleosides, and nucleotides, in: Data for Biochemical Research, Oxford University Press, Oxford, 1969, pp. 145-179.

2. A. Bzowska, E. Kulikowska, D. Shugar, Purine nucleoside phosphorylase: properties, functions and clinical aspects, Pharmacology and Therapeutics 88, 349-425 (2000).

3. E. Kulikowska, A. Bzowska, J. Wierzchowski, D. Shugar, Properties of two unusual and fluorescent substrates of purine nucleoside phosphorylase: 7-methylguanosine and 7-methylinosine, Biochim. Biophys. Acta, 874, 355-363 (1986).

9. Guidance for the assistant

To prepare

1. 200 mM buffer phosphate pH 7.0

2. 20 mM HEPES buffer pH 7.0 phosphate free, approx. 100 ml, phosphate contamination determination with molybdeniate assay (reference)

3. Guanine see table 5 below

4. PNP see below

9.1. Guanine sample

Guanine dissolve poorly in neutral pH, whereas it dissolves well in basic pH.

Dissolve guanine in 0.1N NaOH pH 12 (1st grade), from this solution prepare solution at pH 7.0

Table 5.

Compound	MW [g/mol]	Sample weight [mg]	Solvent volume [ml]	Absorbance at a maximum in a 1 cm cuvette		Concentration [μM]	
				Planed	Real	Planed	Real
Guanine 1st grade In 0.1N NaOH (from sample weight), pH must be 12.0 or above Centrifuge#	151.10	2.7	10	1.2*		1800	
Guanine 2nd grade In 50 mM HEPES buffer pH 7.0		130 μl 1st grade solution	10 ml HEPES buffer	~0.23		~25	

#This solution has to be kept frozen. Thawed, guanine has to dissolved again warmed, the optical density has to be rechecked in 1mm optical pathlength cuvette.

9.2. Desalting calf PNP

Calf PNP, approx. 5mg/ml (150μM), suspended in approx. 3M ammonium sulphate. Desalting in 4ml Amicon filter with 10kDa cut off. Pipette necessary volume of protein solution, e.g. 50μl, dilute with 20mM HEPES buffer pH 7.0 up to 4ml and concentrate on filter. Repeat 6 time (7 dilutions all together), in the last step concentrate to volume similar to initial. Thus desalted protein may be kept 2-3 weeks in the fridge.

Analogical desalting regime for recombinant protein suspended in ammonium sulphate

Sulphate anion is enzyme inhibitor, thus distorts results of interaction with other ligands.

Assay of concentration of obtained protein solution based on absorption spectra: dilute 81x (10μl concentrated PNP into 0.8ml HEPES buffer).

Extinction coefficient at 280nm for 1% solution of calf PNP in 1cm cuvette equals 9.6 cm^{-1}

Concentration of desalted protein solution:

$C[mg/ml] = 81 \times 10 \times A_{280}/(9.6\ [cm^{-1}] \times 1\ [cm])$

For two students group: ~40µl protein solution at a concentration of 5 mg/ml, of which:

a) approx. 20µl for absorption spectra in the range of maximum located at 280nm (20µl into approx. 1ml HEPES buffer); the same solution shall be used for spectra maximum located in the region of 205nm (after approx. 30times dilution), for measurement of specific activity (few µl into 1.2 ml reaction mixture), for 1 or 2 titrations (after approx. 4 times dilution).

b) Remaining 20µl shall be enough for 2-3 more titration (after approx. 150 times dilution).