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"

Is Michealis-Menten model of catalysis applicable to phosphorolysis of 7-methylguanosine by purine nucleoside phosphorylase?

PB11d v1

Variant 1: trimeric mammalian phosphorylase (calf)

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1. Introduction

Enzymes are amazing natural catalysts. Without them the rate of virtually all chemical reactions in the cell would be very low or even negligible. They are exceptionally efficient, some of them are able to increase rate of the reaction up to 10^{17} times, other convert up to 10^6 substrate molecules per second. Enzymes are also exceptionally specific, they usually catalyse one reaction or set of closely related reactions, they accept as substrate one particular molecule, or group of closely similar molecules, some are as specific as to work only on one enantiomer!

It was long thought that only proteins can serve as enzymes, but lately a ribonucleic acids exhibiting enzymatic activity were discovered, they are called ribozymes.

There are molecules, which have influence on catalytic rate of enzymes, they can increase rate of catalysis – activators, or decrease it – inhibitors. Both of them occur naturally in cell, as elements of biochemical regulation of metabolic processes, or can be introduced artificially serving as therapeutic or toxic agents. Many of commonly used drugs are inhibitors of particular enzymes.

Understating molecular mechanism of catalysis of particular enzyme can be very useful for medical and biotechnological application. Detailed knowledge about how the catalysis occur may lead to construction enzymes inhibitors which activity is morbific. For example, a popular antiflu drug Tamiflu, is an inhibitor of viral enzyme neuraminidase, which is necessary for the flu virus particle to release from host cell, therefore its inhibition limits virus particles multiplication and decrease severity of flu infection.

Therefore, many laboratories all over the world, conduct studies with the purpose of understanding molecular mechanism of catalysis of particular enzymes. This kind of research usually demands variety of methods, ranging from genetic engineering, through molecular biology, chemistry to biochemistry and biophysics.

2. Equations describing enzymatic reactions

Velocity of enzymatic reaction (v) increases linearly with increase of enzyme concentration. Whereas velocity versus substrate concentration ($v(c)$) relationship is more complicated. In the simplest case this realationship is described with Michaelis-Menten model:

$$v_0(c_0) = \frac{V_{max}\ c_0}{c_0 + K_M} \qquad \textit{Eq. 1}$$

where: V_{max} is so called maximal reaction velocity, and K_M - Michaelis-Menten constant. The V_{max} is very often expressed in the frequency units and called turnover number. The higher V_{max} the more efficient the enzyme is, the more substrate molecules per minute it convert into product. K_M measures the inverse substrate affinity to enzyme, i.e. the smaller K_M the higher affinity, K_M dependent of enzyme and substrate, it varies for different substrates of the same enzyme, typical values of this constant for enzymes are of range 10^{-3} – 10^{-7}M. In the cells, substrates concentrations are often close to K_M as then the reaction velocity involving this compound is almost straightforward proportional to its concentration, what eases maintaining optimal concentration of particular compound in the cell at constant level.

Michaelis-Menten model is based on three assumptions:

used symbols: E – enzyme, S – substrate, P - product

1. as an effect of enzyme-substrate interaction the enzyme-substrate complex is created

$$E + S \leftrightarrow ES \qquad \textit{Eq. 2}$$

2. conversion of substrate into product by the enzyme is an irreversible reaction

$$E + S \rightarrow E + P \qquad \textit{Eq. 3}$$

3. concentration of enzyme-substrate complex is constant in time, namely

$$\frac{d[ES]}{dt} = 0 \qquad \textit{Eq. 4}$$

The last of these assumptions(Eq. 4) is not fulfilled in the initial and final stage of the reaction. However this initial phase is so fast, that is not measurable by means of traditional methods (with manual mixing of reagents in experimental cuvette). Studying of this initial, rapid stage of reaction demands special equipment (stopped-flow apparatus), enabling very fast mixing of reagents, and thus decreasing dead time to few milliseconds. This kind of measurements, so called pre-steady state measurements, are valuable tool in researching molecular mechanisms of reactions.

Michaelis-Menten equation (Eq. 1) implies some simple conclusions:

- for low substrate concentration reaction velocity is straightforward proportional to substrate concentration

- for high substrate concentration velocity reaches constant value equal V_{max}

- for substrate concentration equal K_M the velocity is equal half of V_{max}

The purpose of this experiment is broadening knowledge about kinetic reactions conducted by enzymes. So far, all performed experiment, were conducted under assumption that Michaelis-Menten model describes well obtained data, whether it was determining of catalytic or inhibition constant (like for hypoxanthine or formycin inhibiting phosphorylase), because the relatively low number of measured points for kinetic curve didn't allow to notice discrepancies from this model.

2.1. The objects

The protein under study will be purine nucleoside phosphorylase (PNP), already known from previous exercises. Here is a brief reminder of its important properties.

PNP is present in almost every living cell, it catalyses reversible reaction of phosphorolysis of glicosidic bond in purine nucleosides.

$$purine\ nucleoside + ortophosphate \leftrightarrow purine\ base + pentosophosphate$$

The substrates are purine nucleoside and phosphate ion. Whereas spontaneous breakdown of glicosidic bond, for example in acidic environment, occurs with participation of water molecule(hydrolysis), in case of reaction catalysed by PNP, the second substrate is phosphate ion, thus occurring reaction is phosphorolysis.

Specificity of nucleoside phosphorylases depends on the organism it is isolated from. Phosphorylases with lower specificity occur mostly in bacteria, they are build from six subunits of identical aminoacid sequence, so called high molecular mass phosphorylases, they can catalyse reactions with adenosine, guanosine and inosine as a substrate, whereas phosphorylases from eukaryotic organisms, mostly build from three subunits of identical sequence, so called low molecular mass phosphorylases, catalyses only phosphorolysis of purine nucleosides with amino group in position C(6) of the purine ring, not oxo(like for adenosine).

In humans, decreasing of PNP activity causes inhibition of a cell-mediated immune response of the organism, but has no influence on humoral immunity. In general this is harmful effect, because it impair immunity of the organism. But in some cases this effect may be used for benefit. For example decreasing of cell-mediated immunity is necessary in patients after organ transplantation, elevated activity of PNP is noticed in some types of T-cell leukaemias, inhibiting of PNP may be helpful in treatment of autoimmune diseases and diseases related to purine metabolism. Whereas less specific hexameric phosphorylases are used as tools for the enzymatic synthesis of nucleosides, and are likely to be used in the treatment of cancer. Therefore phosphorylases are widely studied in laboratories all over the world. Hitherto there is only one inhibitor of PNP accepted as a drug in therapy of gout (metabolic disease associated with accumulating of uric acid).

Mechanism of reaction catalysed by phosphorylases is complex, and only under specific conditions it can be described with Michaelis-Menten model. In most cases to describe kinetic curve of reaction catalysed by PNP a more complicated equation is necessary, containing quadratic terms of substrate concentration in numerator and denominator. Interpretation of constants i, j, l, m depends of the mechanism of the reaction.

$$v_0(c_0) = \frac{j c_0 + i c_0^2}{1 + m c_0 + l c_0^2} \qquad \textit{Eq. 5}$$

For enzymes composed from many subunits, the deviations from Michaelis-Menten model, are often the result of interaction between subunits, therefore each of subunits has to be describe with different set of kinetic parameters. Usually sole kinetic studies are not enough to establish which mechanism is responsible for the observed alterations from Michaelis-Menten model.

The exercise object is a kinetic of 7-methylguanosine phosphorolysis with PNP. The nucleoside is a substrate of varying concentrations, and the second substrat concentration, i.e. orthophosphate, will be constant and very high – saturating (50 mM). As a phosphate ions source the phosphate buffer is used, at the same time it provide constant pH during measurements. Measurements are conducted at constant temperature 25°C. The reaction rate is measured spectrophotometrically. The differences in the electron absorption spectrum of substrate (7-methylguanosine) and product (7-methylguanine) are used (see scheme in Figure 1). The purpose is determination if reaction under study may be correctly described with Michelis-Menten equation.

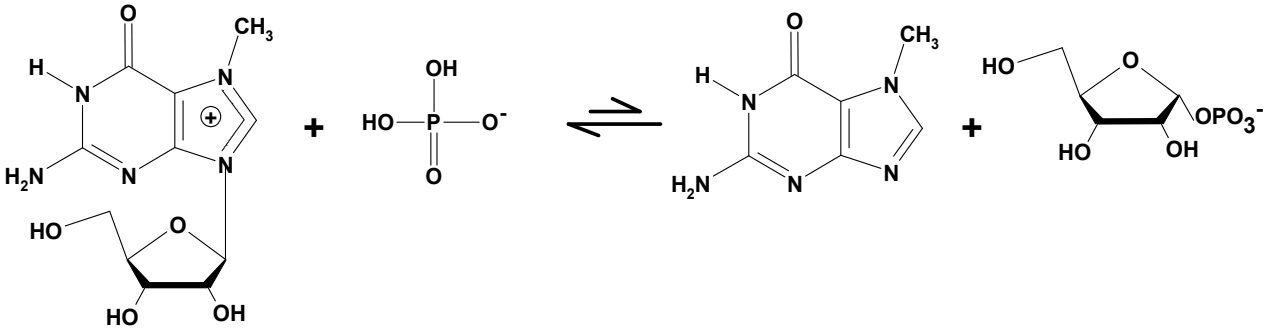

Figure 1. Scheme of phosphorolysis of 7-methylguanosine with PNP

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 lab assistant.

- Basic definitions related to electromagnetic radiation and spectroscopic measurements:
  - wavelength, frequency of vibration, wavenumber,
  - radiation intensity, radiation density,
  - molar extinction coefficient, integral extinction coefficient,
  - Beer–Lambert law.
- Kinetic of enzymatic reactions:
  - Michaelis-Menten model,
  - interpretation of constants defined in this model,
  - the conditions in which the model is satisfied,
  - Michaelis-Menten equation in the presence of inhibitor,
- Relationship between reaction rate and:
  - enzyme concentration,
  - substrate concentration,
  - temperature.
- Methods of determination of the rate of enzymatic reactions:
  - measurement units,
  - continuous method,
  - initial velocities method,
  - stopped-flow method,
  - comparison of above mentioned methods, their advantages and disadvantages.
- Spectrophotometric methods of determination of the rate of enzymatic reactions:
  - continuous method,
  - initial velocities method,
  - stopped-flow method.
- Methods for determining the fundamental kinetic constants (K_M , V_{max}) of the enzyme reactions:
  - fundamental kinetic constants, their units and physical interpretation,
  - determining constant according to $v(c)$ relationship,
  - Lineweaver-Burk transformation,
  - other transformations.
- Equations other than Michaelis-Menten describing enzymatic catalysis:
  - give an example,
  - suggest a method to study whether catalysis is described correctly by the Michaelis-Menten equation,
  - possible reasons of deviation from Michaelis-Menten model.

4. The exercise

4.1. Samples and methods

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

- substrate solution, namely 7-methylguanosine in water, concentration about 200μM (10 ml each group)

- protein solution, namely calf PNP disolved in 20mM HEPES buffer pH 7.0, concentration of the enzyme about 0.045mg/nl (150μl each grup)

4.2. Performing exercise

1. Recording substrate absorption spectra, namely 7-methylguanosine including the range of 200-230nm. To do this, prepare a quartz cuvette of 1 cm pathlength, with solution number 9 from the following table (table 1) and measure its spectrum. In the reference cuvette a phosphate buffer should be placed (solution number 11 in table 1)

2. Then add a PNP solution, in a volume indicated by assistant, and record few spectra of reaction mixture at constant time interval, for example every 60 or 90 seconds.

3. Basing on measurements performed in point 2, determine the best wavelength for mesuring reaction rate.

   Remark: students, who have performed obligatory exercise, wherein the inhibition constant of 7-methylguanosine phosphorolysis with PNP was determined, do not have fulfil points 1-3, as they already have done this.

4. Measuring the PNP enzyme kinetics using the initial velocity method, namely measuring reaction rate v_0 for a series of initial substrate concentration c_0 (according to scheme proposed in table 1) by determining the change in absorbance of reaction mixture at the selected wavelength. Each time, before adding enzyme and starting the reaction, the absorbance of substrate solution has to be recorded, in order to calculate initial concentration of 7-methylguanosine. Reaction curve for each substrate concentration has to be measured twice.

5. If the experiment is performed by more than one group at the same time, each of the groups take all the measurements described in table 1, but in analysis data obtained by all groups have to be included.

6. As a first step of analysis the Michaelis-Menten model should be tested, if the model describes data well a kinetic parameters of the reaction should be determined: maximal velocity (V_{max}) and Michaelis-Menten constant (K_M). Both parameters should be computed applying three methods: Michaelis-Menten equation, Lineweaver-Burk plot and Eadie-Hofstee plot. Obtained parameters shall be presented in following units: K_M in concentration unit [μM], and V_{max} in terms of enzyme specific activity [U/mg] and in terms of number of turnovers [1/s]

   Otherwise a more complicated model with quadratic terms in numerator and/or denominator has to be applied.

   GraphPad Prism or OriginLab Origin are recommended software for data analysis.

7. Comparison of obtained kinetic parameters with published data.

8. Necessary figures:

   a) extinction coefficient (ε) of 7-methylguanosine at pH 7.0 at maximum of absorption (258nm) equals 8 500 $M^{-1}cm^{-1}$, differential extinction coefficient Δε (i.e. difference of extinction coefficient between product and substrate) equals 4 600 $M^{-1}cm^{-1}$.

   b) concentration of PNP will be submitted by assistant,

c) molar mass of subunit of calf PNP equals 32 093 Da,

d) differential extinction coefficient (Δε) for product and substrate at wavelength chosen by students, as the best for measurements, will be submitted by assistant.

Table 1. Measurements scheme for kinetic of 7-methylguanosine phosphorolysis with PNP, variable concentration of one of the substrates (7-methylguanosine), constant concentration of second substrate (phosphate ion).

L.p.	**200μM m7Guo [μl]**	**200mM phosph. b. [μl]**	**water [μl]**	**substr. plan. conc. [μM]**	**PNP vol. [μl]**	**$A_1(\lambda_{obs})$**	**$A_1(360)$**	**reaction velocity [ΔA/min]**	
								v_1	**v_2**
1.	30	300	870	5					
2.	60	300	840	10					
3.	90	300	810	15					
4.	120	300	780	20					
5.	150	300	750	25					
6.	180	300	720	30					
7.	300	300	600	50					
8.	450	300	450	75					
9.	600	300	300	100					
10.	900	300	0	150					
11.	0	300	900	0	0	Kuweta kontrolna			

5. Recommended literature

1. A. Fersht, “Structure and mechanism in protein science” W.H. Freeman and Company, New York

2. R.A. Copeland, “Enzymes” Wiley-VCH

3. B.D. Hames, N.M. Harper “Biochemistry” The Instans Notes series

4. J. L. Tymoczko, J. M. Berg, L. Stryer “Biochemistry : a short course”

6. Sample preparation - guidance for the assistant

6.1. m^7Guo

- 0.5ml m^7Guo + 0.5ml phosphate buffer pH 7.0 should have absorbance at 1cm cuvette at 259nm $A_{259\,nm}^{1\,cm}$ = 0.85, as the extinction coefficient ε = 8 500 $M^{-1}cm^{-1}$ the concentration of 2x concentrated m^7Guo shall be about 200µM
- each group of students shall receive 10ml of 200µM solutions of m^7Guo

6.2. calf PNP

- sample so called from USA,
- 7.3mg/ml, salted out in 3M ammonium sulphate, dilute 161 times in 20mM HEPES buffer pH 7.0 (2.5µl of PNP sample into 400µl of buffer)
- students shall add 3µl of diluted protein sample into 1.2ml of reaction mixture, check the reaction velocity using condition for 4th reaction from table 1, absorbance change at 260nm shall be about 0.035 a.u. per minute. If the change would be smaller, the larger volume of protein sample should be added.
- desalting of protein sample is not necessary, as the kinetic would be studied under saturating concentration of phosphate (50mM),
- each group of students shall receive 150µl of diluted sample

6.3. Buffer

- 200mM phosphate buffer pH 7.0
- each group of students shall receive 15ml of this buffer

6.4. Best conditions

- Best wavelength for studying phosphorolysis is 260nm, differential extinction coefficient at this wavelength $\Delta\varepsilon_{260nm}$ = 4 600 $M^{-1}cm^{-1}$