LABORATORY OF ELEMENTARY BIOPHYSICS

Determination of kinetic parameters with very low K_m value, measurements of deoxycitidine kinase with radioactive labelled substrates.

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The aim of this exercise is to determine kinetic constants of enzymatic reaction catalyzed by deoxycytidine kinase. It is phosphorylation of 2'-deoźycytosine to the corresponding monophosphate. This reaction is particularly easily monitored using a radiolabeled substrate, and for the reasons explained below is somewhat inconvenient (or even impossible) to track using commonly used to monitor enzymatic reactions spectroscopic methods. For this reason, this system was used to familiarize students with the very effective, though sometimes arousing a certain fears, isotopic methods.

Introduction

In case of following the enzyme-catalyzed reaction it is particularly convenient, that the substrate and the reaction product has clearly different spectral characteristics, like absorption in the visible or ultraviolet light. However, measurements becomes much harder, if these spectral differences are small. An additional problem arises with the enzymatic reactions of very low Michaelis constant (of the order of micromol or less). With such low concentrations even substantial change in the extinction coefficient does not translate into meaningful measurable change in absorbance.

Consider a simple example:

change of extinction coefficient while changing from substrate to product 10 000 (v. high);
change in the concentration of substrate (the measurement of reaction rate): 1 mM;
optical path - 1 cm (typical cuvette);
would result in a complete change of the absorbance of the order of 0.01 at complete conversion of the substrate into product. Using the method of the initial velocities (a most popular one) we have to limit conversion to 10% , so is necessary to accurately measure the absorbance in the range of 0.000-0.001. It is feasible, but at the limit of best spectrophotometers (and don't forget that we have very comfortable change of extinction coefficient). But what if a change in extinction coefficient, is for example ten times lower, or simply close to zero?

Many biologically important chemical reactions can combine both of these problems (low MM constant and little change in spectral properties of the substrate-product transition) and then the only solution is to change the method of measurement. One method particularly useful in the above case, is a whole group of isotopic methods, namely the use of radiolabeled substrates and measuring the amount of radiation during the course of the reaction. This measurement is possible even for considerably less than the requirements of spectroscopic methods quantities of reagent. Unfortunately those methods also has its drawbacks, of which the most prominent are:

General:

- Widespread fear of radioactivity. We should add - the purpose of this exercise is to bring them to a reasonable size and teaching safe handling of radioactive materials.

- The high cost of radiolabeled reagents.

And specific - relating to enzyme kinetics measurements:

- We should forget about continuous measurement. The most precise measurements are performed with intake a small aliquots of the reaction mixture at predetermined time points (the more time points – the better), and simpler measurements base on the total change of radioactivity over time when we can assume that reaction rate at this time was constant.

- We have to stop the reaction in the aliquots (with strong heating or drastically changing the pH);

- We must separate radiolabeled product from unreacted substrate, (because both radiate). Generally as simple as possible (although unfortunately often laborious) chromatographic techniques are used.

Radiolabels used in biochemistry are unstable isotopes of elements present in organic compounds, in particular hydrogen, carbon, phosphorus and sulfur. The vast majority of them undergo β^- decay in which a neutron in the nucleus is transformed into a proton, an electron and an electron antineutrino. Proton remains in the nucleus (thus does not change the isotope mass number, and atomic number increases by one), while electron and an antineutrino are emitted. The generic equation is:

$$^{A}_{Z}\mathrm{X} \rightarrow {}^{A}_{Z+1}\mathrm{Y} + \mathrm{e}^{-} + \overline{\nu}_{e}$$

From our point of view, most important is the presence of easy to detection electron of (relatively) high-energy. Detection was accomplished by scintillation phenomenon, that is, the formation of flash light by the passage of radiation of certain substances (hereinafter scintillators). Many of them are semiconductor crystals, but the biochemical quantitative measurements use scintillators in liquid form (solutions of organic scintillators) which are simply mixed with the test sample in a closed vessels with a standard size (typically 20 or 5 ml volume). These vessels are collected in a scintillation counter which automatically places them one after the other in the light and radiation isolated chamber and, using two photomultipliers, counts the pulses of light for a certain period of time. The measurement accuracy is all the greater, the longer the integration period, so commonly used in times of counts one sample is 1 minute (for coarse measurements) and 10 minutes for accurate measurements. It is no wonder that one of the primary functions of a scintillation counter is a reliable automatic download and deposition of samples, since measurements can take many hours (and typically these more accurate - and therefore longer – are set at night).

Here are the three most common biochemical radioactive labels:

Tritium (^{3}H)

half-life - 12.32 years

β^- decay energy - 0.0186 MeV

The maximum distance covered in the air by the electron - 6 mm

(Because relatively low energy of the emitted electrons we do not need any anti-radiation shields – as electrons are not able to penetrate the dead outer layer of the epidermis)

Carbon ^{14}C

half-life - 5730 years

β- decay energy - 0.156 MeV

The maximum distance covered in the air by the electron - 25 cm

(Shields are not necessary - the outer layer of the epidermis is still sufficient protection)

Phosphorus ^{32}P

half-life - 14.3 days

β- decay energy - 1.709 MeV

The maximum distance covered in the air by the electron - 6 m

(This has significantly more energy than in previous cases and shields are required, typically plexiglas ones)

It worth to mention that one of the world's first biochemical measurements with ^{32}P-labeling was carried out immediately before World War II at the University of Lviv by the eminent Polish biochemist Jakub Parnas.

Biochemical basis.

The reaction, which will deal with (and at which we come across problems with detection described above) is a reaction catalyzed by deoxycytidine kinase, a member of the larger family of kinases called generally deoxynucleoside kinases

This kinase catalyzes the phosphorylation of the 5 '2'-deoxycytidine, or form a 5-monophosphate, 2'-deoxycytidine, with the help of ATP or UTP as a phosphate group donor:

2’ -deoxycytidine + ATP (UTP) → 2’-deoxycytidine-5’-monopphosphate-+ ADP (UDP)

This kinase, in a way contrary to its name, can also catalyze the phosphorylation of 2'deoxyadenosine, and 2' deoxyguanosine, however, we concentrate on 2 'deoxycytidine, as the Michaelis constant for this particular substrate (for the human enzyme) is in the range of 0.5 1 μM, so it is a great illustration of the difficulties described in the introduction as the Michaelis constant is low and transition from nucleosicde to 5'-monophosphate does not create any particular UV spectrum changes.
Other natural substrates Michaelis constant are in level of several dozen - several hundred μM and hence are substantially easier to measure.

A word about the entire family of kinases deoksynukleozydowych and purposefulness of their research.

All this family of kinases catalyze the phosphorylation of the 5 'end of 2'-nucleoside monophosphates, with the aid of ATP (in the case of certain kinases also UTP) as a phosphate group donor:

2' -deoksynukleozyd + ATP (UTP) → 5'-monofosforan-2'-deoksynukleozydu + ADP (UDP)

In the human body (and most mammals), there are four deoxynucleoside kinases:

deoxycytidine kinase (dCK) – catalyzes phosphorylation of, as already mentioned,
2 'deoxycytidine, 2' adenosine and 2 'guanosine;
deoxyguanosine kinase (dGK) - catalyzes phosphorylation of 2 'guanosine and 2' inosine;
And two different thymidine kinases:
Cytoplasmic thymidine kinase (TK1) - catalyzes phosphorylation of thymidine only;
Mitochondrial thymidine kinase (TK2) - catalyzes phosphorylation of thymidine and
2 'deoxycytidine.

It should be noted that these kinases are not part of main way of DNA precursors synthesis (this path is called *"de novo"* and operates on ribose form of nucleosides), but form occurring in the eukaryotic organisms nucleoside salvage pathway, which is a sort of recycling of waste arising from degraded DNA. Not being an essential biochemical pathway is, however, closely

examined, because it has a very practical use. Now many potential drugs that act at the DNA level is in the form of nucleotide analogs. But they can not be administered in that form into the body, as a nucleotide, a charged molecule, does not cross the barrier of cell membranes. So nucleoside is given instead with the hope that the salvage pathway kinases phosphorylate it in individual cells into a triphosphate. The first – and the key - stage: the phosphorylation of nucleoside into corresponding monophosphate is performed by deoxynucleoside kinases (the next stages of phosphorylation leading to triphosphate are run by much less substrate demanding enzymes).

dCK activity measurements.

There are possible two main measurement strategies with radiolabelled compounds:

1 We can radioactively label exactly the element that takes part in the reaction - that is, in our particular case, phosphate group, which is translated from ATP (or UTP) into nucleoside. What we need is ATP with ^{32}P at phosphate group furthest from ribose ring. Such a formulation is popular and commercially available under the name of γ-^{32}P-ATP (phosphate groups are numbered sequentially α, β and γ, counting from the sugar ring). The advantage of this method is that we can test any non-radioactive phosphate acceptor (nucleoside), as the following reaction with γ-^{32}P-ATP gives radioactive product. The disadvantage - quite tedious separation of the resulting monophosphate and unreacted ATP (exactly - the solvent used for this purpose TLC is based on the isobutyric acid and smells very unpleasantly), and the need to work with high energy emission of ^{32}P. A typical disadvantage of working with phosphorus is also a need to purchase each time a fresh preparation because of its short half-life.

2 We can use substrate (phosphate acceptor) labeled anywhere ,use non-labelled ATP and measure the amount of the resultant product which of course will also be radioactive. In this case, we can use less radiation-aggressive (and more long-lived) isotopes, like carbon ^{14}C or tritium. Moreover –separation of the product and remaining substrate (both of which are radioactive) is more pleasant - we can use a very simple ion exchange chromatography - product (monophosphate) having a

negative charge (phosphate group) will be adsorbed on special tissue paper filters while uncharged substrate (nucleoside) not, and it would be easy removed through repeated washings. Unfortunately we have to limit ourselves only to those nucleosides that are readily available in a labeled form. In general, they are popular natural nucleosides as cytidine, guanosine, adenosine or thymine. However, even such a limited system can be widely use in examining the effect of various substances on the reaction of a typical nucleoside. We ca add into such reaction another, but unlabelled nucleoside and observe decrease in the reaction rate, either because the unlabeled nucleoside proves to be competitive substrate and will form the unlabelled product, or because added nucleoside is an inhibitor of the reaction. In the first case - on the basis of a competitive inhibitory properties of the substrate, we can speak about its kinetic parameters, in the second we can directly measure the inhibition properties.

The measurement procedure – details:

In our experiment, for security reasons, we will use low-energy isotope-labeled, so tritium, 2'-deoxycytidine (and therefore we will follow the strategy described in item 2). In addition - the quantity (activity) so labeled compound will be small enough (no more than a few dozen µCi at once) that we can safely (and legally!) work in a regular lab, and not in a special isotope laboratory.

At your disposal will be a sample of human deoxycytidine kinase, a sample of tritium labeled 2 'deoxycytidine, and other ingredients, such as the reaction mixture containing the appropriate buffers and ATP - donor of a phosphate group (the detailed composition of the reaction mixture is in Appendix 2), your task will be determination of kinetic parameters of this reaction, and thus the Michaelis constant (Km) and maximum reaction speed (Vmax).

Technically measurements are as follows:

Reaction kinetic parameters will be calculated using the initial velocities method, and so we will have to perform a series (about ten) reactions with various concentrations of

substrate (labeled 2 'deoxycytidine), preferably in the field of 0.3 to 3 times of predicted value of the Michaelis constant. The second substrate - ATP - is in the reaction mixture in a concentration of saturation to changes in its concentration does not affect the reaction.

The reaction mixtures are set in a relatively small volume (recommended 40 ml) in eppendorf tubes. The reaction mixture contain a buffer for optimum pH, ATP and magnesium ions, necessary for the reaction, and the nucleoside with the appropriate concentration and specific activity (i.e. the amount of radiation per unit concentration). The reaction is run at 37C at heat block..

Initialize of reaction - by adding a convenient volume (eg. 10 ml) of appropriately diluted enzyme and mixing with vortex (be careful not to lather the sample). The enzyme at suitable concentration will be are available for students.
The amount of enzyme is chosen that during reaction time (up to a half hour), the loss of the substrate was so small that we can treat the reaction rate at that time as a constant, and thus assume a linear increase in concentration of the product (monophosphate) as a function of time.

The recommended volume of the reaction (40 µl + 10 µl of enzyme added) is enough for pick four 10 µl aliquots. Those aliquots are spotted at appropriated time points onto ion-exchange filter paper Whatman DE-81 discs. These discs must first be labeled (preferably with soft pencil), because they will be mixed at further proceedings. Because we had about 10 reactions and four time points for each of them, we must prevail over about 40 disks, which means that they must be arranged themselves in a reasonable order.

Do not exceed 30 minutes reaction time, due to the limited stability of the enzyme.

Luckily dropping the reaction mixture onto paper terminates the enzymatic reaction, since the paper almost completely stops diffusion.

Since every reaction runs about half an hour, and we need about ten reaction to determine the kinetic parameters, recommended tactic is to prepare all reactions at once and start

them in convenient intervals - for example every minute (or - a bit more difficult, but for some reason, preferred - every 30 seconds) and then sampling the subsequent reaction in the same time interval.

After completion of the reaction (series of reactions) take 5 µl from the residues and drop them on separate discs, which we will not flush, and the resulting radioactivity of them tell us how it is related to the total concentration of nucleoside (do not forget multiplied by two - the regular aliquots were 10 µl!).

All other discs should be washed three times for 5 minutes in a solution of 5 mM ammonium formate, and then once with water (each wash of 300- 400 ml). This procedure removes the unreacted labeled nucleoside from discs, leaving only negatively charged species - especially a labeled reaction product – 2'-deoxycytidine-5 'monophosphate.

The discs should be dried and placed into scintillation tubes. Then pour 0.25 ml of 0.1 N / 0.2M HCl / KCl mixture and stir vigorously 10 minutes to separate the paper charged product and convey it into the solution.

Finally pour into each scintillation tube approx. 4 ml of liquid scintillator, close them and mix carefully, repeatedly turning upside down several times. Now samples are ready to measure in a scintillation counter.

(Just to reflect: why we treat discs with HCl/KCl mixture? We could add the liquid scyntillator onto dry discs, and also obtain counts at scintillation counter.).

Final report

In the report you should take into account the Introduction with some brief statements about monitoring of enzymatic reactions and used enzyme, and the Materials and Methods in which to present the methodology used and equipment, test compounds and measurement conditions. Then, in the Results and Discussion should be present achieved results –

especially calculated kinetic constants of the enzymatic reaction. Obtaining of results should be documented by means of clear calculations and charts. You should also compare the results with literature data (part 3 of Literature) and try to clear up any discrepancies.

Requirements for joining the exercises

Since performance of this experiment require some knowledge of enzyme kinetics the familiarity of Michaelis-Menten kinetics, different methods of graphical presentation of the kinetics (at natural coordinates and at least one, which takes the form of a linear graph) is required.
Moreover, acceding to the experiment requires a basic knowledge about the use of radioactive labels in biochemistry, and their detection. Pos. 4 of Literature is sufficient.
I would also ask you to remember structures of natural nucleosides and 2 'deoxynucleosides.

Literature

1. A.L. Lehninger Biochemistry
2. L. Stryer Biochemistry
Both in the field of enzyme kinetics, Michaelis-Menten equation.
3. Arner ES, Eriksson S (1996). "Mammalian deoxyribonucleoside kinases." Pharmacol. Ther. 67 (2): 155-86 (available free over the Internet – please print it and carry with them).
4. Basic information about the radioactive tracer used in biochemistry, and methods of quantitative measurements of radiation using liquid scintillators are well gathered on the web site (recommended first seven articles):
https://www.nationaldiagnostics.com/liquid-scintillation/articles

Appendix 1 - Human deoxynucleoside kinases.

The cells of mammals (including human) contain four types of deoxynucleoside kinases. These are, taking the name of their basic substrates:

deoxycytidine kinase (dCK)
Natural substrates:
deoxycytidine (Km ~ 1 mM)
deoxyadenosine (Km ~ 40 mM)
deoxyguanosine (Km ~ 150 uM)

deoxyguanosine kinase (DGK)
Natural substrates deoxyguanosine, deoxyadenosine.

thymidine kinase 1 (TK1)
thymidine, deoxyuridine

thymidine kinase 2 (TK2) - mitochondrial
thymidine, deoxycytidine, deoxyuridine.

(The order of the substrates is not accidental - are listed for each enzyme in order from best to weakest, taking into account the efficiency of the enzyme, the ratio Vmax / Km)

To think - why is this quotient - Vmax / Km a good measure of the effectiveness of the substrate? Note: Please consider the Michaelis-Menten kinetics when the substrate concentration is low compared to the Km.

Appendix 2 - the reaction conditions and the composition of the reaction mixtures.

The optimum conditions for the reaction catalyzed by this a bit fragile enzyme, are:

Tris-HCl pH 7.2 50 mM
BSA 0.5 mg / ml
10 mM DTT
1 mM ATP
1 mM MgCl2

buffer provides optimal pH for the reaction, ATP is the second reactant that supply a phosphate group, magnesium ions are required for the reaction (in fact the second substrate is a complex $ATP{\cdot}Mg^{++}$), BSA (bovine serum albumin) is a protein commonly used to stabilize other proteins and DTT (dithiothreitol) is a reagent used to maintain disulfide bridges on the protein surface in a reduced form).

For the convenience and flexibility in the design of the reactions, use the following scheme:

reaction - the total volume of 50 ml, including:

20 ml – a reaction mixture (mixture of above listed substances in concentrations 2.5 times higher than the target because it will be diluted in 20 ml to a final volume of 50 ml, which is just 2.5 times).

20 ml - for additions (in our case labeled substrate at the appropriate concentrations);

10 ml - enzyme.

The reaction mixture is already prepared and available for the students.

Of course, this plan should be treated as a recommendation, if you need to increase the total reaction volume in order to collect more time points please feel free and just do it that way.

Appendix 3 - units of radioactivity

Key Characteristics of a radioactive preparation is its activity, that is, the amount of radioactive disintegrations per unit of time. Currently the legal (SI) unit of radioactivity is the **becquerel**, defined simply:

1 becquerel (1 Bq) = one radioactive decay per second

old and still popular unit is **curie (Ci)**, equal to:

$1\ Ci = 37\ GBq = 3.7 \times 10^{10}\ Bq$

and corresponds to the activity of one gram of radium-226.

The names of all these units are derived from the names of pioneers of the radioactivity research (Henri Becquerel, marriage Pierre Curie and Maria Skłodowska-Curie).

Scintillation counters typically display activity in CPM - counts per minute. In general this value is not equal 60-fold activity expressed in becquerels (60 – because becquerel is disintegration per second), but usually lower. During scintillation not every disintegration results observed flash of light, and this efficiency depends greatly of decay energy (and also of scyntillator itself) and is 20-50% for low energy (^{3}H) and close to 100% for high energy decay (^{32}P). More sophisticated counters can calculate this efficiency and express results in DPM – disintegrations per minute.

Typical parameter of radiolabelled formulation is its specific activity – it is activity of a certain amount (usually expressed in moles) of the formulation. It is of course higher, the more molecules of the preparation (in our case nucleoside) contains the radioactive label. Commercial product of the radioactive label generally contains information on specific activity of the formulation, and the total activity of the sample. On that basis, one can calculate the concentration of the formulation, generally not specified on the label. I warn you that you have to be able to do so during exercise.