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Effect of viscosity on efficiency of enzyme catalysis of bacterial luciferase coupled with lactate dehydrogenase and NAD(P)H:FMN-Oxidoreductase

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ABSTRACT

One of the current trends of the modern biology figures out cellular enzyme behaviour. Numerous researches look more closely at the chemical composition of creating *in vivo* simulated media conditions. The aim of this work was to find out a thermodynamic cooperativity of enzymes in a triple-enzyme chain (lactate dehydrogenase + NAD(P)H: FMN-oxidoreductase + bacterial luciferase) under *in vivo* simulated condition. The thermodynamic cooperativity effects were found out based on the influence of the viscogens (glycerol and sucrose) on the thermal stability of the triple-enzyme system. The results showed that the viscogens do not lead to an increase in the thermal stability of the triple-enzyme system. In addition, organic solvents (sucrose and glycerol) added as viscous agents to the reaction medium altered the kinetics of this triple-enzyme chain, including changing the light emission decay constant (k_{dec}) and quantum yield of luminescence (Q). Plus, sucrose was found to be more efficient in limiting the flexibility of enzymes than glycerol. The high sensitivity of the triple-enzyme system to the viscogens may be connected with a fact that lactate dehydrogenase does not bound with couple enzyme system NAD(P)H: FMN-oxidoreductase + bacterial luciferase inside the real cell. Since this approach may be used as a method to understand the real connection between enzymes in cellular multi-enzyme metabolic chains inside the luminous bacteria cell.

1. Introduction

To date, there is a widespread belief that the real environmental conditions inside the cell differ from the conditions in which enzymes usually investigated in a test tube. This belief occurs from a massive receiving data that are showing that the intracellular compartment comprises a variety of biological molecules such as proteins, amino acids and polysaccharides [1]. These biological molecules make up 5–40% of the entire volume of the cell [2]. Since that, there is an obvious fact that investigation enzyme kinetics under *in vitro* conditions do not lead to any relevant acquiring of knowledge regarding enzymatic behaviour inside the cell. This could be a reason that *in vitro* and *in vivo* simulated conditions have different research tasks. The main task of *in vitro* conditions is investigating and understanding the catalytic mechanisms of an enzyme, and in this case, enzymes should show the greatest activities they can. Moreover, a relevant question arises here: Do enzymes show such high constant activities in a real cell, or do optimal experimental conditions lead to their extremely high activity? In contrast, the main task of *in vivo* simulated conditions is to understand the mechanisms of cellular enzyme dynamics.

To obtain *in vivo* simulated conditions, organic solvents and/or crowding agents are utilised [1–6]. Also, the additives that help to simulate the crowded nature and structural environment in the cell are used to gain a functional understanding of the biochemical processes in it. The additives simulate two different factors, namely changes in solution viscosity and the excluded volume effect [4]. Unfortunately, at present time, numbers of researcher mainly focus on the impact of molecular crowding (or the excluded volume effect) on enzyme kinetics [1–6]. Probably, this attention to molecular crowding is connected with the speculations that understanding molecular crowding is a one-of-a-kind feature of the development of cell biology and figure out of enzyme behaviour in the cell [7]. However, the recently published article showed that the investigation only effects of macromolecular crowding do not provide accurate and high informative knowledge about *in vivo* surroundings and enzyme behaviour under it [8]. In addition, as has been shown previously, catalytic activities of enzymes depend on a number of factors [1,2].

Another significant factor that can also result in data obtained during *in vivo* simulated testing is the reaction media viscosity [1,2]. There is no doubt that cell viscosity differs from that of a buffer; this

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could be attributed to certain enzymes. Considering Kramer's theory, it can be stated that many enzymatic reactions depend on the reaction mixture viscosity. It was also reported that the enzymatic reaction rate constant is inversely proportional to the environmental medium viscosity [9,10]. Unfortunately, viscous organic solvents such as glycerol and sucrose are frequently used only for an application manner task (*i.e.* for increasing activity of enzymes under unfavourable experimental conditions and enhancing its sensitivity of determination of different toxicants or other metabolites) [11]. Few authors have detailed speculation about increasing of enzyme activity or shifting of optimum temperature of an enzyme system in the presence of organic solvents. Basically, a variation of thermal stability of an enzyme system could represent a level of efficiency of an enzyme catalysis [12]. Furthermore, studies have confirmed that the viscosity dependence of enzyme reactions is frequently accompanied by enzyme conformational changes [13]. It seems that an increased enzyme activity in the presence of viscogens under temperatures that are higher than optimum temperature could represent advantages for enzyme reactions. Probably, this increased enzyme activities in the presence of viscous agents are connected with natural intracellular conditions (*i.e.* altering of structural conformations of proteins, which are caused by viscogens, makes the protein catalysis closer to a cell mode. In addition, some authors notice sophistication and harmonization of enzyme reaction in a cell. This harmony and preciosity of metabolic chains inside the cell bring about the cooperativity of biological systems. Kinetic and thermodynamic cooperativity go up the efficiency of enzymatic reactions. Thus, if a multi-enzyme system reaches the thermodynamic cooperativity, which brings about some conformational changes, this system should have resistant to any adverse conditions (*i.e.* temperature, pH *etc.*) [12]. The aim of this work was to investigate kinetic and thermodynamic cooperativity of enzymes in the triple-enzyme system under *in vivo* simulated condition. Since the effect of the reaction mixture viscosity on the kinetic features and thermal stability of a bioluminescent multi-enzyme system was tested. The multi-enzyme system was presented as a coupled triple enzyme system, which consisted of lactate dehydrogenase + NAD(P)H:FMN-oxidoreductase + bacterial luciferase (LDH + R + L):

$$\text{L-lactate} + NAD^+ \xrightarrow{\text{lactate dehydrogenase}} NADH + H^+ + \text{Pyruvate} \quad (1)$$

$$NADH + H^+ + FMN \xrightarrow{\text{NADH:FMN-oxidoreductase}} NAD^+ + FMNH_2 \quad (2)$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + hv \quad (3)$$

These enzymes were selected for the following reason: all outgoing researches regarding modelling *in vivo* simulated conditions involve NAD^+-dependent enzymes. The R + L enzyme system is also an NAD^+-dependent system. In addition, the R + L enzyme system has one unique feature. This feature is that different metabolic pathways could be constricted by coupling NAD^+-dependent enzymes with R + L enzyme system [14]. Since that such constricted bioluminescent multi-enzyme systems could be a good experimental tool for investigation of the intracellular behaviour of a variety of metabolic pathways, by which enzymes cooperate with each other inside the cell.

2. Experimental

2.1. Chemicals and solutions

The lyophilized preparations of highly purified enzymes were produced at the Laboratory of Nanobiotechnology and Bacterial Bioluminescence of the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). Each vial of the lyophilized preparation contained 0.5 mg of luciferase (L) (EC 1.14.14.3) from the recombinant strain *Escherichia coli* and 0.15 units (0.15 μmole of flavin mononucleotide (FMN) will be reduce per one minute at pH 6.8 at +25 °C) of NADH:FMN-oxidoreductase (R) (EC 1.5.1.29) from *Vibrio fischeri*. LDH (EC 1.1.1.27) from rabbit muscle was procured from Sigma (Type XI, catalogue no. L1254, 5000 units (five mmole of pyruvate to L-lactate will reduce in one minute at +37 °C and pH 7.5)). For preparing the LDH enzyme solution, 0.5 mL of potassium phosphate buffer was added to a vial. NAD^+ (AppliChem), DL-lactic acid (Sigma-Aldrich), FMN (Serva) and myristic aldehyde (Merck) were used as the substrates of LDH, R and L. Glycerol (Gerbu) and sucrose (Gerbu) were used as viscogens. Myristic aldehyde solution [0.0025% (v/v)] was prepared by mixing 50 μL of 0.25% (v/v) ethanol solution of aldehyde and 5 mL of 0.05 M potassium phosphate buffer (pH 7.1). The NAD^+ solution was prepared in 0.05 M potassium phosphate buffer (pH 7.1). Samples of FMN, lactic acid, glycerol [5–50% (v/v)] and sucrose [10–40% (w/v)] were dissolved in distilled water. The concentrations of glycerol and sucrose are expressed as viscosity values, according to the chemical handbooks [15,16].

2.2. Measuring bioluminescent activity of multi-enzyme system

The activity of the coupled LDH + R + L enzyme system was measured using a reaction mixture containing: 300 μL of 0.05 M potassium phosphate buffer (pH 7.1), 5 μL of LDH solution, 5 μL of enzyme (R + L) solution, 50 μL of 0.0025% (v/v) myristic aldehyde solution, 10 μL of 15 mM lactic solution, 10 μL of 0.5 mM FMN solution and 50 μL of 0.5 mM NAD^+ solution. At the beginning, the control luminescence intensity of the enzyme system in the buffer (I_b) was registered. For I_b registration, all components of the reaction mixture were added to the luminometer cuvette and quickly mixed, and the luminescence intensity values were subsequently measured. For registration of the experimental luminescence intensity (I_{exp}) in the presence of viscogens, 300 μL of 0.05 M potassium phosphate buffer at pH 7.1 was replaced by 300 μL of the viscogen. The luminescence intensity of the coupled LDH + R + L enzyme system was measured using a Glomax 20/20^n luminometer (Promega, USA). A VT-8 liquid circulation ultra-thermostat (Therex-2, Russia) was used to study the effect of temperature on the activity of the multi-enzyme system. All experiments were performed in triplicate and the data were subjected to variation statistics.

The decline in bioluminescence determines the rate of decay of the enzyme-substrate complex in time and follows the exponential law. The light emission decay constant (k_{dec}) is calculated using the following formula:

$$k_{dec} = (\ln I_{80} - \ln I_{20}) / \Delta t \quad (4)$$

where I_{80} and I_{20} are the luminous intensity values that were registered during decrease of the luminescence from 80 to 20% of maximum intensity [17].

The quantum yield (Q) is the total quantity of emitted light quanta during a bioluminescent reaction. The quantum yield can be calculated using the following expression [17]:

$$Q = I_{max} / k_{dec} \quad (5)$$

Thermal stability of the multi-enzyme system was measured by collecting the I_b and I_{exp} values after incubation of the enzyme preparations at different temperatures (15–80 °C) for 5 min. Activation energy (E_a) was graphically determined in the Arrhenius coordinates (ln k_{dec} against 1/T) [17]. k_{dec} is the luminescence decay rate constant, and is calculated as the decrease from 80% to 20% in the I_{max}.

3. Results

3.1. Viscosity effects on kinetic parameters of LDH + R + L multi-enzyme bioluminescent system

The effects of the viscogens on two kinetic parameters of the LDH + R + L multi-enzyme bioluminescent system, k_{dec} and Q, were