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A Method for the Determination of Bi-substrate Kinetic Coefficients: the Example of the β-D-glucose- NAD-GDH Enzymatic Reaction

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Abstract: Colorimetric detection of glucose in sample liquids such as human plasma is made by using enzymatic reactions. Either glucose oxidase (GOX) or glucose dehydrogenase (GDH) can be used to convert glucose. In the multi reactional scheme, the first enzymatic reaction is determinant. We focused here on the study of the enzyme GDH together with the enzymatic cofactor NAD (nicotinamide adenine dinucleotide). This reaction falls in the category of ternary enzymatic reactions. Such reactions depend on four parameters. A method to determine these four parameters is presented in this work, based on a comparison between a series of experiments and the theory. The best values of the parameters are indicated. *Copyright © 2015 IFSA Publishing, S. L.*

Keywords: Ternary enzymatic reaction, Michaelis-Menten kinetics, Bi-substrate kinetics, Glucose.

1. Introduction

Nowadays, blood glucose self-monitoring is widely spread on the market and is routinely used by people suffering from diabetes. However, the accuracy of such tests can still be improved.

Historically, the first detection method employed was reflectance photometry [1-2]. In the 1980's, the first strips using electrochemical detection were launched [3]. For both methods—colorimetry and electrochemistry—glucose detection in human blood is achieved by the means of enzymatic reactions.

Either glucose oxidase (GOX) [4] or glucose dehydrogenase (GDH) [5] can be used to convert glucose and generates an appropriated signal. An important remark at this point is that, in a multi reactional scheme the first enzymatic reaction is determinant.

We focused here on the study of the enzyme GDH together with the enzymatic cofactor NAD (nicotinamide adenine dinucleotide). The reaction can be written as

$$\beta - D - Glu\cos e + NAD + GDH \leftrightarrow Glucono - lactone + NADH + GDH \quad (1)$$

This reaction falls in the category of ternary enzymatic reactions (Fig. 1). In a first reactional step, the coenzyme NAD (nicotinamide adenine

dinucleotide) binds to the GDH (glutamate dehydrogenase) and in a second step, the β-glucose binds to the enzymatic complex. The reaction then occurs, and the product (glucono-lactone) is released together with the NADH (nicotinamide adenine dinucleotide dehydrogenase). The GDH is not affected by the reaction: its concentration stays constant during the reaction.

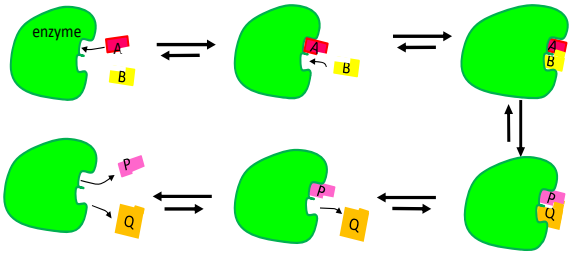

Fig. 1. Principle of enzymatic ternary reaction: A=β-glucose, B=NAD, P=NADH, Q= glucono-lactone.

Ternary reaction models—also called bi-substrate models, because one enzyme and one coenzyme intervene - depend on four parameters [6-9].

In order to optimize the reaction, these parameters must be determined. A method to determine these four parameters is presented in this work. The determination of the four reaction parameters stems from a comparison between a series of experiments and the theory. The best values of the parameters are indicated.

2. Theory

The theory of ternary enzymatic reaction is well documented in the literature. It is derived from the Michaelis-Menten law for enzymatic reactions. The derivation of the ternary reaction velocity is not straightforward: it can be found in the book of Stan Tsai [10]. In our particular case, the reaction is ordered (not the ping-pong type of reaction) and its velocity is given by

$$V = K_0 [E_0] \ldots \frac{[A][B]}{[A][B] + [A]K_{MB} + [B]K_{MA} + K_{SA} K_{MB}}, \quad (2)$$

where $[E]$ is the concentration of enzyme (GDH), $[E_0]$ is the initial enzyme concentration; $[A]$ is the β-D-glucose concentration, $[B]$ the NAD concentration, and the coefficients K's are the Michaelis-Menten constants. In (2), K_{MA} is the Michaelis coefficient for $[A]$ when $[B]$ is saturating, and K_{MB} is the Michaelis coefficient for $[B]$ when $[A]$ is saturating. K_{SA} is the affinity of $[A]$ for the free enzyme (GDH).

Dividing by $[B]$ the numerator and denominator of (2) yields

$$V = \frac{K_0 [E_0]}{1 + \frac{K_{MB}}{[B]}} \frac{[A]}{[A] + \frac{K_{MA}[B] + K_{SA}K_{MB}}{[B] + K_{MB}}} \quad (3)$$

In our case the NAD concentration is fixed and equal to $[B_0] = 0.4$ mM. Relation (3) generalizes the Michaelis-Menten law: if we note

$$V_{\max,app} = \frac{K_0 [E_0]}{1 + \frac{K_{MB}}{[B]}} \quad (4)$$

and

$$K_{M,app} = \frac{K_{MA}[B] + K_{SA}K_{MB}}{([B] + K_{MB})} \quad (5)$$

relation (3) reduces to the Michaelis-Menten law.

For the concentration $[B]$ the reaction can be simply characterized by the two Michaelis-Menten constants V_{max} and K_M. But the knowledge of the four ternary reaction constant bares a larger generality.

Reaction kinetics (3) is valid if both concentrations $[A]$ and $[B]$ are such that $[A] >> [E_0]$ and $[B] >> [E_0]$, which is our case for reaction (1) and which has been reproduced in the experimental set-up.

3. Model

Relation (3) has three unknowns V, $[A]$ and $[B]$, and four parameters K_0, K_{MA}, K_{MB} and K_{SA}. However, a solution for the kinetics of the product $[Q]$ (glucono-lactone) can be derived, from the determination of the consumption of $[A]$ (β-glucose) [11].

By definition the reaction velocity is

$$V = \frac{d[A]}{dt} \quad (6)$$

Using the law of mass action [6-7], we can write

$$[B] = [B_0] - [A_0] + [A] \quad (7)$$

Relation (3) can then be cast in the form of a differential equation in $[A]$, with the initial values $[E_0]$, $[B_0]$

$$\frac{d[A]}{dt} = \frac{K_0 [E_0]\{[A]^2 + [A]([B_0] - [A_0])\}}{[A]^2 + [A]([B_0] - [A_0] + K_{MA} + K_{MB}) + K_{SA}K_{MB} + K_{MA}([B_0] - [A_0])} \quad (8)$$

Equation (8) is of the form:

$$\frac{d[A]}{dt} = -cste \ \frac{P_2([A])}{Q_2([A])}, \tag{9}$$

where P_2 and Q_2 are two second order polynomials.

We have programmed the integration of (9) with the software MATLAB, and used a conventional Runge-Kutta approach to solve for the NAD concentration.

Finally, the product (glucono-lactone) concentration $[Q]$ is given by

$$[Q] = [A_0] - [A] \tag{10}$$

4. Experiments

A series of six kinetics using increasing concentrations of β-D-glucose has been performed (Fig. 2). Briefly, final concentrations of β-D-glucose, ranging from 0 mM to 25 mM, were incubated together with NAD and glucose dehydrogenase (*Pseudomonas sp* from Sigma Aldrich) at final concentrations of 0.4 mM and 0.5 U/mL respectively, using a 25 mM Tris, 192 mM Glycine buffer pH 8.05 at 37 °C. For each β-D-glucose concentration, the reactions have been done in triplicates. Kinetics were obtained by following the OD (optical density) at 340 nm, corresponding to NADH spectrum.

In a Michaelis-Menten type of reaction, the reaction is characterized by the affinity of the substrate (β-D-glucose) for the enzyme (k_M) and the enzyme turn over (k_{cat}), which represent a global view of the reaction.

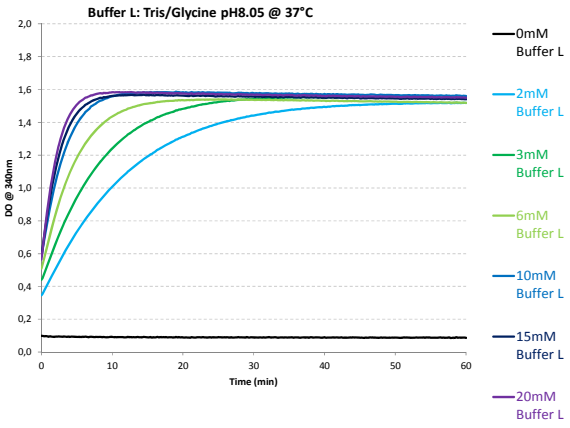

Fig. 2. Reaction kinetics for different concentration of β-D-glucose {2, 3, 6, 10, 15, 20 mM} in Tris/Glycine buffer.

On the basis of the Lineweaver-Burk plot (Fig. 3), the affinity of the β-D-glucose for the enzyme can be determined: k_M = 9.0 mM. In addition, the enzyme turn over has the value k_{cat} = 89 s^{-1}.

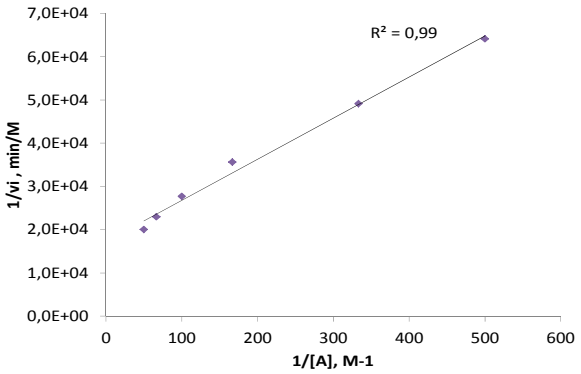

Fig. 3. Lineweaver-Burke plot used for the determination of k_M and k_{cat}. The slope of the linear fit is k_M/V_{max}, and the intercept with the vertical axis is $1/V_{max}$. The notation [*A*] stands for the β-D-glucose concentration.

The ratio k_{cat} / k_M, reflecting enzyme efficiency, equals to 9.8 × 10^3 $M^{-1}{\cdot}s^{-1}$. These results are in the same order of magnitude with that obtained in previous works at pH 8.0, performed on wild type and mutants of GDH isolated from *Bacillus megaterium*, known to be specific for glucose determination in body fluids [12]. In these studies, k_M values ranged from 2.7 to 55 mM and k_{cat} values are comprised between 23 and 430 s^{-1} [13-15]. Depending on the mutants, the enzyme efficiency previously reported varies from 3.6 × 10^3 to 42.8 × 10^3 $M^{-1}{\cdot}s^{-1}$.

The results that we obtain for the enzyme efficiency using GDH from *Pseudomonas sp* compare well with the previous ones reported on the various forms of GDH from *Bacillus megaterium*.

5. Determination of the Reaction Parameters

In reality, the reaction is a Michaelis-Menten ternary reaction. In order to characterize the reaction in details, we follow a double reciprocal plot approach. The velocities *V* are calculated from the kinetic curves of Fig. 2. In a {*1/[A]*, *1/V*} coordinates plot, the experimental data points are satisfactorily aligned (Fig. 4).

The four bi-substrate Michaelis-Menten parameters (K_0, K_{MA}, K_{MB} and K_{SA}) can be determined by comparison with experimental results.

A linear fit with the experimental results produces the values of the four reaction constants (Fig. 5). The fitted values of the four bi-substrate reaction constants are: K_0=1·10^5 s^{-1}, K_{MA}=1 mM, K_{MB} = 2.9 mM, K_{SA}=12 mM.

Reporting these values in (4) and (5), with [*B*]=0.4 mM, we find $K_{M,app}$ = 10.7 mM, and $V_{max,app}$ = 0.003 mM/s, and $k_{cat,app}$=$V_{max,app}$/[E_0]=222 s^{-1}. The values of apparent K_M and k_{cat} deduced from the ternary model are in the same range as the corresponding experimental Michaelis-Menten kinetic constants.

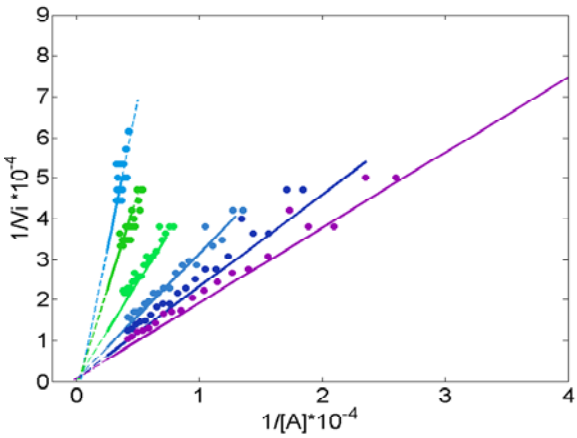

Fig. 4. Representation of the experimental velocities in a Lineweaver-Burke diagram.

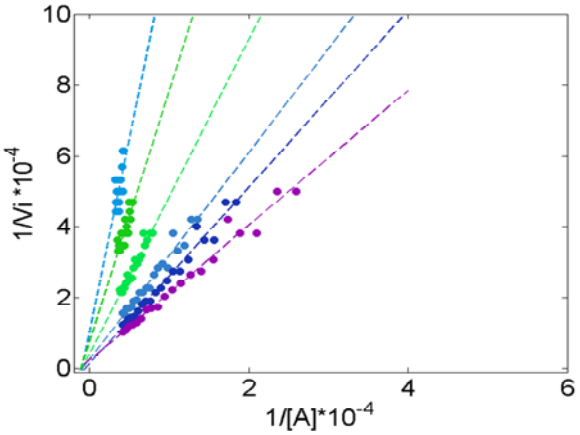

Fig. 5. Fit of the theoretical model on the experimental results in the double Lineweaver-Burke diagram.

Reversing the approach, and reporting the fitted parameters in relation (3), we obtain a very satisfactory comparison of the kinetics between experiments and model (Fig. 6), confirming the approach.

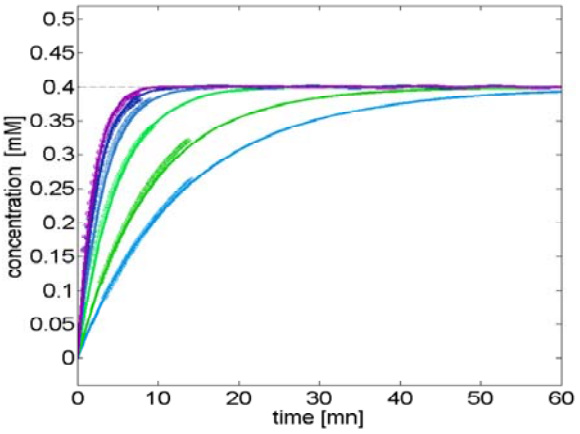

Fig. 6. Kinetics comparison between model and experiments: continuous lines correspond to the experimental measures and the dots to the model.

6. Colorimetric Detection

In fact, reaction (1) is the first step of a two stages reaction process. The second enzymatic reaction uses a tetrazolium salt and creates a colored product (formazan). This reaction can be symbolically written as

$$NADH + MTT + Diaphorase \rightarrow NAD + Formazan + Diaphorase \quad (11)$$

The reaction uses another enzyme called diaphorase together with a yellow colored salt (MTT). In general the dye needs to fulfill several characteristics. First of all, it has to be reduced by NADH, in order to regenerate the NAD. Secondly, its reduced form should present some optical properties compatible with the biological sample which is subjected to the detection test. Typically, if one wants to perform a colorimetric detection using whole blood, the absorption properties of the dye to be used should be very different from that of hemoglobin, i.e. above 600 nm. The tetrazolium salt MTT and its reduced form MTT-formazan [16] satisfy those two characteristics. The formazan produced by the reaction has a strong violet color indicating that the reactions have taken place.

The second enzymatic reaction has a very rapid kinetics. The whole process is then limited by the first reaction. Interestingly, the NAD is restored by the second reaction. Hence, one can consider that the set of the two reactions is performed with a nearly constant NAD concentration. Remembering that in relation (3) $[B]$ is the NAD concentration, a constant NAD concentration results in a Michaelis-Menten kinetics defined by

$$\frac{d[F]}{dt} = V = \frac{K[E_0][A]}{[A]+K_M} = \frac{V_0[A]}{[A]+K_M}, \quad (12)$$

where $[F]$ denotes the formazan concentration and

$$V_0 = \frac{K_0[E_0]}{1+\frac{K_{MB}}{[B]}} = \frac{K_0[E_0]}{1+\frac{K_{MB}}{[NAD]}} \quad (13)$$

and

$$k_M = \frac{K_{MA}[B]+K_{SA}K_{MB}}{[B]+K_{MB}} = \frac{K_{MA}[NAD]+K_{SA}K_{MB}}{[NAD]+K_{MB}} \quad (14)$$

Using the data found in the preceding section for the different constants, we end up with the value $k_M \sim 11$ mM.

Fig. 7 shows the characteristic Michaelian reactions for five different typical concentrations of β-D-glucose. As expected, the asymptote of the signal occurs later for higher concentrations.

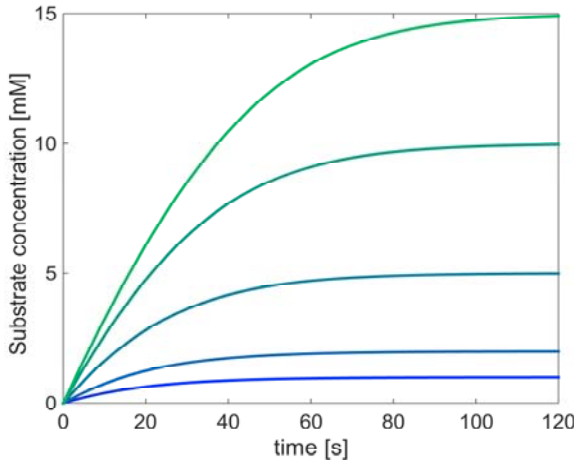

Fig. 7. Michaelis-Menten kinetics for the two coupled reactions (assuming that the second reaction is instantaneous); the initial glucose concentrations are 1, 2, 5, 10 and 15 mM (from bottom to top).

7. Conclusions

In this work, we propose a method for the determination of the kinetic coefficients for the β-D-glucose-NAD-GDH enzymatic ternary reaction, based on the comparison between the theory of bi-substrate enzymatic reaction and experiments.

In order to determine the four constants of the reaction, we place ourselves in a double reciprocal plot approach {*1/[A], 1/V*} where the kinetic curves are linear. Such an approach facilitates the fit of the constants. A reverse reconstruction reproduces the kinetic signals.

The knowledge of the coefficients of the reaction is essential to optimize glucose detection systems. Moreover, the method can be generalized to other bi-substrate reactions.

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