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A method to describe enzyme-catalyzed reactions by combining steady state and time course enzyme kinetic parameters

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ABSTRACT

Background: Complete analysis of single substrate enzyme-catalyzed reactions has required a separate use of two distinct approaches. Steady state approximations are employed to obtain substrate affinity and initial velocity information. Alternatively, first order exponential decay models permit simulation of the time course data for the reactions. Attempts to use integrals of steady state equations to describe reaction time courses have so far met with little success.
Methods: Here we use equations based on steady state approximations to directly model time course plots.
Results: Testing these expressions with the enzyme β-galactosidase, which adheres to classical Michaelis–Menten kinetics, produced a good fit between observed and calculated values.
General significance: This study indicates that, in addition to providing information on initial kinetic parameters, steady state approximations can be employed to directly model time course kinetics.
Integrated forms of the Michaelis–Menten equation have previously been reported in the literature. Here we describe a method to directly apply steady state approximations to time course analysis for predicting product formation and simultaneously obtain multiple kinetic parameters.

1. Introduction

Two general kinds of mathematical relationships have traditionally been employed to describe the nature and progress of enzyme-catalyzed reactions [1]. The first approach involves determining the initial rates of reaction for a variety of known starting substrate concentrations and assumes rapid development of steady state conditions. The relationship between rate and substrate concentration was established in the Michaelis–Menten equation [2] [Eq. (1)].

$$v = V_{max}\left(\frac{[A]_0}{K_m + [A]_0}\right) \tag{1}$$

This classical expression has the advantage of being easily expanded to take into account the effects that modifiers (inhibitors or activators) have on the rate of enzyme-catalyzed reactions [3]. The major disadvantage of this analysis is that, with its focus on initial rates and substrate concentrations, it does not lend itself to the practical aspect of monitoring the time course for substrate decline and product appearance. Attempts have been made to obtain enzyme kinetic time course information such as through integration of the Michaelis–Menten equation [1] [Eq. (2)].

$$V_{max}t = K_A ln\frac{A}{A_0} - (A - A_0) \tag{2}$$

The above integral form of the Michaelis–Menten equation uses the natural logarithm to describe change in substrate concentration as an exponential decrease. This can also be modeled using an exponential decay equation such as the first order chemical kinetic equation [Eq. (3)].

$$A_t = A_0 e^{-kt} \tag{3}$$

In Eq. (3), the exponential decline in substrate concentration (A) with time (t) is related through two constants, the rate constant (k), and Euler's constant ($e = 2.718\ldots$), the base of the natural logarithm. The simplicity of Eq. (3) with its single definitive constant (k) makes it the preferred way to describe reactions that undergo exponential decay. The disadvantage of using Eq. (3) to describe enzyme kinetics is that the rate constant (k) does not relate directly to the important steady state parameters (K_m and V_{max}) described in the Michaelis–Menten equation [Eq. (1)]. Furthermore, attempts to obtain a time course relationship, like that of Eq. (2), by integration of the Michaelis–Menten equation [Eq. (1)] have failed to provide

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expressions that fit experimentally observed data, unless the "constants" are assumed to vary with substrate concentration [4]. Here we present a way of incorporating steady state approximations directly into exponential decay equations and successfully test these equations using, as a model, β-galactosidase, an enzyme that obeys Michaelis–Menten kinetics.

2. Materials and methods

2.1. Materials

Imidazole and o-nitrophenyl β-D-galactopyranoside (ONPG) were from Sigma-Aldrich. β-Galactosidase (EC 3.2.1.23 from *Lactis kluveromyces*), brand name Lactaid, was from McNeil Consumer Healthcare.

2.2. β-Galactosidase activity

The activity of the enzyme β-galactosidase was determined using a modification of the method described by Russo and Moothart [5]. The β-galactosidase stock solution was prepared by dissolving 0.15 mL (3 drops) of Lactaid in 25 mL of distilled water. A 4 M imidazole stock solution was prepared with 0.02 M phosphate, and the pH was then adjusted to 7.5 to account for buffering effects of the imidazole. The assay solution consisted of 1.4 mL of ONPG (0.35 –3.5 mM, final concentration) in 0.02 M phosphate buffer (pH 7.5), 0.05 mL of the same phosphate buffer or 0.05 mL of imidazole (5 –136 mM, final concentration). The reactions were preformed in a quartz cuvette of 1 cm path-length. The reaction was initiated by the addition of 0.05 mL of β-galactosidase stock solution. The rate of change of absorbance (ΔA/min), reflecting the rate of formation of product, o-nitrophenolate, was recorded every 5 s for 1 min using a Milton–Roy uv–visible spectrophotometer set at λ = 420 nm. The extinction coefficient of the product at 420 nm, in 0.02 M phosphate buffer (pH 7.5) was determined via standard curve to be 3658.1 M^{-1} cm^{-1}.

2.3. Global data fitting to test equations

Global data fitting to equations has been shown to be a robust method for testing the ability of a particular equation to describe observed data [6].

Full reaction time course data was fitted to proposed equations using the solver feature of Microsoft Excel [7,8] a program that utilizes root solving methods. The solver feature of Excel was used to vary the values of enzyme constants (K_m and V_{max}), to determine the minimum difference between the observed and calculated values. That is, obtaining the minimum sum of absolute residual values that maximize global data fitting.

3. Results

3.1. Blending steady state and exponential decay equations

Useful information is provided by the steady state approach and equations used to examine time course kinetics, but the extreme diversity and complexity of steady state relationships do not easily lend themselves to integration into time course relationships [4].

Since an enzyme only increases the rate of a chemical reaction, the time course of such a reaction should be described using a first order kinetic equation such as Eq. (3). This exponential equation has more than one constant, e and k, that can just as easily be represented with a single combined constant, $`k = e^{-k}$ as in Eq. (4).

$$A_t = A_0 `k^t \tag{4}$$

The constant ($`k$) in Eq. (4), represents the proportion of the substrate that persists after a defined time interval (t) and would be expressed as a value less than one. While it may be useful to define the remaining reactant after each time period, defining the amount of substrate consumed, which directly relates to product formed, is of more practical value in monitoring enzyme catalysis. To obtain this information, Eq. (4) can be expanded to a form that defines the rate constant ($`k$) in Eq. (4) as the proportion of substrate altered in the initial time interval ($`k$ goes to $1-k$) to give Eq. (5). The constant (k) in this equation can be substituted with an expression involving the initial rate and the initial substrate concentration (k goes to $v/[A]_0$), as defined under the Michaelis–Menten steady state conditions of Eq. (1), to yield Eq. (6).

$$A_t = A_0(1-k)^t \tag{5}$$

$$[A]_t = [A]_0\left(1-\left(\frac{v}{[A]_0}\right)\right)^t \tag{6}$$

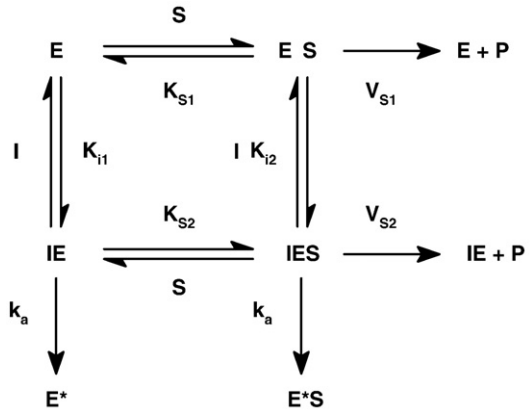
Thus, Eq. (6) represents the substrate concentration at a particular time (A_t) in terms of the initial substrate concentration (A_0) multiplied by a term representing the proportion of the substrate converted to product per time period, raised to the time interval. Since the rate of substrate consumption is defined as the proportion of the initial substrate consumed under steady state conditions, the initial rate term (v) in Eq. (6) can be replaced by the corresponding parameters from the Michaelis–Menten equation [Eq. (1)], to provide an expanded version of Eq. (6), namely, Eq. (7).

$$[A]_t = [A]_0\left(1-\left(\frac{V_{max}\left(\frac{[A]_0}{K_m+[A]_0}\right)}{[A]_0}\right)\right)^t \tag{7}$$

This expression contains the Michaelis–Menten substrate affinity constant (K_m) and maximum velocity (V_{max}), under the conditions, representing steady state parameters, as well as the potential time course evaluation of the enzyme-catalyzed reaction.

3.2. β-Galactosidase time course analysis

The validity of Eq. (7) for monitoring substrate decay/product buildup was first tested using an enzyme, β-galactosidase, that strictly obeys Michaelis–Menten kinetics and is inhibited by imidazole. The equation previously developed [3] to describe this enzyme/inhibitor



Reaction Scheme 1. Reaction schematic for the hydrolysis of substrate (S) by the enzyme β-galactosidase (E). K_{S1} and V_{S1} represent the substrate affinity and maximum reaction velocity in the absence of inhibitor (I), imidazole, while K_{S2} and V_{S2} represent the substrate affinity and maximum reaction velocity induced by the noncovalent interaction of the inhibitor and the enzyme ($K_{i1} = K_{i2}$, represented as K_i in Eqs. (8) and (9)). k_a represents the rate of irreversible inactivation of the enzyme (E*) by imidazole.

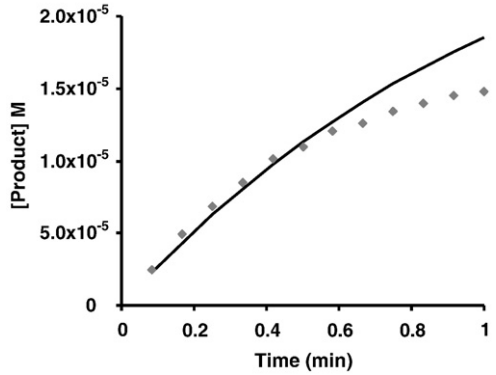


Fig. 1. Hydrolysis of 3.58 mM ONPG by β-galactosidase in the presence of 114 mM imidazole displaying increased inhibition of product formation with time.

system was incorporated into Eq. (6) in a manner similar to that for Eq. (7), but incorporating inhibitor terms, to generate Eq. (8).

$$[S] = [S]_0\left(1 - \frac{\left(V_{S1} - \left((V_{S1} - V_{S2})\frac{[I]}{[I] + K_i}\right)\right)\left(\frac{[S]_0}{[S]_0 + K_{S1} - \left((K_{S1} - K_{S2})\frac{[I]}{[I] + K_i}\right)}\right)}{[S]_0}\right)^t \quad (8)$$

Upon testing, Eq. (7) was able to describe substrate hydrolysis by β-galactosidase in the absence of inhibitor but Eq. (8) was not able to accurately describe the inhibited enzyme activity in the presence of imidazole. An examination of the literature suggested that the failure could be due to imidazole being able to irreversibly inhibit β-galactosidase [9] (Reaction Scheme 1).

This inactivation was also suggested experimentally by a plateau in product formation well below expected values in assays of low substrate and high inhibitor concentration (Fig. 1).

Incorporation of an irreversible inhibition component into Eq. (8) was accomplished by rationalizing the use of the stoichiometry parallel reactions [10]. In parallel reactions, the ratio of the original rates equals the ratio of product formation via each reaction pathway. This required the division of the initial substrate concentration into a fraction that would be hydrolyzed before the enzyme was irreversibly inhibited [Component 1] and a fraction that would not be hydrolyzed [Component 2]. Both of these terms are comprised of the rate of substrate hydrolysis represented by the Michaelis–Menten-like component, and the term for the rate of irreversible enzyme inhibition.

$$[S]_0\left(\frac{\left(V_{S1} - \left((V_{S1} - V_{S2})\frac{[I]}{[I] + K_i}\right)\right)\left(\frac{[S]_0}{[S]_0 + K_{S1} - \left((K_{S1} - K_{S2})\frac{[I]}{[I] + K_i}\right)}\right)}{[S]_0}\right)$$
$$\left(\frac{\left(V_{S1} - \left((V_{S1} - V_{S2})\frac{[I]}{[I] + K_i}\right)\right)\left(\frac{[S]_0}{[S]_0 + K_{S1} - \left((K_{S1} - K_{S2})\frac{[I]}{[I] + K_i}\right)}\right)}{[S]_0} + \left(k_a\frac{[I]}{[I] + K_i}\right)\right) \quad \text{(Component 1)}$$

$$[S]_0\left(\frac{\left(k_a\frac{[I]}{[I] + K_i}\right)}{\left(\frac{\left(V_{S1} - \left((V_{S1} - V_{S2})\frac{[I]}{[I] + K_i}\right)\right)\left(\frac{[S]_0}{[S]_0 + K_{S1} - \left((K_{S1} - K_{S2})\frac{[I]}{[I] + K_i}\right)}\right)}{[S]_0} + \left(k_a\frac{[I]}{[I] + K_i}\right)\right)}\right) \quad \text{(Component 2)}$$

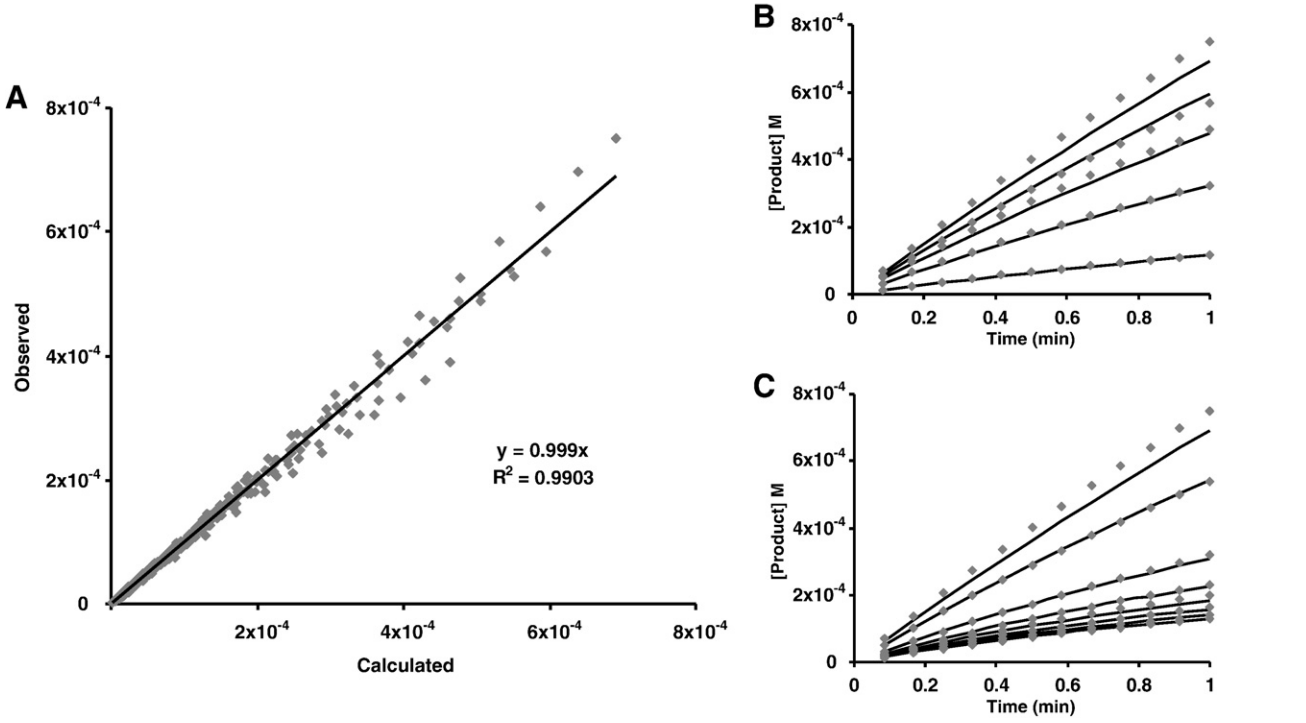


Fig. 2. Global fitting of Eq. (9) to observed experimental data for product formation over time, based on 480 time course points representing ONPG hydrolysis by β-galactosidase in the presence and absence of the enzyme inhibitor imidazole. A) Plot of all the observed product formation data points against their respective calculated product formation values, based on global fitting to the tabulated values (Table 1). The linearity of the best fit line and *R* squared values were used to assess the ability of Eq. (9) to fit the data set as a whole. B) Plot of the observed product formation in the presence of 0.36, 1.16, 1.97, 2.77 and 3.58 mM ONPG over time, overlaid with lines representing the values predicted by the global fitting of Eq. (9). C) Plot of the effect of increasing concentrations of imidazole (0, 5.44, 27.2, 48.9, 70.2, 92.5, 114 and 136 mM) on the hydrolysis of 3.58 mM ONPG over time, overlaid with lines representing the values predicted by the global fitting of Eq. (9).

Table 1
β-Galactosidase imidazole kinetic constants: K_{S1} substrate affinity constant, V_{S1} maximum reaction velocity, K_i inhibition constant, K_{S2} inhibitor induced substrate affinity, V_{S2} inhibitor induced maximum reaction velocity, and k_a irreversible inhibition constant for enzymatic activity.

K_{S1}	V_{S1}	K_i	K_{S2}	V_{S2}	k_a
4.42 mM	1.54 mM/min	33 mM	10.89 mM	0.208 mM/min	0.689A/min

Inserting these components [1 and 2] into Eq. (8) resulted in an equation [Eq. (9)] that divides the initial substrate concentration into the fraction that is hydrolyzed by the enzyme before enzymatic inactivation (Eq. (8) multiplied by Component 1) and the amount of substrate remaining after enzyme inactivation [Component 2]. This equation was found to accurately model β-galactosidase in the absence and presence of imidazole (Fig. 2, Table 1).

$$[S] = [S]_0\left(\frac{\left(V_{S1}-\left((V_{S1}-V_{S2})\frac{[I]}{[I]+K_i}\right)\right)\left(\frac{[S]_0}{[S]_0+K_{S1}-\left((K_{S1}-K_{S2})\frac{[I]}{[I]+K_i}\right)}\right)}{[S]_0}}{\frac{\left(V_{S1}-\left((V_{S1}-V_{S2})\frac{[I]}{[I]+K_i}\right)\right)\left(\frac{[S]_0}{[S]_0+K_{S1}-\left((K_{S1}-K_{S2})\frac{[I]}{[I]+K_i}\right)}\right)}{[S]_0}+\left(k_a\frac{[I]}{[I]+K_i}\right)}\right)^t \times \left(1-\frac{\left(V_{S1}-\left((V_{S1}-V_{S2})\frac{[I]}{[I]+K_i}\right)\right)\left(\frac{[S]_0}{[S]_0+K_{S1}-\left((K_{S1}-K_{S2})\frac{[I]}{[I]+K_i}\right)}\right)}{[S]_0\left(\frac{\left(V_{S1}-\left((V_{S1}-V_{S2})\frac{[I]}{[I]+K_i}\right)\right)\left(\frac{[S]_0}{[S]_0+K_{S1}-\left((K_{S1}-K_{S2})\frac{[I]}{[I]+K_i}\right)}\right)}{[S]_0}+\left(k_a\frac{[I]}{[I]+K_i}\right)\right)}\right) + [S]_0\left(\frac{\left(k_a\frac{[I]}{[I]+K_i}\right)}{\frac{\left(V_{S1}-\left((V_{S1}-V_{S2})\frac{[I]}{[I]+K_i}\right)\right)\left(\frac{[S]_0}{[S]_0+K_{S1}-\left((K_{S1}-K_{S2})\frac{[I]}{[I]+K_i}\right)}\right)}{[S]_0}+\left(k_a\frac{[I]}{[I]+K_i}\right)}\right) \quad (9)$$

4. Discussion

Eq. (7), that combines the steady state assumptions of the Michaelis–Menten equation [Eq. (1)] with the first order chemical kinetic equation [Eq. (3)], provides a basis for describing the steady state parameters (K_m and V_{max}) of an enzyme-catalyzed reaction that obeys Michaelis–Menten kinetics, as well as the time course of product formation in such an enzyme-catalyzed reaction (Fig. 2A and B). Furthermore, this blended expression [Eq. (7)] can be readily expanded to describe the more complex effects of enzyme modifiers [Eq. (8)], and even the effect of irreversible inhibition as seen with the effect of imidazole on β-galactosidase [Eq. (9)] (Fig. 2A and C). This method of equation building may even lend itself to the description of catalysis by enzymes that express more complex kinetic behaviors.

While the first order chemical kinetic equation [Eq. (3)] is believed to be empirically represented by its integration into a form that involves the Euler constant, traditional empirically-derived relationships are not always accurate. The rate of a first order chemical reaction is defined by a constant multiplied by the initial concentration of the reactant [Eq. (10)].

$$v = kA \quad (10)$$

Therefore, this constant is a fraction or percent of the reactant population which will chemically react to produce a product in an observed time period [Eq. (11)].

$$k = \frac{v}{A} \quad (11)$$

The standard integral of this process produces an equation with a base of Euler's constant raised to the rate constant [Eq. (3)]. Therefore, the result of "e" raised to the "$-k$" should be the fraction of the reactant left after an observed time period. This, however, is not the case since inserting the rate constant (k) into the equation containing Euler's constant does not equal the percent reactant left after the observed time period [Eq. (12)].

$$(1-k) \neq e^{-k} \quad (12)$$

This can be demonstrated by calculating the observed change in substrate if one assumes that the consumption rate is 5% ($k=0.05$). After one time period one would assume that 95% of the original substrate would be left using the first order kinetic decay equation [Eq. (3)]. However, this turns out not to be the case since a remaining value of 95.12% is observed. To properly fit to the data using Euler's constant requires an artificially increased "rate constant" $k=0.05129$. While this may not be significant for very low turnover rate the problem becomes more magnified the higher the observable rate. For example if the turnover rate were taken to be 95% ($k=0.95$) the expected substrate remaining after one time period would be 5% of the original. However, when this value is inserted into Eq. (3), a result of 38.67% remains. Indeed, the rate constant (k) must be increased to 2.99573 to produce a remainder of 5% after one time period, or an observable rate of approximately 300%. While Euler's constant can be used in exponential decay equations to model time course kinetics it seems to introduce a systematic error into observed rate constants. This has hindered the use of traditional enzyme kinetic equations which have, until now, been treated as steady state approximations in time course analysis of data. Indeed, the integral form of the Michaelis–Menten equation produces a complex equation that is difficult to solve and has generated much work into developing efficient ways for its modeling [11]. However, the correctness of this integration remains in doubt since data fitting to this equation has consistently been observed to require adjustment of kinetic parameters over the time course of the reaction modeling [4]. Furthermore, non-corrected models of this integrated treatment tend to intercept the time axis rather than approaching it as a limit. This difficulty in modeling, and general inability to predict the behavior of more complex systems, limits the usefulness of the integrated Michaelis–Menten equation.

The equations we propose, which are based on steady state models, are versatile in that they can be adapted to model the effects of enzyme activity modification by inhibitors. The ease with which these non-integrated equations can be used to model the time course of complex enzyme-catalyzed reactions suggests many advantages over integral equations which incorporate Euler's constant. For such reactions, Euler's constant appears not to be a necessity for accurate modeling and may, in fact, obscure relationships between reaction rates and mechanisms.

In conclusion, the non-integrated equation [Eq. (7)] described here is able to model both steady state and time course data in enzyme-catalyzed reactions obeying Michaelis–Menten kinetics. Furthermore, this equation can be expanded to model a variety of conditions, such as those taken into account in Eq. (9), that influence the activity of such enzymes.

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