On a Nonelementary Progress Curve Equation and Its Application in Enzyme Kinetics

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The analytical equation describing progress curves of an enzyme catalyzed reaction acting upon the Michaelis−Menten mechanism has been known for the case in which only the free enzyme incurs a loss of its activity, either spontaneously or as a result of an irreversible inhibitor action. The solution of differential equations which defines the rates of enzyme inactivation and substrate utilization is expressed by a nonelementary function in equation of an implicit type that precludes direct calculation of the extent of reaction at any time. Previously, the implicit equations have been rearranged to the alternative formulas and solved by the Newton−Raphson method, but this procedure may fail when used upon the presented equation. For this reason the other root-finding numerical method was applied, and the enzyme kinetic parameters of such numerically solved implicit equation for the reaction mechanism of irreversibly inhibited acetylcholinesterase were fitted to the experimental data by a nonlinear regression computer program.

1. INTRODUCTION

A system of differential equations must be solved in order to know the time variation of substrate remaining or product formed during the enzyme catalyzed reaction. Such system of differential equations is not readily solved because it is usually nonlinear and in most cases no analytical solution can be obtained.[1−4] Accordingly, an alternative approach must be acquired, and there are two main possibilities which could be taken into account. The transformation of the differential equations describing the reaction mechanism can be achieved in a linear form under adequate conditions,[5,6] but when even this approximately method fails only a numerical integration treatment remains available.[4,7,8] The latter method has been widely used, but it is computationally intensive and therefore relatively slow.[9] Besides, another problem could arise when applying numerical integration calculations in enzyme kinetics. Very low concentrations of the enzyme, the modifier, and the substrate, on one hand, and very high values of the reaction rate constants, on the other hand, make the system of differential equations extremelly stiff.[10] It has been demonstrated recently that the widely used standard Runge−Kutta algorithm could be unsuccessful in such cases.[7]

For these reasons solved analytical solutions of differential equations are still an object of the interest, but it is also true that even the simplest rate equation cannot be integrated to express the dependent variable as an explicit algebraic function of all independent variables.[11] A variety of linear transformations and graphical methods has been proposed to test the applicability of these implicit equations,[12] but the introduction of nonlinear regression has enabled to fit these equations directly on the experimental data using various root-finding numerical methods for calculation of time dependent variable.[13,14] The most widely used root-solving computer program routines are based on the Newton−Raphson method which seems very efficient especially when an alternative formulation of the implicit progress curve equation is applied.[13] In some cases the implicit function's derivative cannot be easily computed, and therefore one of the other root-finding algorithms given in standard text[15] must be availed. Fortunately, the development of highly efficient algorithms makes new approaches in progress curves analysis which are an attractive alternative for parameter estimation in enzyme kinetics.[9]

In the present paper the relation of reaction amounts with respect of time for the reaction catalyzed according to the Michaelis−Menten mechanism with declining free enzyme concentration is defined by the analytical equation and an efficient root-solving numerical method is applied and tested. Both, the mathematical solution and the numerical method used allow progress curve analysis to be applied to unstable enzyme systems. The analysis is verified using the experimental data obtained by the reaction catalyzed by acetylcholinesterase, measured in the presence of an irreversible phosphorylating inhibitor.

2. METHODS AND MATERIALS

Theoretical. The theory is based upon a formal analysis of the case in which only the free enzyme incurs a loss of its activity, either spontaneously or as a result of an irreversible active site directed inhibitor competing with a substrate for the active center. The reaction sequence for the interaction of a substrate with an enzyme following Michaelis−Menten kinetic behavior, where free enzyme concentration decreases with an invariable rate constant, is found in Scheme 1 where E is the enzyme, E' is the inactivated

Scheme 1

$$E' \xleftarrow{i} E + A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EA \xrightarrow{k_{+2}} E + P$$

enzyme, A is the substrate, EA is the enzyme−substrate complex, and P is the product. k_{+1} is a second-order rate

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constant, k_{-1} and k_{+2} are first-order rate constants, and j is a first-order or a second-order rate constant when the conversion of the free enzyme to the inactivated form is spontaneous or the effect of an irreversible inhibitor action, respectively. The mechanism shown in Scheme 1 is described by the following coupled differential equations which define the rates of enzyme inactivation and substrate utilization[11]

$$\frac{d[E']}{dt} = \frac{j([E]_T - [E'])}{\left(1 + \frac{[A]}{K_M}\right)} \quad (1)$$

$$\frac{d[A]}{dt} = \frac{-k_{+1}([E]_T - [E'])[A]}{\left(1 + \frac{[A]}{K_M}\right)} \quad (2)$$

where K_M is the Michaelis constant and $[E]_T$ is the total enzyme concentration. It should be emphasized at this place that the underlying eqs 1 and 2 are valid only when the steady-state treatment to *EA* is assumed. These means that $j \ll k_{+1}[A]$, k_{-1}, k_{+2} and substrate−enzyme ratio is 1000 or greater.[11] While these equations cannot be integrated with respect to time, eq 1 can be divided by eq 2 to give

$$\frac{d[E']}{d[A]} = -\frac{j}{k_{+1}[A]} \quad (3)$$

Eq 3 may be integrated to give an expression relating inactivated enzyme concentration and substrate remaining concentration

$$[E'] = -\frac{j}{k_{+1}} ln\left(\frac{[A]}{[A]_T}\right) \quad (4)$$

where $[A]_T$ stands for the initial concentration of substrate. If the expression on the right side of the latter equation is substituted in eq 2, then the following rate equation is obtained

$$\frac{d[A]}{dt} = -\frac{k_{+1}\left([E]_T + \frac{j}{k_{+1}} ln\left(\frac{[A]}{[A]_T}\right)\right)[A]}{\left(1 + \frac{[A]}{K_M}\right)} \quad (5)$$

Upon the integration, the analytical solution of this differential equation is an implicit function[11]

$$F_1 = ln\left(z + \frac{\beta}{\alpha}\right) - ln\left(\frac{\beta}{\alpha}\right) + \frac{[A]_T}{K_M} e^{-\beta/\alpha}\left(Ei\left(z + \frac{\beta}{\alpha}\right) - Ei\left(\frac{\beta}{\alpha}\right)\right) + \alpha t = 0 \quad (6)$$

where α is j, β is $k_{+1}[E]_T$, z is $ln(1 - [P]_t/[A]_T)$, and $Ei(x)$ is the exponential integral function (see Appendix). $[P]_t$ is the product concentration at time t. It should be emphasized that eq 6 cannot be used when j is equal to zero. In that case the well-known integrated Michaelis−Menten equation is valid[11]

$$F_1^* = K_M\, ln\left(1 - \frac{[P]_t}{[A]_T}\right) - [P]_t + k_{+2}[E]_T t = 0 \quad (7)$$

which is usually rearranged to more appropriate equation defined along the entire substrate concentration interval[13]

$$F_2^* = \left(1 - \frac{[P]_t}{[A]_t}\right) - e^{([P]_t - k_{+2}[E]_T t)/K_M} = 0 \quad (8)$$

Eqs 6 and 7 preclude direct calculation of substrate remaining or product formed at any time. For this reason the dependent variable has to be numerically approximated. The Newton−Raphson method is very efficient when eq 8 is applied as described previously.[13] The integrated Michaelis−Menten equation can be also rewritten such that the resulting expression is explicit in $[A]_t$ and is related with Lambert ω function.[9] Unfortunately, the same procedures are impossible in the case of eq 6. Therefore, two rearrangements have to be done to avoid problems because of logarithmic term and exponential integral function in eq 6:

$$F_2 = z + \frac{\beta}{\alpha}(1 - e^{-[A]_T/K_M e^{-\beta/\alpha}(E_i(z+\beta/\alpha) - E_i(\beta/\alpha)) - \alpha t}) = 0 \quad (9)$$

and

$$F_3 = e^{F_2} - 1 = 0 \quad (10)$$

Only eq 10 is defined on the entire substrate concentration interval (see Figure 1), but instead of the Newton−Raphson method, a root bracketing numerical method must be used for the computation.

The reaction mechanism for the substrate hydrolysis by acetylcholinesterase in the presence of the substrate, in concentration low enough to avoid the substrate inhibition,[7] and the phosphorylating agent can be represented by Scheme 2.

Scheme 2

$$E' \xleftarrow{j} I + E + A \underset{}{\overset{k_{+1}}{\rightleftarrows}} EA \xrightarrow{k_{+2}} E + P_2$$
$$\downarrow$$
$$P_1$$

In this case *EA* stands for the acylated enzyme, *I* is the irreversible inhibitor, *E′* represents the phosphorylated enzyme, kinetic parameter j is a bimolecular rate constant, and k_{-1} is neglibible small and therefore discarded[7,16,17] in comparison with Scheme 1. It should be remembered that the substrate is metabolized into two products P_1 and P_2 during the reaction catalyzed by AChEs. All derived equations are valid for the mechanism shown in Scheme 2, but kinetic parameter j must be multiplied by the inhibitor concentration.

Experimental. Materials. Electric eel acetylcholinesterase (AChE), acetylthiocholine (ATCh), and 5,5′-dithio-bis-nitrobenzoic acid (DTNB) were purchased from Sigma Chemicals Co. (St. Louis, U.S.A.). 7-(Methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) was synthesized according to Levy and Ashani[18] and supplied as a gift from Dr. Didier Fournier (Laboratoire de Synthese et Physicochimie des Molecules d'Interet Biologique, Universite Paul Sabatier, Toulouse, France). The concentration of MEPQ stock solution was determined by absorbance ($\epsilon_{406} = 10.1$ mM^{-1} cm^{-1} in 0.01 M KOH[18]).The initial enzyme active sites concentration was estimated independently by the so-called pseudoirreversible titration, according to the procedure

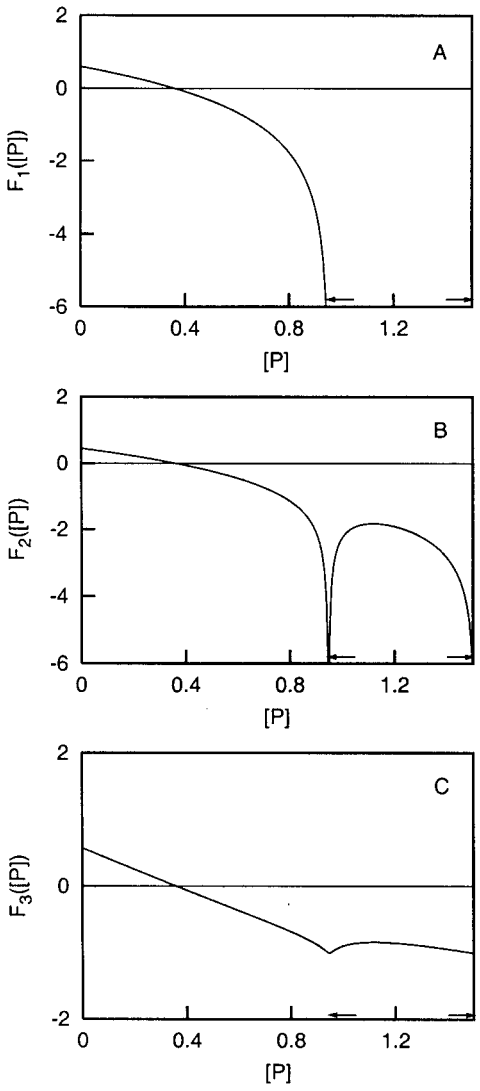

Figure 1. Dependence of F_1, F_2, and F_3 upon [P]. The function which represents (A) F_1 as defined by eq 6, (B) F_2 as defined by eq 9, or (C) F_3 as defined by eq 10 is plotted against [P] for the case where $\alpha = 1$, $\beta = 1$, $K_M = 1.5$, $[A]_T = 1.5$, and $t = 0.6$. Left-handed and right-handed arrows mark concentration values $[A]_T(1 - e^{-k_{+1}[E]_T/j[I]})$ and $[A]_T$, respectively (see Results and Discussion).

described previously.[19] All salts and buffers were of analytical grade. The experiments were carried out at 25 °C in a buffer solution prepared according to Britten and Robinson[20] with a pH of 8.0 and a total ionic strenght 0.2 M, obtained by the addition of NaCl.

Kinetic Measurements. The hydrolysis of ATCh catalyzed by AChE was recorded spectrophotometrically[21] on a stopped-flow apparatus. Equal amounts of the two solutions, one containing the enzyme and the other one inhibitor, ATCh and DTNB, were mixed together in the mixing chamber of the apparatus. Progress curves were recorded until the rate of increase in absorbance became negligible. The concentration of substrate at the begining of the reaction was 50 μM, low enough to prevent substrate inhibition. The concentration of MEPQ at the reaction start were from 0.6 nM to 5 nM and it always exceeded the enzyme concentration (0.06 nM). The inhibitor−enzyme ratios were always high enough that the inhibitor concentrations did not change appreciably as a function of time ($[I]_t \approx [I]_T$).

Data Analysis. The Newton−Raphson method and the root-finding algorithm according to Van Wijngaarden-Dekker-Brent (WDB)[15] have been used for evaluating roots of eq 8 (cf. 13, 14) and eq 10 (cf. 11), respectively. The latter method is efficient and guaranted to converge, so long as the function can be evaluated within the interval for which is known to contain the root. The values of nonelementary function $Ei(x)$ which exists in eq 10 and rapidly converge were calculated with the summation of sufficient number of terms in developed power series to satisfy sufficient accuracy (see Appendix). Data analysis was performed using a modified nonlinear regression computer program.[22] The kinetic parameters were independently calculated also by numerical integration treatment using nonlinear regression program written by Stojan.[7] The double precision arithmetic was used in both cases, and the CPU times needed for the calculation according to one or the other numerical method were compared.

3. RESULTS AND DISCUSSION

The dependence of implicit functions F_1, F_2 and F_3 upon $[P]$ (see eqs 6, 9, 10) is illustrated in Figure 1. It is obviously at the first glance that the Newton−Raphson method based on the derivative calculation is not appropriate root-solving algorithm which could guarantee the convergence and the right root evaluation because of the following reasons: (i) Function F_1 (label A) is not defined for product concentrations greater than $[A]_T(1 - e^{-k_{+1}[E]_T/j[I]})$ (see eq 4), and the derivatives of function in the proximity of this value also becomes undefined. (ii) Function F_2 (label B) is otherwise defined along the entire substrate concentration range except in two points where product concentration is $[A]_T(1 - e^{-k_{+1}[E]_T/j[I]})$ or $[A]_T$. Besides the calculation of the derivatives at values close to these two points is critical, and the problem arises also in local extrema where the derivative is equal to zero. (iii) There is no point within the substrate concentration range where function F_3 (label C) is not defined, but the critical points are local extrema where the derivative is either noncontinuoes and undefined or zero, respectively. It is true that F_3 is close to linear function of $[P]$ around the root of eq 10, and therefore the Newton−Raphson method should converge rapidly to the solution when starting at the right initial estimated value, but the problems could arise when $[P]$ approaches the value $[A]_T(1 - e^{-k_{+1}[E]_T/j[I]})$.

For all these reasons, the divergence-proof root bracketing WDB's method was applied for the root approximation calculated from eq 10. WDB's algorithm is the method of choice to find a bracketed root of a general one-dimensional function (eqs 6, 9, 10) when function's derivative cannot be easily computed (see Figure 1) but the function itself is defined within the interval containing the root (see Figure 1, panel C). The latter method combines the sureness of bisection with the speed of a higher-order method[15] that makes it even much more justified. The accuracy of the WDB's method in describing the product concentration was tested and the relative error was computed as previously described.[9] The experimental determination of product concentration in our study was unlikely to be accurate to more than 100 ppm but the achieved numerical accuracy of computer program using WDB's algorithm was of 10^{-16}. This is not surprising because for most of the root-finding

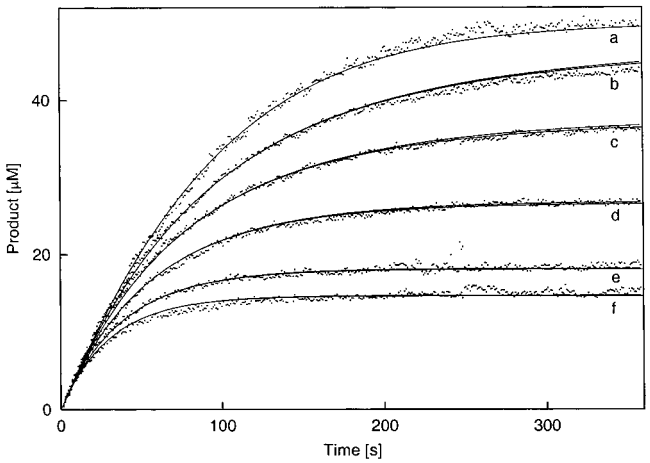

Figure 2. Progress curves for the hydrolysis of acetylthiocholine catalyzed by electric eel AChE in the absence and in the presence of MEPQ. The enzyme concentration is 0.06 nM and the concentrations of MEPQ are as follows: (a) 0, (b) 0.6, (c) 1.2, (d) 2.2, (e) 3.8, and (f) 4.9 nM. Lower solid curves at each experimental progress curve are theoretical obtained by fitting eq 8 to the uppermost experimental curve and eq 10 to all other curves, simultaneously. Nearby upper solid curves (at each experimental curve) represent simulated theoretical reaction time course computed by numerical integration method which take into consideration also depletion of the inhibitor.

methods the accuracy is adjustable parameter and is limited by the lower range of computer precision arithmetic (double precision in the present case). The more convincing reason which argue the advantage of complex mixed analytical/numerical approach (WDB's method) over direct numerical integration is the speed of this method. It has been demonstrated that the computational cost of the direct numerical integration based on a semiimplicit midpoint rule extrapolation method[10] is approximately seven times higher than those of root-finding method according to WDB. The latter numerical integration method has been developed for solving numerical solutions of stiff systems of differential equations[7,10] and due to the nature of this integration method, the stepsize in the integration had to be 10 times smaller than in the original program using WDB's algorithm (see Appendix in ref 7). Unfortunately, for this reason the data analysis applying numerical integration treatment became computationally more intensive and consequently also slower.

The experimental data are presented in Figure 2. A plateau is reached in each individual progress curve but only in the uppermost curve the level of the plateau corresponds to the initial concentration of the substrate used. A special attention should be paid when the integrated Michaelis−Menten equation (eq 7) is fitted to such progress curve because the assumptions for the derivation of steady-state rate equations become violated at complete conversion of substrate.[7,11] This problem can be avoided by very low enzyme concentration as in our experiments. The plateau levels of progress curves recorded in the presence of MEPQ decrease with increasing inhibitor concentrations but it should be emphasized that these plateaus are achieved faster at higher MEPQ concentrations. Another important characteristics of the experimental progress curves is that the initial rate is inhibitor concentration independent. This is the evidence that the reversible enzyme−inhibitor complex does not accumulate and is therefore kinetically invisible.[23,24] This confirms the rightness of the assumed mechanism presented by Scheme 2 for our experimental system. The values for k_{+1}, k_{+2}, and j determined as described in **Data Analysis** are $(2.8 \pm 0.7) \times 10^8$ M^{-1} s^{-1}, $14\,460 \pm 60$ s^{-1}, and $(9.3 \pm 0.3) \times 10^6$ M^{-1} s^{-1}, respectively. It is obvious from Figure 2 that the fitted curves agree very well with the experimental data. The evaluated parameters are also in very good agreement with the values found in the literature.[7,18] Although the constant inhibitor concentration assumption were violated especially at the lowest MEPQ concentration used (0.6 nM $= 10 \cdot [E]_T$), the computation by both numerical treatments according to WDB or numerical integration which took into consideration also the depletion of MEPQ concentration gave nearly the same results. It is also shown in Figure 2 that simulated progress curves calculated by one or the other numerical method do not vary obviously. This fact legitimates mentioned assumption ($[I]_t \approx [I]_T$) and consequently the application of eq 6 in the present studied case.

The kinetic parameter j can be also determined at substrate concentrations high enough to avoid substrate depletion during the measurements when only appropriate initial portions of progress curves are recorded. In this case the progress curves are linear or exponential functions of time when obtained in the absence or presence of the inhibitor, respectively.[23−25] The latter is the consequence of differential equation transformation in the simplified linear form when the substrate concentration can be set constant.[5] However, two main problems must be realized when the experiments are carried on at high substrate concentrations: (i) the Michaelis constant must be known or otherwise experiments must be done at various substrate concentrations and (ii) the substrate inhibition at high substrate concentration can appear as by AChEs and therefore the assumed mechanism shown by Scheme 2 is violated. It should be remembered that the inhibitor concentration and for this reason also inactivation rate must be kept constant in all cases.

The unstability and consequently inactivation is realistic situation by various enzymes. They spontaneously incur a loss of activation, but their substrates in many cases protect them from inactivation. The inactivation rate constant j for spontaneous loss of free enzyme may, of course, be determined also independently by following the loss of enzymatic activity in the absence of substrate, but there is still another reason the progress curve equation (eq 6) seems to be justified. This is the estimation of Michaelis constant. A theoretical approach for describing unstable enzymes has been proposed by Duggleby.[3] It is based on graphical procedures and the secondary so-called $[A]_f$ and J plots are very useful for diagnostic interpretation of individual mechanisms. The advantage of these plots is that the only information needed to construct them are initial and final substrate concentrations (see plateaus in Figure 2). Unfortunetely, the inactivation rate constant j in the mechanism shown by Schemes 1 or 2 can be calculated from J plot only if K_M is known and independently determined.[3,26] The latter is not easy procedure because inactivation of the free enzyme is present also by initial rate measurements especially at low substrate concentrations. Therefore the final results relied on initial velocities may be weak and can lead to the wrong estimation of K_M. Moreover, omitting the entire progress curve, however, means to leave off the most informative portion of the experimental data which enable simultaneous

analysis of both parameters, inactivation rate constant j as well as the Michaelis constant, from the most reliable primary data.

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APPENDIX

Exponential integral function is defined as

$$Ei(x) = \int_{-\infty}^{x} \frac{e^t}{t} dt = C + ln|x| + \sum_{n=1}^{\infty} \frac{x^n}{n \cdot n!} \qquad (11)$$

where C is Euler's constant. The series on the right side is convergent because for every given $\epsilon > 0$ we can find an $N$where the remainder R_N of this series after the Nth term is smaller than ϵ (Cauchy convergence principle). The remainder R_N is smaller than ϵ when the following relation is satisfied

$$N \cdot N! > \frac{x^N}{\left(1 - \frac{x}{N+1}\right) \cdot \epsilon} \qquad (12)$$

and this is also the criterion for number of sufficient terms in partial sum to attain appropriate accuracy.

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