Determination of Michaelis parameters from differentials of progress curves

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First differentials of progress curves are easily obtainable in many enzyme assay systems. Such curves may be more readily applicable to kinetic analysis than are the usual progress curves. The theory for this approach is developed, and simple graphical procedures for the determination of Michaelis parameters are indicated. By using an electronic differentiator device the application of the method is demonstrated on the kinetics of three different serine proteinases with various synthetic substrates. Whenever the steady-state concentration of an intermediate of the reaction is proportional to the rate, the transition of this intermediate in substrate-depletion experiments may be analysed in similar terms. This is demonstrated with cytochrome *c* oxidase kinetics. A number of other possible applications are discussed.

Measurements of initial rates are an established practice in enzyme kinetic studies. This approach is advantageous because of the relative simplicity of the rate equations, and for other reasons such as the benefit of avoiding complicating effects due to the accumulation of products. The use of the entire progress curve in enzyme kinetic studies is, however, attractive, since it should result in greater accuracy, and because, in theory, more information is available from analysis of the curve throughout its full length. It is thus possible, in simple cases, to obtain information about both V and K_m from a single progress curve. The integrated Michaelis–Menten equation, which takes the form of the Henri equation (Henri, 1903) (eqn. 1):

$$V \cdot t = (s_0 - s) + K_m \cdot \ln \left(\frac{s_0}{s} \right) \quad (1)$$

may be used for this purpose. Here s_0 denotes the initial substrate concentration and s is the substrate concentration at time t . The use of eqn. (1) and more elaborate equations of this sort in kinetic studies has been reviewed by Orsi & Tipton (1979). Despite extensive theoretical studies on the progress curve, examples of its use in practice are relatively sparse. It is likely that the cumbersome form of the Henri equation (eqn. 1) and similar integrated rate equa-

tions provides an obstacle to a more widespread exploitation of the progress curve.

In the present paper it is proposed that recording of the first derivative of the progress curve may provide an alternative that is more readily applicable to kinetic analysis than is the usual progress curve. The theory for this approach is developed, and simple graphical procedures for the determination of Michaelis parameters are indicated. The application of the method is demonstrated with a few examples.

Materials and methods

Human Glu-plasminogen was prepared by affinity chromatography on lysine–Sepharose followed by gel filtration on Ultrogel AcA-44 as described by Thorsen *et al.* (1981). Human urokinase was from Leo Pharmaceuticals, Copenhagen, Denmark, and pig trypsin was from Novo, Bagsvaerd, Denmark. Human thrombin [3200 NIH (National Institutes of Health) units/mg] was a gift from Dr. W. Lawson (New York State Department of Health, Albany, NY, U.S.A.) to Dr. I. Clemmensen of this laboratory. Val-Leu-Lys-Nan, 2HCl (substrate S2251) and Phe-Pip-Arg-Nan, HCl (substrate 2238) was from Kabi A.B., Stockholm, Sweden. Tosyl-Gly-Pro-Arg-Nan, CH₃CO₂H (Chromozym-TH) was from Boehringer, Mannheim, Germany. Ox heart cytochrome *c* oxidase was prepared by the procedure described by van Buuren (1972) (Hartzell *et al.*, 1978). Horse heart cytochrome *c* was type VI from Sigma Chemical Co., St. Louis, MO, U.S.A. Benz-

Abbreviations used: Val-Leu-Lys-Nan, D-valyl-L-leucyl-L-lysine 4-nitroanilide; Phe-Pip-Arg-Nan, D-phenylalanyl-L-pipecolyl-L-arginine 4-nitroanilide; Tos-Gly-Pro-Arg-Nan, tosylglycyl-L-prolyl-L-arginine 4-nitroanilide.

amidinium chloride and ascorbic acid were from Merck, Darmstadt, Germany.

The cleavage of the chromogenic peptide substrate was measured at 410 nm, and the steady-state oxidation of cytochrome *c* was measured at 550 nm with a Beckman Acta VI spectrophotometer. First differentials of progress curves were obtained by using the electronic differentiator module of the spectrophotometer.

Proteinase experiments were performed in 0.1 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, at 25°C. Cytochrome *c* oxidase kinetics were studied in 100 mM-potassium phosphate buffer, pH 7.4, containing 0.5% Tween 80, at 25°C. A manual mixing device described in a previous paper (Petersen & Clemmensen, 1981) was used to obtain rapid and efficient mixing. Areas under curves were determined by weighing graph paper.

Theory

Michaelis-Menten kinetics

The first derivative of the progress curve is obtained by insertion of the Michaelis equation (eqn. 2):

$$v = \frac{Vs}{K_m + s}; \quad s = K_m \cdot \frac{v}{V-v} \quad (2)$$

in eqn. (1), which yields eqn. (3):

$$\frac{V}{K_m} \cdot t = \frac{V}{V-v_0} - \frac{V}{V-v} + \ln \left(\frac{v_0}{V-v_0} \cdot \frac{V-v}{v} \right) \quad (3)$$

A graphical presentation of eqn. (3) is shown as the full continuous line in Fig. 1. Two features of this curve may be used to determine kinetic parameters, as follows. (i) The amount of substrate left at any moment of the reaction is equal to the area described by the integral:

$$s = \int_t^\infty v \cdot dt \quad (4)$$

and (ii) the slope of the curve, $\dot{v}$ (the second derivative of the progress curve), is related to v by eqn. (5), derived by differentiation of eqn. (2) with respect to time:

$$\dot{v} = -v(V-v)^2/V \cdot K_m \quad (5)$$

At $v = V/3$ the slope can be shown to have its maximum value:

$$\dot{v}_{\max.} = -\frac{4}{27} \cdot \frac{V^2}{K_m} \quad (6)$$

A satisfactory accurate estimate of this value may be obtained graphically by drawing the tangent through the inflexion point, as shown in Fig. 1. The area

enclosed by this line and the rate curve (indicated on Fig. 1 by the hatched area) can be shown to be:

$$\Delta A^* = K_m/8 \quad (7)$$

Competitive inhibition

The first and second derivatives of the progress curve are obtained by replacing K_m in eqns. (3) and (5) with $K_m(i+K_i)/K_i$, which yields eqns. (8) and (9):

$$\frac{K_i}{(K_i+i)} \cdot \frac{V}{K_m} \cdot t = \frac{V}{V-v_0} - \frac{V}{V-v} + \ln \left(\frac{v_0}{V-v_0} \cdot \frac{V-v}{v} \right) \quad (8)$$

$$\dot{v} = -v \cdot \frac{(V-v)^2}{V \cdot K_m} \cdot \frac{K_i}{(K_i+i)} \quad (9)$$

It follows that:

$$\dot{v}_{\max.} = -\frac{4}{27} \cdot \frac{V^2}{K_m} \cdot \frac{K_i}{K_i+i} \quad (10)$$

and

$$\Delta A^* = \frac{K_m}{8} \cdot \frac{K_i+i}{K_i} \quad (11)$$

Competitive product inhibition

Accumulation of competitive inhibitory products is often met in experiments where the consumption of substrate is followed to depletion. The rate equation for competitive product inhibition is given by eqn. (12):

$$v = \frac{V \cdot s}{K_m[1 + (s_0-s)/K_i] + s} \quad (12)$$

and the corresponding progress curve is described by eqn. (13) (see, e.g., Orsi & Tipton, 1979):

$$V \cdot t = \frac{K_i - K_m}{K_i} \cdot (s_0 - s) + \frac{K_i + s_0}{K_i} \cdot K_m \cdot \ln \left(\frac{s_0}{s} \right) \quad (13)$$

Differentiation of eqn. (12) yields eqn. (15):

$$\dot{v} = -v \cdot \left(V - v \cdot \frac{K_i - K_m}{K_i} \right)^2 \cdot \frac{K_i}{K_i + s_0} \cdot \frac{1}{V \cdot K_m} \quad (14)$$

with a maximum slope [at $v = V \cdot K_i/3(K_i - K_m)$] of:

$$\dot{v}_{\max.} = -\frac{4}{27} \cdot \frac{V^2}{K_m} \cdot \frac{K_i}{K_i + s_0} \cdot \frac{K_i}{K_i - K_m} \quad (15)$$

and

$$\Delta A^* = \frac{K_m}{8} \cdot \frac{K_i + s_0}{K_i - K_m} \quad (16)$$

Results

First differentials of progress curves in proteinase kinetics

Fig. 1 shows an experiment where the plasmin-catalysed cleavage of Val-Leu-Lys-Nan is followed by measuring the rate of 4-nitroaniline formation at 410nm. Direct monitoring of the first differential of the progress curve is obtained by means of an electronic differentiator device. Thus the curve shown in Fig. 1 represents the rate as a function of time. The decline in rate as a consequence of substrate depletion attains a maximum value, $\dot{v}_{\max}$, that can be estimated graphically as indicated. Measurement of the area enclosed by this line and the rate curve provides an estimate of K_m ($\Delta A^* = K_m/8$). Also shown in Fig. 1 is the theoretical curve of rate plotted against time obtained from eqn. (3) assuming $V = 2.9 \mu\text{M/s}$ and $K_m = 0.123 \text{ mM}$.

First differentials of progress curves were recorded at a fixed concentration of substrate, but with various concentrations of enzyme. The total area enclosed by the rate curve is equal to s_0 . This area remains constant, whereas the sharpness of the rate curve increases with an increasing enzyme concentration. Values of ΔA^* and $\dot{v}_{\max}$, determined from such recording of first differentials of progress curves of the thrombin-, trypsin- and plasmin-catalysed cleavage of chromogenic peptide substrates are shown in Fig. 2. With all three enzymes the value of ΔA^* remains essentially constant over the enzyme concentration range tested, and the K_m values determined this way are compared with those

Fig. 2. Curve area, ΔA^* , and slope maximum, $\dot{v}_{\max}$, obtained from first differentials of progress curves measured at various concentrations of proteolytic enzymes

First differentials of the progress curves of the plasmin-catalysed hydrolysis of $205 \mu\text{M}$ -Val-Leu-Lys-Nan (O), of thrombin-catalysed hydrolysis of $50 \mu\text{M}$ -Tos-Gly-Pro-Arg-Nan (□) and of trypsin-catalysed hydrolysis of $120 \mu\text{M}$ -Tos-Gly-Pro-Arg-Nan (Δ) were recorded as described in Fig. 1. From these curves ΔA^* and $\dot{v}_{\max}$ were determined graphically as described in the text. (a) ΔA^* as a function of the initial rate, v_0 , obtained at various concentrations of proteolytic enzymes. (b) v_0^2 as a function of $\dot{v}_{\max}$. Plasmin concentration was varied from 0.4 to $1.0 \mu\text{M}$, thrombin concentration from 7.3 to 29 nM and trypsin concentration from 22 to 56 nM . Other conditions were as described in the text.

Fig. 1. First differential of the progress curve of plasmin-catalysed Val-Leu-Lys-Nan degradation. Buffer (1 ml) containing $1.2 \mu\text{M}$ -plasmin was mixed with an equal volume of buffer containing 0.36 mM -Val-Leu-Lys-Nan. The rate was monitored and recorded as described in the text. The curve produced in this way is compared with the theoretical curve (full continuous line) described by eqn. (3) assuming $V = 2.9 \mu\text{M/s}$, $v_0 = 1.8 \mu\text{M/s}$ and $K_m = 0.123 \text{ mM}$.

determined by initial-rate methods (Table 1). Also shown in Fig. 2 is a plot of the square of the initial rate (v_0^2) against $\dot{v}_{\max}$, obtained at various

Table 1. Comparison of K_m values obtained from first differentials of progress curves with those obtained by initial-rate methods

Substrate	Enzyme	K_m (μM)		K_i (mM)
		Initial-rate methods	Present study*	Present study
Val-Leu-Lys-Nan	Plasmin	120†	123 ± 3	>2
Tos-Gly-Pro-Arg-Nan	Trypsin	5.0‡	9.5 ± 0.6	0.15
Phe-Pip-Arg-Nan	Thrombin	1.3§	1.3 ± 0.6	0.015
Tos-Gly-Pro-Arg-Nan	Thrombin	5.0§	14 ± 1	0.070

* Estimated values obtained from plots such as that shown in Fig. 4.

† Christensen & Ipsen (1979).

‡ L. C. Petersen (unpublished work).

§ Lottenberg *et al.* (1982).

enzyme concentrations. The data fit a straight line through the origin, as expected from eqn. (6). The theory predicts that the slope of this line converges towards a limiting value of $6.75 K_m$ with increasing s_0 as v_0 approaches V .

Effect of competitive inhibition by benzamidine

Benzamidine is known to be a competitive inhibitor of various serine proteinases, including trypsin (Mares-Guia & Shaw, 1965; Markwardt *et al.*, 1968). In theory, the presence of benzamidine should therefore affect the first differential of the progress curve as predicted from eqns. (10) and (11). This is confirmed by the results shown in Fig. 3, where data from progress curves obtained at various concentrations of benzamidine are analysed. The plots of ΔA^* and $1/\dot{v}_{\max}$ against inhibitor concentration each fit a straight line with an intercept on the horizontal line of $-K_i = 17 \mu\text{M}$, in agreement with the K_i value obtained by initial-rate methods (Mares-Guia, 1968). Higher apparent K_i values for benzamidine inhibition are obtained with high s_0 values, probably because of competition between inhibitor and product.

Effect of product inhibition

In the absence of product inhibition first differentials of progress curves obtained at various values of s_0 are expected to be superimposable. Experimentally it is found that product inhibition is insignificant by this criterion with plasmin-catalysed cleavage of Val-Leu-Lys-Nan. However, significant effects of products on the shape of the curve are observed with the trypsin-catalysed cleavage of Tos-Gly-Pro-Arg-Nan and the thrombin-catalysed cleavage of Phe-Pip-Arg-Nan and Tos-Gly-Pro-Arg-Nan. The effects of s on both ΔA^* and $1/\dot{v}_{\max}$ are shown in Fig. 4 for the thrombin-catalysed cleavage of Tos-Glu-Pro-Arg-Nan. As predicted from eqns. (15) and (16), the data fit a straight line, and it is possible to obtain the extrapolated value of

Fig. 3. Effect of benzamidine on the first differential of the progress curve of trypsin-catalysed Phe-Pip-Arg-Nan hydrolysis

The reaction mixture contained $40 \mu\text{M}$ -Phe-Pip-Arg-Nan, 40 nM -trypsin and various concentrations of benzamidine. Other conditions were as described in the text. ΔA^* (O) and $1/\dot{v}_{\max}$ (Δ) are plotted as a function of the concentration of benzamidine.

K_m in the absence of product inhibition, and also to obtain K_i for this inhibition. These values are listed in Table 1.

Transients in the steady-state cytochrome *c* oxidation level on oxygen depletion in cytochrome *c* oxidase kinetics

The application of the method to measure the apparent K_m for oxygen of cytochrome *c* oxidase is demonstrated in Figs. 5 and 6. The steady-state kinetics of the system:

are studied by measuring the steady-state oxidation level of cytochrome *c* during an oxygen-depletion experiment (Fig. 5). Addition of $120 \mu\text{M}$ -oxygen to an anaerobic solution containing oxidase, cytochrome *c* and ascorbate produces a transient in the steady-state oxidation of cytochrome *c* monitored at 550 nm . The oxidation of cytochrome *c* on addition

of oxygen is much faster than can be observed with the present set-up, but the aerobic-anaerobic transition of the system on oxygen depletion is apparent as a sigmoidal curve. Such curves obtained at various concentrations of oxidase are shown in Fig. 5. The presence of ascorbate at relatively high concentrations in the enzyme assay system involves two important implications, as follows.

(i) The reducing substrate is regenerated and accumulation of product is strongly diminished owing to the reaction:

(ii) The rate of cytochrome *c* reduction is given by eqn. (17):

$$v = k_r \cdot [\text{ascorbate}][\text{cyt.}c^{3+}] \quad (17)$$

which means that in practice the rate is proportional to the cytochrome *c* oxidation level monitored at 550 nm.

The curves presented in Fig. 6 are therefore equivalent to first differentials of progress curves such as that shown in Fig. 1. The maximal rate, V , can be calculated from $\Delta A_{550}(\text{max.})$, or the anaerobiosis time, and $\dot{v}_{\text{max.}}$ can be obtained as the maximal slope of the aerobic-anaerobic transition at 550 nm. The anaerobiosis time, t_a , is here defined as the time interval from oxygen addition to the initiation of the transient in the cytochrome *c* steady-state level ($V = 120/t_a \mu\text{M/s}$).

Fig. 4. Effect of product inhibition on first differentials of progress curves of thrombin-catalysed degradation of Tos-Gly-Pro-Arg-Nan

The reaction mixture contained 22 nM-thrombin and various concentrations of chromogenic substrate. ΔA^* (○) and $1/\dot{v}_{\text{max.}}$ (Δ) are plotted as a function of the initial concentration of substrate, s_0 .

Fig. 5. Transient in cytochrome *c* steady-state oxidation level during oxygen depletion in cytochrome *c* oxidase kinetics

Buffer (1.1 ml) containing 73 nM-ascorbate, 73 μM -cytochrome *c* and (a) 0.13 μM -, (b) 0.25 μM -, (c) 0.51 μM - or (d) 1.0 μM -cytochrome *c* oxidase was allowed to go anaerobic. It was then mixed with an equal volume of air-equilibrated buffer, and the aerobic-anaerobic transition measured at 550 nm was followed.

Fig. 6. Square of the maximal rate, V^2 , as a function of the maximal slope, $\dot{v}_{\text{max.}}$, of the aerobic-anaerobic transition in cytochrome *c* steady-state oxidation level V^2 and $\dot{v}_{\text{max.}}$ were obtained from a number of experiments, such as those shown in Fig. 5, where the concentration of cytochrome *c* oxidase was varied. Results obtained with both 37 mM- (○) and 73 mM-ascorbate (●) are shown. The final concentration of cytochrome *c* oxidase after mixing was varied between 0.035 and 0.70 μM . Other conditions were as described in Fig. 5.

Discussion

An electronic differentiator circuit, which is easily constructed, provides a simple data-processing unit that can be applied in studies of a great number of enzyme reactions to obtain a direct monitoring of the catalytic rate. Such devices have only been applied previously for restricted purposes, for ex-

ample to observe respiratory-control phenomena in mitochondrial respiration (Holian *et al.*, 1977) and in kinetic studies on the progressive inhibition of tight-binding inhibitors (Petersen & Clemmensen, 1981; Petersen & Jørgensen, 1983). The present study suggests a more general application of such a differentiator device as a tool to allow a better exploitation of the information contained in the progress curve. Application of digital computers for data collection and data processing opens the possibility for curve-smoothing and differentiation, for example in accordance with the procedure suggested by Savitzky & Golay (1964). Such improvements are likely to increase the potential of the method in practice.

A priori the enzymes investigated in the present study are known to obey Michaelis–Menten kinetics. This is also indicated by the close fit of the experimentally obtained data to the theoretical curve shown in Fig. 1. Procedures for a convenient test for the fit of experimental data to the derivatives of the Michaelis–Menten equation have not been developed here; however, with on-line computation the fit may be evaluated by means of polynomial regression analysis by using, for example, eqns. (5) and (14). It is demonstrated that the first differential of the progress curve can be used to obtain a graphical determination of Michaelis–Menten parameters. It should be stressed that it is not usually satisfactory to rely on a single set of initial-rate conditions with respect to enzyme concentration and substrate concentration. Thus the advantage of the method compared with the conventional initial-rate method in terms of the number of experiments required may be marginal. The method is rather a supplement to initial-rate methods, and may be considered clearly advantageous under special conditions. A few such examples are considered in the following.

The determination of initial rates may be associated with considerable error, particularly in kinetic experiments on enzymes with very high affinity for substrate. This is because it takes a certain amount of substrate to measure an accurate rate, and that this amount may not be small compared with the K_m of the enzyme. This situation is characterized by considerable curvature of rate curves, and naturally calls for analysis in terms of progress curves. One such analysis is the direct-linear-plot method (Cornish-Bowden, 1975), which may be used under these conditions to obtain more reliable initial-rate estimates (see, e.g., Lottenberg *et al.*, 1982). The method based on first differentials of progress curves provides a different approach to the problem, which could be used as an alternative or a supplement to the present available methods. The results presented in Table 1 indicate that it is possible with nitro-anilide substrates to obtain estimates of K_m values down to about 10^{-6} M, where it is difficult to

establish the kinetics with conventional methods (Griffith *et al.*, 1979, 1980; Lottenberg *et al.*, 1982). The K_m values obtained from first differentials of progress curves agree reasonably well with K_m values obtained by initial-rate methods. The fact that some discrepancy is observed with Tos-Gly-Pro-Arg-Nan may be due to differences in the experimental conditions, or due to substrate impurities with a lower affinity for the enzyme. The method of estimating K_m from ΔA^* is likely to be quite sensitive to the latter type of artifacts.

It is important also with the present method to consider the effect of product inhibition. Product inhibition affects the shape of the first differential of the progress curve (eqn. 14). With $K_i > K_m$ it is possible to correct for this effect, as indicated in Fig. 4, but with $K_i \leq K_m$ the curve is not sigmoid any more, and the graphical procedure of drawing the tangent through the point of inflexion cannot be applied.

Usually the purpose of a coupled enzyme assay is to transform the primary products of the reaction under investigation into compounds that are more readily monitored. Auxiliary enzymes for this transformation are present at concentrations that imply the removal of primary products in an apparent first-order reaction. Thus the steady-state concentration of product is proportional to the reaction rate. This means that, in addition to the conventional application in a coupled enzyme assay, auxiliary enzymes may also be used as chemical differentiators (in the case of primary products that can easily be monitored), and the time course of the steady-state concentration of products may be analysed as indicated in the present study.

An example of such transitions in the steady-state concentration of product is given by the results obtained with the cytochrome *c* oxidase system (Figs. 5 and 6), although the removal of product in a first-order reaction is in this case obtained by means of a reducing agent, not by an auxiliary enzyme system. Because cytochrome *c* oxidase is a two-substrate enzyme, it is of importance to aim at a set of experimental conditions such that the concentration of one substrate, reduced cytochrome *c*, is essentially constant when measuring the progress curve of the activity during the depletion of the other substrate, oxygen. This is obtained by the addition of relatively high concentrations of ascorbate and cytochrome *c*. The reaction with ascorbate brings about the regeneration of reduced cytochrome *c*, and it ensures that the steady-state concentration of reduced cytochrome *c* is decreased maximally by 25% as a consequence of the oxidase activity in the presence of oxygen. Since water is the second product of the reaction, product-inhibition effects should therefore be of minor importance. The fact that s_0 is about 100 times K_m provides an accurate estimate of

V , which can be used to obtain K_m according to eqn. (6) (Fig. 6).

The plot shown in Fig. 6, which yields a K_m for oxygen of $1.1\mu\text{M}$, suggests that this procedure of obtaining the K_m value is in fact practicable under these conditions. However, the ΔA^* procedure is difficult to apply to the cytochrome *c*-transient results, primarily because the area corresponding to $K_m/8$ is very small compared with the total area; the procedure is also complicated by a slow but relatively small change in absorbance at 550 nm, the origin of which is unclear at the moment.

Data obtained by calorimetry of enzyme-catalysed reactions may be treated in a similar fashion. In all cases it is important that the transition time of the auxiliary system is short compared with that induced by substrate depletion.

Metabolic systems *in vivo* may have properties that make them kinetically similar to coupled enzyme assay systems; and this could provide a framework for the determination of Michaelis-Menten parameters from the measurement of progress curves of intermediates during a substrate-depletion experiment.

In enzyme kinetic studies it is possible with a small number of enzymes to take advantage of the fact that Nature has endowed them with spectral properties, such that the complex-formation between substrate and enzyme can be monitored directly. Recording of progress curves for these enzyme species provides the basis for the method proposed by Chance (1943). After mixing with substrate the enzyme-substrate complex is monitored until exhaustion of substrate takes place. The method has been applied to study the kinetics of peroxidase (Chance, 1943) and glucose oxidase (Gibson *et al.*, 1964). In principle, the curves obtained in this way are closely similar to the first differentials of substrate/product curves, and the theory in the present study can be easily modified to account for the progress curves of such enzyme species.

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