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A Theoretical Validation of the Substrate Depletion Approach to Determining Kinetic Parameters

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Abstract

The substrate depletion approach is an increasingly popular alternative to the traditional method (observing product formation) of determining the kinetic parameters (K_M and V_{\max}) of an enzyme. Obach and Reed-Hagen (2002) used an empirical relationship between substrate depletion rate constants and initial substrate concentration to determine kinetic parameters for a number of cytochrome P450-catalyzed reactions. We present a proof that this relationship can be derived from the Michaelis-Menten equation, and therefore that kinetic parameters obtained by the substrate depletion approach are equivalent and comparable to those obtained by the traditional product formation approach. Analysis of a simulated data set produced similar kinetic parameters regardless of which approach was used, confirming the theoretical result.

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Introduction

Traditionally, the kinetic behavior of an enzyme is characterized by monitoring rates of product formation at various substrate concentrations. However, it is also possible to determine the kinetic parameters of an enzymatic reaction by monitoring depletion of substrate as a function of time, for use in situations where experimental considerations make it impossible or impractical to monitor product formation. Substrate depletion is increasingly widely used to characterize cytochrome P450 kinetics (Obach and Reed-Hagen, 2002; Isoherranen *et al.*, 2004; Jones and Houston, 2004; Komura and Iwaki, 2005; Mohutsky *et al.*, 2006). Obach and Reed-Hagen proposed the following empirical equation to obtain the Michaelis constant, K_M :

$$k_{dep} = k_{dep([S] \rightarrow 0)} \cdot \left(1 - \frac{[S]}{[S] + K_M} \right) \quad (1)$$

where k_{dep} is the apparent first-order rate constant of substrate depletion, $[S]$ is the substrate concentration and $k_{dep([S] \rightarrow 0)}$ is the theoretical k_{dep} at infinitesimally low substrate concentrations. Since this is an empirical relationship and not derived from first principles, the K_M values obtained from the substrate depletion approach are not necessarily equivalent to those obtained from the traditional product formation approach. Here, we present a proof of this formula from the Michaelis-Menten equation, and thereby demonstrate that kinetic parameters obtained from the substrate depletion approach can be meaningfully compared to those obtained by monitoring product formation.

Scheme 1 shows the conversion of a substrate S to a product P by enzyme E , via an intermediate enzyme-substrate complex ES . The following rate equations apply to this system:

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$$\begin{aligned}\frac{d[S]_t}{dt} &= -k_1[E]_t[S]_t + k_{-1}[ES]_t \\ \frac{d[ES]_t}{dt} &= k_1[E]_t[S]_t - k_{-1}[ES]_t - k_{cat}[ES]_t \\ \frac{d[P]_t}{dt} &= v = k_{cat}[ES]_t\end{aligned}\quad (2)$$

Note that $[S]_t$, $[E]_t$, $[ES]_t$ and $[P]_t$ refer to the transient concentrations of each species at time t , while unsubscripted symbols refer to their initial concentrations. When substrate depletion displays apparent first-order kinetics, substrate concentration as a function of time can be described by the following single exponential:

$$\begin{aligned}[S]_t &= [S]e^{-k_{dep}t} \\ \ln[S]_t &= \ln[S] - k_{dep}t\end{aligned}$$

Taking the first derivative:

$$\begin{aligned}\frac{d[S]_t}{[S]_t} &= -k_{dep}dt \\ \frac{d[S]_t}{dt} &= -k_{dep}[S]_t\end{aligned}\quad (3)$$

We now assume that the concentration of the ES complex is constant. Under this steady-state approximation,

$$\begin{aligned}\frac{d[S]_t}{dt} &= -\frac{d[P]_t}{dt} \\ -k_{dep}[S]_t &= -v \\ k_{dep} &= \frac{v}{[S]_t}\end{aligned}\quad (4)$$

Close to the start of a reaction, when $[S]_t \approx [S]$, equation (4) gives us:

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$$k_{dep} = \frac{v}{[S]_t} \approx \frac{v}{[S]} \quad (5)$$

Note that this assumption, that transient substrate concentration is almost equal to the initial substrate concentration, corresponds to the assumption in the traditional approach that the rate of product formation is linear at the beginning of a reaction (Segel, 1975). This assumption means that only data from the beginning of each reaction (where $[S]_t$ is close to its initial value) should be used in the calculation of k_{dep} . Substituting equation (3) into the rate equations gives us:

$$\begin{aligned} -k_{dep} [S]_t &= -k_1 [E]_t [S]_t + k_{-1} [ES]_t \\ \Rightarrow k_{dep} &= k_1 [E]_t - \frac{k_{-1}}{[S]_t} [ES]_t = k_1 [E]_t - \frac{k_{-1}}{[S]_t} \cdot \frac{k_1 [E]_t [S]_t}{k_{-1} + k_{cat}} \\ \Rightarrow k_{dep} &= k_1 [E]_t \left(1 - \frac{k_{-1}}{k_{-1} + k_{cat}} \right) = k_1 [E]_t \left(\frac{k_{cat}}{k_{-1} + k_{cat}} \right) = \frac{k_{cat} [E]_t}{\frac{k_{-1} + k_{cat}}{k_1}} = \frac{k_{cat} [E]_t}{K_M} \end{aligned} \quad (6)$$

where $K_M = (k_{-1} + k_{cat})/k_1$ (Segel, 1975). At infinitesimally low substrate concentrations, the transient free enzyme concentration will be equal to the initial concentration of total enzyme:

$$\begin{aligned} k_{cat} [E]_t &= k_{cat} [E] = V_{\max} \\ k_{dep([S] \rightarrow 0)} &= \frac{V_{\max}}{K_M} \end{aligned} \quad (7)$$

The Michaelis-Menten equation typically takes the form:

$$v = \frac{V_{\max} [S]}{K_M + [S]}$$

This can be rearranged to give a form analogous to equation (1):

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$$\frac{v}{[S]} = \frac{V_{\max}}{K_M} \cdot \left(\frac{K_M}{K_M + [S]} \right) = \frac{V_{\max}}{K_M} \cdot \left(1 - \frac{[S]}{[S] + K_M} \right) \quad (8)$$

Substituting (5) and (7) into equation (8) gives us Obach and Reed-Hagen's original equation:

$$k_{dep} = k_{dep([S] \rightarrow 0)} \cdot \left(1 - \frac{[S]}{[S] + K_M} \right) \quad (1)$$

This demonstrates that the substrate depletion approach to determining reaction parameters is equivalent to the traditional approach of monitoring product formation: therefore, values for K_M and V_{\max} obtained from the two different approaches can be meaningfully compared. In order to verify this equivalence, we analyzed a simulated data set using the substrate depletion approach and the traditional product formation approach, and compared the recovered kinetic parameters. Using a simulated data set instead of experimental data allows the rigorous comparison of these two approaches in the absence of the complexities inherent in a real enzymatic reaction.

Methods

Simulations of the system described in Scheme 1 were performed using a script in the Python programming language. Transient concentrations of the various species were governed by the differential rate equations (2), with $k_I = 10^5 \text{ M}^{-1}\text{s}^{-1}$, $k_{-I} = 1 \text{ s}^{-1}$ and $k_{cat} = 0.1 \text{ s}^{-1}$, and an initial enzyme concentration of 100 nM. The resulting (calculated) K_M and V_{\max} for this system are 11 μM and 10 nM s^{-1} respectively. Six substrate concentrations, from 10 nM to 1 mM, were simulated, and each simulation covered 4000 seconds.

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Substrate Depletion Approach:

The equation $[S]_t/[S] = e^{-k_{dep}t}$ was fit to the first 100 seconds of the transient substrate concentration curves (see Fig. 1 a)) to obtain k_{dep} values for the various initial substrate concentrations. (The transient substrate concentration was within 10% of the initial concentration for all six simulations over this time period.) Equation (1) was fit to the k_{dep} values to obtain $k_{dep([S] \rightarrow 0)}$ and K_M (see Fig. 1 b)). V_{max} was calculated using equation (7).

As an alternate method of analysis, equation (5) was used to calculate velocity values for the various substrate concentrations. These data were then fit to the Michaelis-Menten equation to obtain K_M and V_{max} directly (see Fig. 1 c), black circles).

Product Formation Approach:

The slope of the transient product concentration curves over the first 100 seconds of each simulation was used to obtain the reaction velocities at the various substrate concentrations. These values were fit to the Michaelis-Menten equation to obtain K_M and V_{max} directly (see Fig. 1 c), grey squares).

Results and Discussion

Substrate depletion curves can be used to obtain first-order substrate depletion rate constants (k_{dep}) for various initial substrate concentrations (see Fig. 1 a)). The relation between these rate constants and the substrate concentration (equation (1)) depends on the Michaelis constant K_M , and the theoretical rate constant at infinitesimally low substrate concentrations $k_{dep([S] \rightarrow 0)}$. Substrate depletion rate constants at a range of substrate concentrations (Table 1) were used to

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determine K_M and V_{\max} for a simulated data set. When k_{dep} data were fit to equation (1) (see Fig. 1 b)), the recovered values for K_M and V_{\max} were 11.4 μM and 10.2 nM s^{-1} respectively (compared to the true values of 11 μM and 10 nM s^{-1}). When reaction velocities were calculated from substrate depletion rate constants (equation (5)) and then fit to the Michaelis-Menten equation (see Fig. 1 c), black circles), the recovered values for K_M and V_{\max} were 11.0 μM and 10.0 nM s^{-1} respectively. In comparison, when reaction velocities were obtained from product formation curves and fit to the Michaelis-Menten equation (see Fig. 1 c), grey squares), the recovered values for K_M and V_{\max} were 11.4 μM and 10.0 nM s^{-1} respectively. For this simulated system, both the substrate depletion approach and the traditional product formation approach recover predicted kinetic parameters with about the same degree of fidelity.

There is one important constraint that must be kept in mind when using the substrate depletion approach: only time points for which transient substrate concentration is relatively close to the initial substrate concentration should be used to determine k_{dep} . To reiterate, this constraint corresponds to the assumption in the traditional approach that the rate of product formation is linear at the beginning of a reaction. As substrate is depleted, the assumption that $[S]_t \approx [S]$ (in equation (5)) becomes less and less accurate, and the resulting error will affect the values of the recovered kinetic parameters. The deviation of a substrate depletion curve from a single exponential is especially evident when the initial substrate concentration is close to the K_M . If data from later time points (where much of the substrate has been consumed) are used for fitting, the obtained k_{dep} values will be erroneously high, which in turn results in erroneously high K_M and V_{\max} values (Fig. 2). As a rule of thumb, k_{dep} values should only be calculated from time points where no more than 10% of the substrate has been consumed in order to minimize this

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error; however, recovered kinetic parameters are only about 15% above their true values even when the data used to obtain k_{dep} values include points where 50% of the substrate has been consumed.

Conclusion

We have shown that Obach and Reed-Hagen's method of kinetic analysis by monitoring substrate depletion is equivalent to the traditional product formation approach. Kinetic parameters obtained by one approach can indeed be compared with those obtained by the other, as long as the assumptions inherent in each method (that transient substrate concentration is close to its initial value, for the substrate depletion approach; that the rate of product formation is linear, for the traditional approach) are not violated.

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References

Isoherranen N, Kunze KL, Allen KE, Nelson WL, Thummel KE (2004) Role of Itraconazole Metabolites in CYP3A4 Inhibition. *Drug Metab Dispos* **32**:1121-1131.

Jones HM and Houston JB (2004) Substrate Depletion Approach for Determining *in vitro* Metabolic Clearance: Time Dependencies in Hepatocyte and Microsomal Incubations. *Drug Metab Dispos* **32**:973-982.

Komura H and Iwaki M (2005) Nonlinear Pharmacokinetics of Propafenone in Rats and Humans: Application of a Substrate Depletion Assay Using Hepatocytes for Assessment of Nonlinearity. *Drug Metab Dispos* **33**:726-732.

Mohutsky MA, Chien JY, Ring BJ, Wrighton SA (2006) Predictions of the *In Vivo* Clearance of Drugs from Rate of Loss Using Human Liver Microsomes for Phase I and Phase II Biotransformations. *Pharm Res* **23**:654-662.

Obach RS and Reed-Hagen AE (2002) Measurement of Michaelis Constants for Cytochrome P450-Mediated Biotransformation Reactions Using a Substrate Depletion Approach. *Drug Metab Dispos* **30**:831-837.

Segel IH (1975) *Enzyme Kinetics*. John Wiley & Sons, New York.

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Footnotes

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Legends for Figures

Scheme 1: The system under consideration: the conversion of a substrate S to a product P by enzyme E , via an intermediate enzyme-substrate complex ES .

Figure 1 a): Transient substrate concentrations (grey lines) for six simulations at varying initial substrate concentrations, along with fits (black dashes) of single exponentials to the first 100 seconds of each simulation.

b): k_{dep} values (recovered from the exponential fits) as a function of substrate concentration, fit to equation (1).

c): Velocity (black circles: obtained from the substrate depletion approach; grey squares: obtained from the traditional product formation approach) as a function of substrate concentration, fit to the Michaelis-Menten equation.

Figure 2: Error in the recovered values for K_M (squares) and V_{max} (circles) using the substrate depletion approach, as a function of the maximum amount of substrate depleted in the curves used to obtain k_{dep} . As data from later timepoints (when more substrate has been consumed) are included in the curve-fitting, the recovered kinetic parameters deviate from their true values.

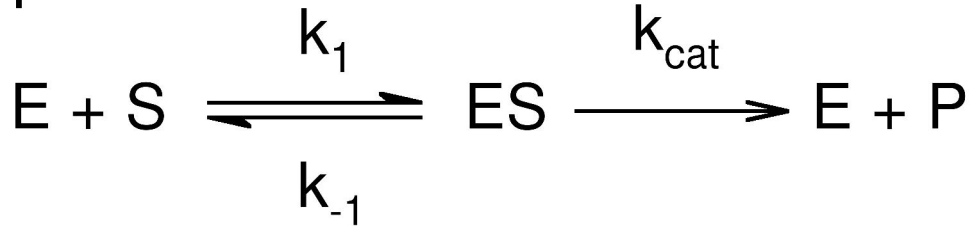
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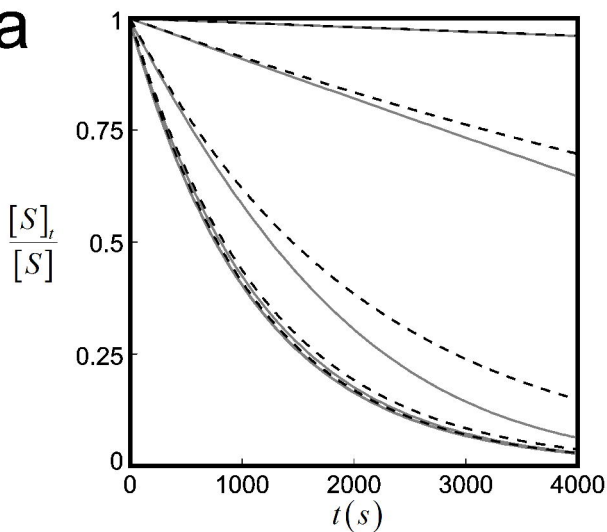
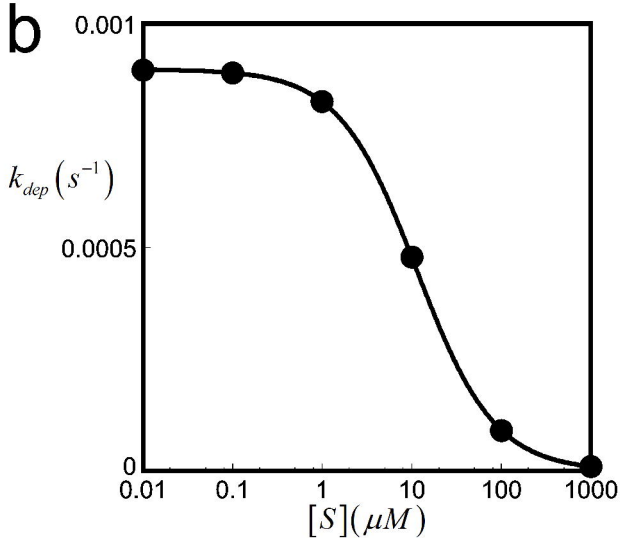
Table 1: Recovered k_{dep} and reaction velocities obtained from fits to simulations.

[S]	k_{dep}	Reaction velocity	
		$v = k_{dep} [S]$	$v = d[P]/dt$
0.01 μM	$8.96 \times 10^{-3} \text{ s}^{-1}$	$8.96 \times 10^{-12} \text{ M s}^{-1}$	$8.54 \times 10^{-12} \text{ M s}^{-1}$
0.1 μM	$8.89 \times 10^{-3} \text{ s}^{-1}$	$8.89 \times 10^{-11} \text{ M s}^{-1}$	$8.48 \times 10^{-11} \text{ M s}^{-1}$
1 μM	$8.26 \times 10^{-3} \text{ s}^{-1}$	$8.26 \times 10^{-10} \text{ M s}^{-1}$	$7.90 \times 10^{-10} \text{ M s}^{-1}$
10 μM	$4.78 \times 10^{-3} \text{ s}^{-1}$	$4.78 \times 10^{-9} \text{ M s}^{-1}$	$4.67 \times 10^{-9} \text{ M s}^{-1}$
100 μM	$9.03 \times 10^{-5} \text{ s}^{-1}$	$9.03 \times 10^{-9} \text{ M s}^{-1}$	$9.00 \times 10^{-9} \text{ M s}^{-1}$
1000 μM	$9.89 \times 10^{-6} \text{ s}^{-1}$	$9.89 \times 10^{-9} \text{ M s}^{-1}$	$9.89 \times 10^{-9} \text{ M s}^{-1}$

Table 1 shows the substrate depletion rate constant obtained by fitting the first 100 seconds of each substrate depletion curve to a single-exponential decay and reaction velocities calculated from those rate constants, as well as the reaction velocities obtained by monitoring product formation. These values were used to generate Figures 1 b) and c).

1



a**b****c**