A 'hit-and-run' kinetic mechanism for the analysis enzyme progress curves under low substrate concentrations

Petr Kuzmič*

BioKin Ltd.

15 Main Street Suite 232, Watertown, Massachusetts 02472, U.S.A

Daniel J. Sexton and Diana Martik

Dyax Corp., Discovery Research

300 Technology Square, Cambridge, Massachusetts 02139, U.S.A

Abstract

For enzymatic progress curves conforming to the Michaelis–Menten mechanism ($E + S \rightleftharpoons ES \rightarrow E + P$) under the experimental conditions where the substrate concentration is at least several times smaller than the Michaelis constant, the minimal fitting model cast as a system of numerically integrated differential equations is the simple 'hit-and-run' mechanism, $E + S \rightarrow E + P$. The best-fit value of single relevant rate constant is identical to the specificity constant, $k_{\mathrm{cat}}/K_{\mathrm{M}}$. An illustrative example involves a fluorogenic continuous assay of the matrix metalloprotease MMP12, analyzed by the differential-equation oriented software package DYNAFIT [P. Kuzmic (1996) Anal. Biochem. 237, 260].

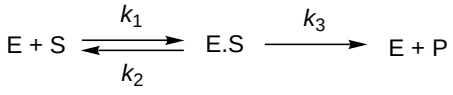
Key words:
Enzyme kinetics; Theory; Mathematics; Statistics; Regression; Ordinary differential equations; Michaelis–Menten; human MMP12 protease; Mechanism; DYNAFIT

This note addresses the following problem. Our task is to extract as many unique rate constants as possible from the experimental time-course of an enzyme reaction. We wish to use a software package that allows a *symbolic* rather than algebraic description of mechanisms, such as KINSIM [1], DYNAFIT [2], or COPASI [3]. What, then, is the most appropriate theoretical model when the initial substrate concentration, $[S]_0$, is significantly lower than the Michaelis constant, K_{M}? The answer is in the "hit-and-run" reaction mechanism, $E+S \rightarrow E+P$, in which the enzyme-substrate complex never appears.

Under typical enzyme assay conditions (conducted on the time scale of seconds, minutes, or even hours) the experimental progress curves can never fully support the Michaelis–Menten (MM) mechanism [4], $E + S \rightleftharpoons ES \rightarrow E + P$, even though it is the "true" reaction mechanism in most cases. The problem is that the association rate constant ($E + S \rightarrow ES$) can be determined only under rapid-kinetic conditions, on the time scale of microseconds to milliseconds.

To get around this problem of parametric redundancy, we [5] have recently invoked the simpler Van Slyke–Cullen (VSC) mechanism [6], $E + S \rightarrow ES \rightarrow E + P$, which only contains two microscopic rate constants instead of three. However, when $[S]_0$ is significantly lower than K_{M}, the enzyme progress curve is simply an exponential, and the relevant first-order rate constant is equal to $k_{\mathrm{cat}}/K_{\mathrm{M}}$. In other words, *only a single rate constant* can be determined from the experimental progress curve if and when $[S]_0 << K_{\mathrm{M}}$.

Many simple software tools allow the fit of exponential data to the *algebraic* equation $y = A_0 e^{-k\,t}$. However, what if we wish to use an advanced software tool based on the automatic construction of *differential* equations [1–3] ? Similar software systems indeed have many advantages (for review, cf. [7]). Our challenge then is to reduce the MM mechanism in *Scheme 1* such that only a single constant remains, while both the enzyme (E) and the substrate (S) still appear in the reaction scheme.



Scheme 1

As a solution, we propose the hypothetical "hit-and-run" (HR) reaction mechanism shown in *Scheme 2*. The designation "hit-and-run" originates in that the enzyme-substrate complex, ES, is formed so fleetingly that its concentration is always essentially zero when compared with the concentration of the free enzyme. The single rate constant k_1^* appearing in *Scheme 2* is an "apparent" bimolecular association rate constant, exactly equivalent to the specificity constant $k_{\mathrm{cat}}/K_{\mathrm{m}}$ [5].

*Corresponding author
Email address: pksci01@biokin.com (Petr Kuzmič)
URL: http://www.biokin.com (Petr Kuzmič)

$$\mathrm{E + S} \xrightarrow{k_1^*} \mathrm{E + P}$$

Scheme 2

Scheme 2 is conceptually similar to the Theorell-Chance mechanism for bisubstrate enzymes [8, p. 594] ($E + A \rightarrow EA; EA + B \rightarrow EQ + P; EQ \rightarrow E + Q$). No ternary molecular complex is postulated, even though at least one such complex is *implied*. In the HR mechanism, we also know that the Michaelis complex ES must be physically present. We are choosing to ignore it, so that we can produce a *practically useful* minimal model of the reaction progress.

The reaction scheme in *Scheme 2* is used by software systems such as KINSIM or DYNAFIT to derive a system of differential equations (1) and (2). Note that $\mathrm{d}[E]/\mathrm{d}t = 0$. These differential equations then serve as the mathematical model for the the progress of enzyme reactions conforming to the MM mechanism, under the special experimental conditions where $[S]_0 << K_\mathrm{M}$.

$$\mathrm{d}[S]/\mathrm{d}t = -k_1^*[E][S] \quad (1)$$
$$\mathrm{d}[P]/\mathrm{d}t = +k_1^*[E][S] \quad (2)$$

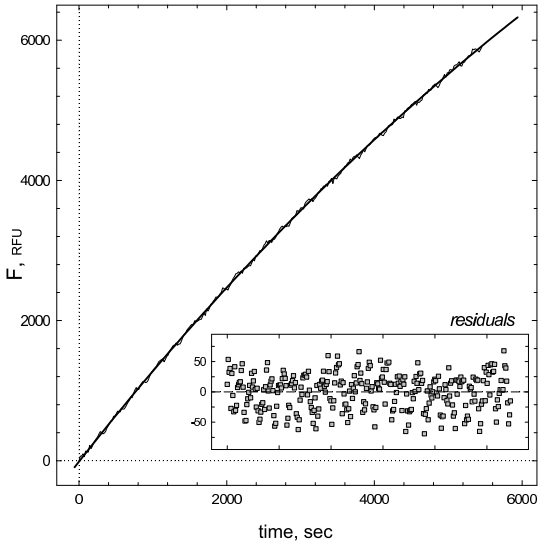


Figure 1: *Jagged curve*: Experimental data from a typical assay of the human MMP12 protease ($[E]_0$ = 0.3 nM determined by active-site titration) using the fluorogenic substrate Mca-Lys-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH_2 [9] ($[S]_0 = 10\ \mu$M). *Smooth curve*: Least-squares fit either to the MM mechanism (*Scheme 1*) or to the HR mechanism (*Scheme 2*). The Michaelis constant was determined independently at approximately $K_\mathrm{M} = 90\ \mu$M (data not shown). Thus, the substrate $[S]_0$ is approximately nine times lower than K_M and therefore the progress curve is clearly an exponential.

A representative experimental data set, from a continuous fluorogenic assay of the human matrix metalloprotease MMP12, is shown in *Fig. 1*. The smooth model curve corresponds to the best-fit values $k_1^* = (0.269 \pm 0.005)\ \mu\mathrm{M}^{-1}\mathrm{s}^{-1}$ The full listing of a DYNAFIT [2] input file is shown in the Appendix.

Exactly identical model curve (within eight significant digits) was obtained by fitting the same data to the MM model (*Scheme 1*), in which k_1 was held constant at $k_1 = 10\ \mu\mathrm{M}^{-1}\mathrm{s}^{-1}$. The best-fit values of the adjustable rate constants were $k_2 = (4460 \pm 47000)\ \mathrm{s}^{-1}$ and and $k_3 = (128 \pm 1300)\ \mathrm{s}^{-1}$, from which $k_\mathrm{cat}/K_\mathrm{m} = k_1k_3/(k_2 + k_3) = 0.27\ \mu\mathrm{M}^{-1}\mathrm{s}^{-1}$. This value is identical to k_1^* in the HR model; note that k_3 and k_2, considered individually, are very poorly determined by the experimental data. This is yet another expression of the fact that only a single rate constant can be determined when $[S]_0 << K_\mathrm{M}$.

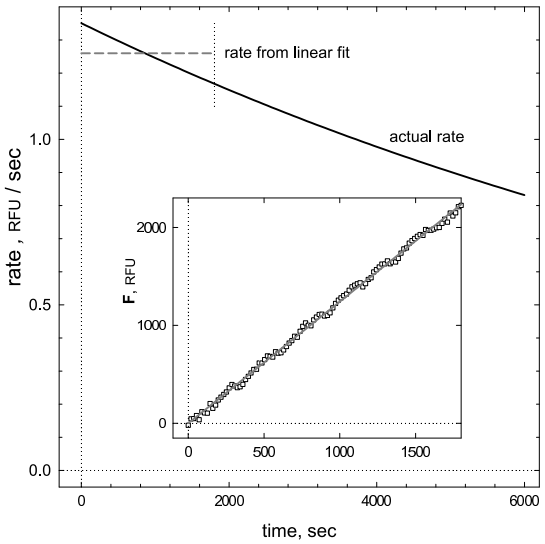


Figure 2: *Main panel*: *Solid curve* – Instantaneous reaction rate (the derivative of the observable signal with respect to time) changing over time. Plotted values were obtained by the numerical differentiation of the solution to ODE system (1) and (2) [2]. *Dashed horizontal line* – Fixed reaction rate determined by linear fit of the first 30 minutes (see inset). Note that the actual rate changes significantly during this period. *Inset*: Linear least-squares fit of the first 30 minutes. Note that a slight but *systematic* deviation from linearity is virtually impossible to spot.

As a side note, it is worth addressing the possibility of a *linear fit* of only the initial portion of the progress curve. This is frequently the standard operating procedure, especially in the pharmaceutical and biotechnology industries, because most software packages preloaded with 96-well plate readers cannot perform nonlinear regression of the reaction time course.

Fig. 2 displays the actual reaction rate changing over time. At time zero, the instantaneous rate is 1.35 relative fluorescence units per second (RFU/s). However, at the end of the assay the instantaneous rate has dropped to 0.86 RFU/s – by almost 40%. Most researchers looking at the data plot in *Fig. 1* would probably notice that the reaction rate is changing slightly (in other words, that the time-course is somewhat nonlinear), but how many would be able to "eye-ball" correctly the extent of the reaction slowing down? An informal survey suggests that most biochemists would guess a 10%–20% drop in reaction rate, instead of 40%.

The highly deceptive nature of a "linear fit of the initial portion", excised from the overall data trace, is shown in the inset to *Fig. 2*. This is the first 30 minutes of the assay fit to a straight line, giving the initial rate of 1.26 FRU/s. However, note that

the actual initial rate is 1.35 RFU/s, a value *systematically* lower by 7%. Experience shows that the linear fit of arbitrarily defined "initial portion" to the overall reaction progress introduces *systematic* errors on the order of 10–20%. Of course, the data analyst should always avoid any preventable systematic errors, and/or understand fully the propagation of such errors into the final results (e.g., inhibition constants).

A famous statistician once quipped that "essentially, all models are wrong, but some are useful." [10, p. 424] In this brief note we have identified yet another physically "wrong" but mathematically very useful kinetic model – the "hit-and-run" reaction mechanism shown in *Scheme 2*.

Perhaps the most important conclusion has to do with the continuing need to think deeply about the essential nature of each particular biochemical system under kinetic investigation. It is not enough to have available exquisitely convenient computer programs such as KINSIM [1], DYNAFIT [2], or COPASI [3], even though these tools do automate the construction mathematical equations from highly intuitive input. For example, if we are working with an enzyme/substrate system that presumably conforms to the MM mechanism, it is very tempting simply to type `E + S <==> ES ---> E + P` on the keyboard, and let the machine derive the corresponding system of differential equations.

However, we still have to understand the fundamental nature of *saturating behavior* in enzyme kinetics. In particular, we do have to understand that when the substrate concentration is very low relative to K_M, the amount of the Michaelis complex is always negligibly small when compared to the amount of the free enzyme. To express this particular biochemical idea in a symbolic notation, we must use the truncated "hit-and-run" kinetic model, `E + S ---> E + P`, instead of the full MM mechanism. Only then the automatically generated system of differential equation will contain only as many rate constants (namely, a single one) that can be supported by the experimental data.

References

[1] B. A. Barshop, R. W. Wrenn, C. Frieden, Analysis of numerical methods for computer simulation of kinetic processes: Development of KINSIM – a flexible, portable system, Anal. Biochem. 130 (1983) 134–145.

[2] P. Kuzmič, Program DYNAFIT for the analysis of enzyme kinetic data: Application to HIV proteinase, Anal. Biochem. 237 (1996) 260–273.

[3] S. Hoops, S. Sahle, R. Gauges, C. Lee, J. Pahle, N. Simus, M. Singhal, L. Xu, P. Mendes, U. Kummer, COPASI a COmplex PAthway SImulator, Bioinformatics 22 (2006) 3067–3074.

[4] L. Michaelis, M. L. Menten, Die kinetik der invertinwirkung, Biochem. Z. 49 (1913) 333–369.

[5] P. Kuzmič, Application of the Van Slyke - Cullen irreversible mechanism in the analysis of enzymatic progress curves, Anal. Biochem. 394 (2009) 287–289.

[6] D. D. V. Slyke, G. E. Cullen, The mode of action of urease and of enzymes in general, J. Biol. Chem. 19 (1914) 141–180.

[7] P. Kuzmič, DynaFit – A software package for enzymology, Meth. Enzymol. 467 (2009) 247–280.

[8] I. H. Segel, Enzyme Kinetics, Wiley, New York, 1975.

[9] U. Neumann, H. Kubota, K. Frei, V. Ganu, D. Leppert, Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme, Anal. Biochem. 328 (2004) 166–173.

[10] G. E. P. Box, N. R. Draper, Empirical Model-Building and Response Surfaces, Wiley, New York, 1987.

Appendix

The following DYNAFIT [2] script is used to fit experimental data in a two-column (time vs. fluorescence intensity) text file A01.txt to the "hit-and-run" mechanism in *Scheme 2*, to generate the best-fit model curve displayed in *Fig. 1*.

```
[task]
   task = fit
   data = progress
[mechanism]
   E + S ----> E + P   :    k1*
[constants]
   k1* = 0.3 ?
[concentrations]
   E = 0.0003
   S = 10
[responses]
   P = 1000 ?
[output]
   directory ./project/hit-and-run/output
[data]
   directory ./project/hit-and-run/data
   extension txt
   delay     60
   file A01 | offset = auto ?
[end]
```