Integrated Michaelis-Menten equation in DynaFit.
3.
Application to HIV protease

BIOKIN TECHNICAL NOTE TN-2016-03

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Abstract

The DynaFit software package (http://www.biokin.com/dynafit/) contains a built-in ("hard-coded") implementation of the integrated Michaelis-Menten equation formulated in terms of the Lambert omega function. This built-in regression model can be used conveniently to determine the substrate kinetic parameters either from a single progress curve or from a collection of enzymatic progress curves analyzed globally. An illustrative example includes previously published data on the substrate kinetics of HIV protease. It is shown that even a single reaction progress curve can be used to determine K_M and k_{cat} as long as two conditions are met simultaneously: (1) the initial substrate concentration must be higher than the K_M; and (2) the enzyme reaction must be allowed to proceed to full completion.

Key words:
enzyme progress curves; global fit; HIV protease; integrated Michaelis-Menten equation; Lambert omega function; substrate kinetics;

Contents

1. Introduction

In a preceding Technical Note (http://www.biokin.com/TN/2016/02), we demonstrated that in a specific case of the 5α-ketosteroid reductase [1] it is possible to determine the Michaelis constant K_M and the turnover number k_{cat} from a *single* enzyme assay, utilizing a *single* initial substrate concentration. In the present Note we explore in greater detail under what experimental conditions such a time-saving and labor-saving kinetic determination becomes possible.

Based on previously published kinetic experiments with the HIV protease [2, 3], we now show that a necessary prerequisite for a successful determination of K_M and k_{cat} from a single progress curve is that the initial substrate concentration is at least twice larger than the Michaelis constant. This also implies that in the case of a global fit [4] of multiple combined progress curves, at least one kinetic trace must be obtained at $[S]_0 \geq 2 \times K_M$.

When the initial substrate concentrations are *not* sufficiently high for a successful K_M determination using the present method ($[S]_0 < 2 \times K_M$), we show that progress curve analysis can be used to determine at least the specificity number, $k_S \equiv k_{cat}/K_M$. A successful direct determination of k_S, even at relative low substrate concentrations, is made possible by utilizing a newly derived algebraic form of the integrated Michaelis-Menten rate law [5], in which k_S explicitly appears as one of the model parameters.

2. Methods

The software package DynaFit [6, 7] contains a family of two closely related, predefined mathematical models, which can be used to fit the time course of enzyme enzyme reactions following the Michaelis-Menten kinetic mechanism [8, p. 19].

The first of two algebraic forms is represented by Eqn (1) [9–11], where F is some appropriate experimental variable, such as for example fluorescence, recorded at the reaction time t; F_0 is the experimental signal observed at $t = 0$ (i.e., baseline offset – essentially a property of the instrument); $[S]_0$ is the initial substrate concentration; K_M is the Michaelis constant; and r_P is the specific molar response coefficient of the reaction product; $[E]_0$ is the concentration of the enzyme active sites; t is the reaction time; and k_{cat} is the turnover number. The symbol ω represents the value of the Lambert omega function, also referred to as Lambert W function [9–11]. Eqn (2) represents the instantaneous observed reaction rate, i.e., the first derivative with respect to time t of the physical variable F being monitored.

$$F = F_0 + r_P \left\{ [S]_0 - K_M \, \omega \left[\frac{[S]_0}{K_M} \, \exp\left(\frac{[S]_0}{K_M} - \frac{k_{cat}}{K_M} \, [E]_0 \, t \right) \right] \right\} \quad (1)$$

$$\frac{dF}{dt} = r_P \, k_{cat} \, [E]_0 \frac{\alpha}{1+\alpha} \quad (2)$$

An alternate, and algebraically equivalent way of expressing the integrated rate law is given by the recently proposed [5] Eqn (3). In this case, the integrated rate equation does not contain k_{cat} as a model parameter, but rather k_S (and K_M, as before). Note that k_S is the *specificity number* defined as k_{cat}/K_M. Note that k_S has the dimension of a second-order (bimolecular association) rate constant. The derivative of the model function with respect to time, i.e., the instantaneous observed reaction rate, is defined by Eqn (4).

$$F = F_0 + r_P \left\{ [S]_0 - K_M \, \omega \left[\frac{[S]_0}{K_M} \, \exp\left(\frac{[S]_0}{K_M} - k_S \, [E]_0 \, t \right) \right] \right\} \quad (3)$$

$$\frac{dF}{dt} = r_P K_M \, k_S \, [E]_0 \frac{\beta}{1+\beta} \quad (4)$$

The alternate use of Eqn (1) or Eqn (3) depends on which combination of steady-state parameters (K_M or k_{cat} along with k_S) is of greater interest to the investigator.

For details on how Eqns (1)–(3) can be invoked in the DynaFit software package, see ref. [12].

3. Results and discussion

3.1. Raw experimental data

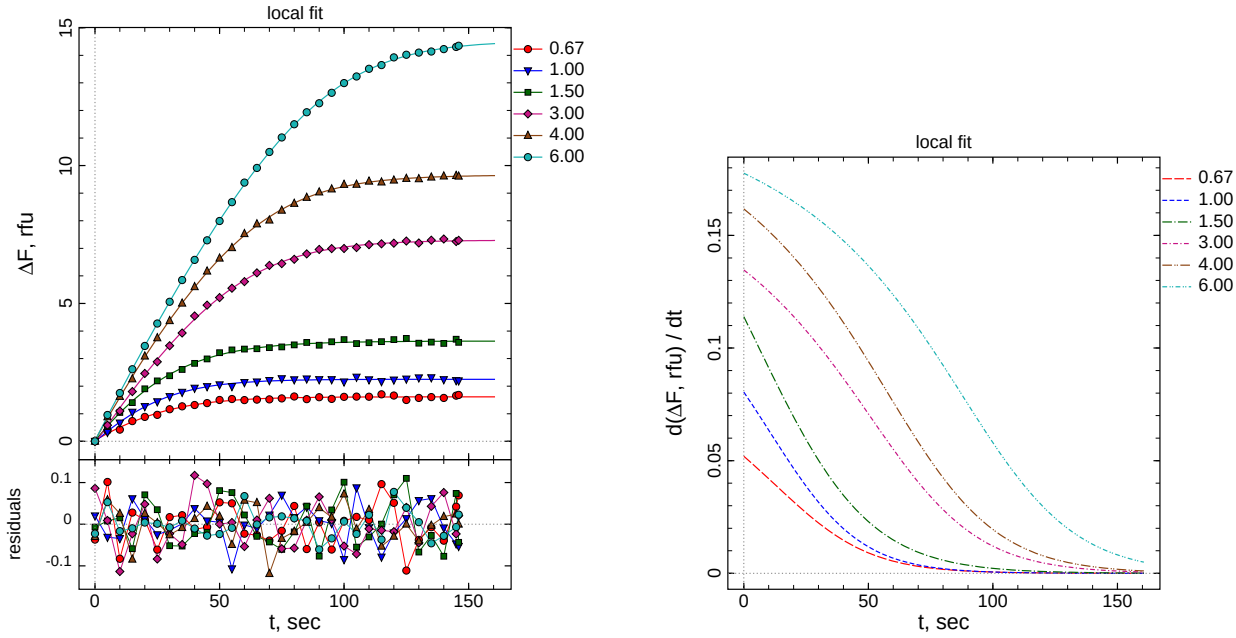
The HIV protease has been assayed by using a continuous fluorogenic assay as described in ref. [2]. The experimental data utilized in this Technical Note are those that were originally published in Figure 1 of the above reference. The only difference is that, in this document, we assumed zero mixing delay time, utilizing the experimental data exactly as it was generated by the instrument. In contrast, in the original ref. [2], all time points were corrected by adding $\Delta t = 4.0$ sec to all raw instrument readings.

3.2. Nonlinear least-squares fit to Eqn (3)

The experimental data originally published in [Fig. 1][2] are analyzed in this report in two different way. First, we performed the "local" fit of individual progress curves. The twofold purpose of this "local" analysis was (a) to determine the molar response coefficient of the final reaction product; and (b) to assess at which individual substrate concentrations it might be possible to determine K_M and k_{cat} values from a single kinetic trace. Second, we performed the global [4] fit of combined progress curves, in order to determine the final values of substrate kinetic parameters.

3.2.1. "Local" fit of individual progress curves

In this first round of nonlinear least squares fit, each individual progress curve was fit separately to Eqn (3). The only *fixed* parameter appearing in Eqn (3) was the enzyme concentration $[E]_0$ = 10 nM. The remaining parameters were optimized such their best-fit values were specific to each individual progress curve, at a given substrate concentration $[S]_0$. The requisite DynaFit input script is listed in Appendix A.1. The results are summarized graphically in *Figure 1* and numerically in *Table 1*. For brevity, he best-fit values of the optimized offsets on the signal axis (F_0) are omitted.



(a) **(b)**

Figure 1: Results of local fit of individual progress curves to Eqn (3). Initial substrate concentrations are shown in the legend (right margin). **(a)**. Symbols: raw experimental data. Smooth curves: best-fit model according to Eqn (3). **(b)**. Best-fit instantaneous rate curves according to Eqn (4).

The residual panel in the bottom panel in *Figure 1*(a) shows that the distribution of residuals is apparently random ("log" shape, as opposed to a "rainbow" or a "wave" shape). The amplitude for residual plot is approximately 0.2 fluorescence units (from -0.1 to +0.1); the amplitude of the experimental signal is approximately 15 fluorescence units. Thus the random noise level is approximately 0.2/15 = 1.3%. It should be noted that for reliable determination substrate kinetic parameters from individual progress curves, the random noise level should be kept below approximately 3%.

The rate plot in *Figure 1*(b) shows that the instantaneous velocity of the enzyme reaction decreases prominently immediately from the start of the assay. This means that strictly speaking there is no "linear initial portion" to any of the six progress curves displayed in *Figure 1*(a). The

parameter	$[S]_0$, μM	final ± std.err.	cv,%	low	high
K_m, μM	0.666	0.85 ± 0.52	61.2	0.19	> 1000
	1	1.34 ± 0.59	44.0	0.50	19.3
	1.5	2.58 ± 0.82	31.8	1.22	11.2
	3	1.64 ± 0.16	9.8	1.27	2.18
	4	1.98 ± 0.15	7.6	1.62	2.46
	6	1.95 ± 0.11	5.6	1.67	2.29
k_S, $\mu M^{-1}s^{-1}$	0.666	5.90 ± 1.10	18.6		
	1	6.19 ± 0.81	13.1		
	1.5	4.97 ± 0.40	8.0		
	3	5.15 ± 0.26	5.0		
	4	5.07 ± 0.21	4.1		
	6	4.99 ± 0.19	3.8		
r_P, rfu/μM	0.666	2.35 ± 0.07	3.0		
	1	2.27 ± 0.05	2.1		
	1.5	2.42 ± 0.03	1.3		
	3	2.46 ± 0.02	0.6		
	4	2.41 ± 0.01	0.5		
	6	2.42 ± 0.01	0.4		

Table 1: Results of "local" fit of individual progress curves to Eqn (3). For details see text.

rate plot indicates that the instantaneous reaction velocity changes by less than approximately 10-15% only if we consider the first approximately 10 seconds of each assay.

The results summarized in *Table 1* show three different behaviors for the optimized model parameters, depending on the extent to which the given parameter varies with the initial substrate concentration, $[S]_0$.

- **Response coefficient.** The molar response coefficient of the fluorogenic product, r_P, shows only a negligible degree of variation. The average and standard deviation is $r_P = (2.39 \pm 0.07)$ rfu/μM, which corresponds to less than 3% coefficient of variation. Note that the amplitude each progress curves (and therefore also the best-fit value of r_P depends on the the *actual* (as opposed to nominal) substrate concentration in each assay. Thus, from the high reproducibility of r_P across the range for substrate concentrations, we can conclude that the titration error as lower than 3%. The average value of the molar response coefficient ($r_P = 2.39$) is used below, in the global fit of combined progress curves as fixed model parameter, while at the same time allowing the substrate concentrations to vary individually.
- **Specificity number.** The middle block of rows in *Table 1* shows that the specificity number k_S is also largely independent on the initial substrate concentration $[S]_0$. The best-fit values range approximately from 5 to 6 $\mu M^{-1}s^{-1}$. However, between $[S]_0 = 1.5$ and 6.0 μM, the best-fit values of k_S differ by less than 3%. Within this range of substrate concentrations, the coefficient of variation of each best-fit value for k_S is lower than 10%. Thus, we can conclude that the specificity number k_S can be determined with good degree of reliability from *any of the six individual progress curves*.

- **Michaelis constant.** In contrast with the response coefficient and the specificity number, the Michaelis constant values listed in *Table 1* vary significantly, depending on the substrate concentration utilized in each assay. Onlytwo values, determined at the two highest substrate concentration, differ by less than 10%. In addition, the formal standard error from nonlinear regression is relatively large, corresponding to coefficient of variation greater than 25% or even 50% for the three lowest substrate concentrations.

The "low" and "high" values listed for K_M in *Table 1* were computed by using the empirical method proposed by Johnson [13–15]. In particular, in this case, the lower and upper limits of the parameter confidence intervals are determined by searching the parameter space until the residual sum of squares increased by 5%, relative to the best-fit minimum value. By using this 5% empirical criterion, the upper limit of K_M is undefined (de facto indistinguishable from infinity) at $[S]_0 = 0.666\ \mu M$. Even at $[S]_0 = 1.0$ and $1.5\ \mu M$, the confidence interval for K_M is extremely wide, encompassing at least one order of magnitude. Only at $[S]_0 \geq 3.0\ \mu M$ we start to see that the confidence interval narrows significantly, around the best-fit value near $1.9\ \mu M$.

An important conclusion is that reliable determination of K_M from a *single* individual progress curve appears to be possible only *if the substrate concentration is higher than approximately two-fold multiple of Michaelis constant*, $[S]_0 \geq K_M$.

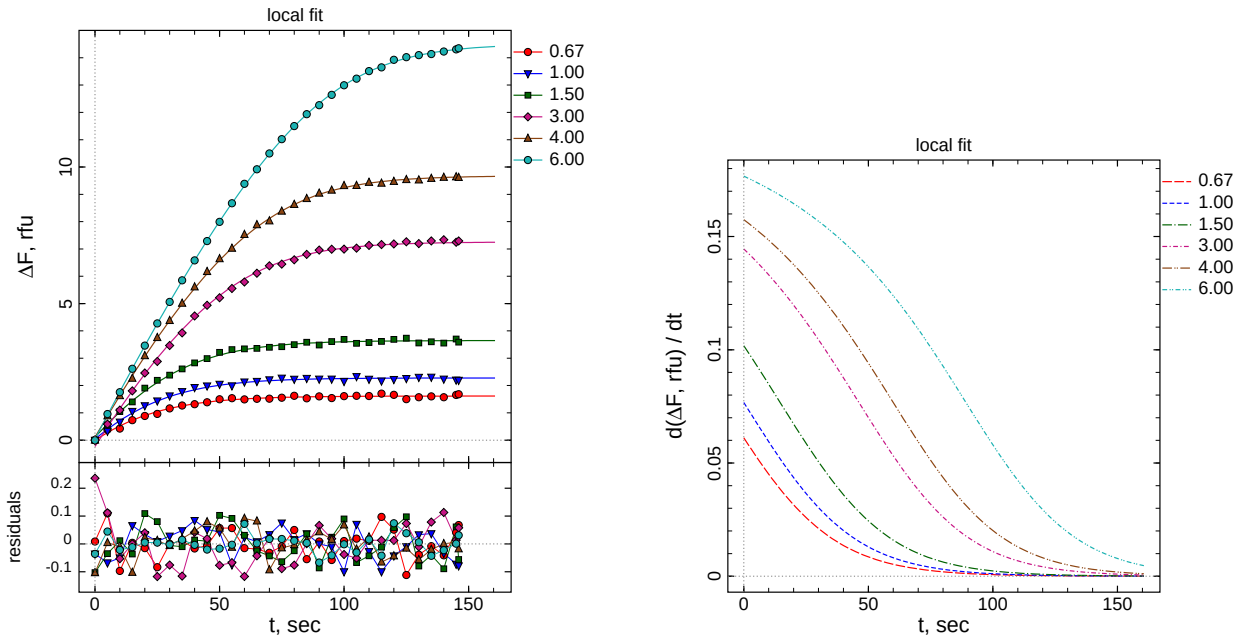
3.2.2. Global fit of combined progress curves

In the second round of kinetic analysis, we performed global fit [4] of combined enzymatic progress curves, in order to arrive at a refined estimate of the substrate kinetic parameters K_M and k_S. Importantly, in this case the molar response coefficient of the reaction product was treated as a fixed constant equal to an average of the six individual values of r_P determined by the local fit, see in section 3.2.1. In the local fit, all individual substrate concentrations were treated as fixed constants where as the response coefficient was locally optimized. In contrast, in the global fit all substrate concentrations are treated as (locally) optimized model parameters. The results are summarized graphically in *Figure 2* and numerically in *Table 2*. For brevity, he best-fit values of the optimized offsets on the signal axis (F_0) are omitted.

parameter	initial	final ± std.err.	cv,%	low	high
k_S, $\mu M^{-1}s^{-1}$	5	5.11 ± 0.09	1.7	4.86	5.38
K_m, μM	2	1.90 ± 0.06	2.9	1.75	2.07
$[S]_0^{(1)}$, μM	0.666	0.68 ± 0.02	2.4		
$[S]_0^{(2)}$, μM	1	0.94 ± 0.02	1.6		
$[S]_0^{(3)}$, μM	1.5	1.48 ± 0.01	0.9		
$[S]_0^{(4)}$, μM	3	3.13 ± 0.01	0.4		
$[S]_0^{(5)}$, μM	4	4.01 ± 0.01	0.3		
$[S]_0^{(6)}$, μM	6	6.06 ± 0.02	0.3		

Table 2: Results of global fit of combined progress curves to Eqn (3). For details see text.

The best-fit values of locally adjusted substrate concentrations listed in *Table 2* differ from their nominal values (listed in the column labeled "initial") by less than 10%. This means that the titration error in these experiments was lower than 10%. The "low" and "high" values of kinetic



(a) **(b)**

Figure 2: Results of global fit of combined progress curves to Eqn (3). Initial substrate concentrations are shown in the legend (right margin). **(a)**. Symbols: raw experimental data. Smooth curves: best-fit model according to Eqn (3). **(b)**. Best-fit instantaneous rate curves according to Eqn (4).

constants in *Table 2* were determined by Johnson's empirical method [13–15] specifically at 5% increase in the residual sum of squares, relative to the least-squares minimum. Note that, not surprisingly, the confidence intervals are (a) not symmetrical around the best-fit values and (b) significantly wider than is suggested by the formal standard errors (column "std. err."). This illustrates the generally valid observation that *formal standard errors from nonlinear regression nearly always underestimate the true uncertainty of nonlinear regression parameters*.

The residual plot in the bottom panel in *Figure 2*(a) again shows essentially a random pattern ("log" shape as opposed to a "rainbow" or "wave" shape). This confirms that the simple Michaelis-Menten reaction mechanism does describe the time course of these assays sufficiently well. In particular, the randomness of residuals in *Figure 2*(a) proves that either product inhibition or enzyme deactivation do not play a statistically significant role.

In conclusion, the Michaelis constant value determined by the global fit method is K_M = (1.9 ± 0.1) μM, as shown in *Table 2*. This confirms that the *locally* determined K_M values listed in *Table 1* are indeed correct for substrate concentrations $[S]_0$ = 4 μM and 6 μM. Note that both concentrations are at least twice higher than the consensus K_M value. As a general rule, in order to determine substrate kinetic constants from progress curves, the global data set must contain *at least one progress curve collected at a substrate concentration twice as high as the Michaelis constant, or higher*: $[S]_0 \geq 2 \times K_M$.

3.3. *Fit of initial reaction rates*

In the process of performing the local fit of individual progress curves, as described in section 3.2.1, the DynaFit software package automatically created a log file of initial reaction rates. In the DynaFit script listed in Appendix A.1, the automatic creation of the initial rate file was arranged by the following code:

```
[output]
...
   rate-file ./proj/IMM/HIVprot/data/rates-local.txt
```

The initial rates are those values of the instantaneous rate displayed in *Figure 1*(b) at the initial reaction time ($t = 0$). The auto-generated numerical values of the initial rates vs. the corresponding substrate concentration were subsequently utilized in a DynaFit script listed in Appendix A.3. The results are shown graphically in *Figure 3*. The best-fit values of the Michaelis constant and the associated formal standard error was $K_M = (1.9 \pm 0.4)$ mM. The nonsymmetrical confidence interval determined by using the profile-t method [16, 17] spanned from 1.2 to 3.3 μM, at the 95% confidence level. Note that the best-fit value $K_M = 1.9\ \mu$M is essentially identical with the results obtained either by the global fit of combined reaction progress curves, or by the local fit of individual progress curves collected at $[S]_0 = 4$ and 6 μM, i.e., at $[S]_0 \geq 2 \times K_M$.

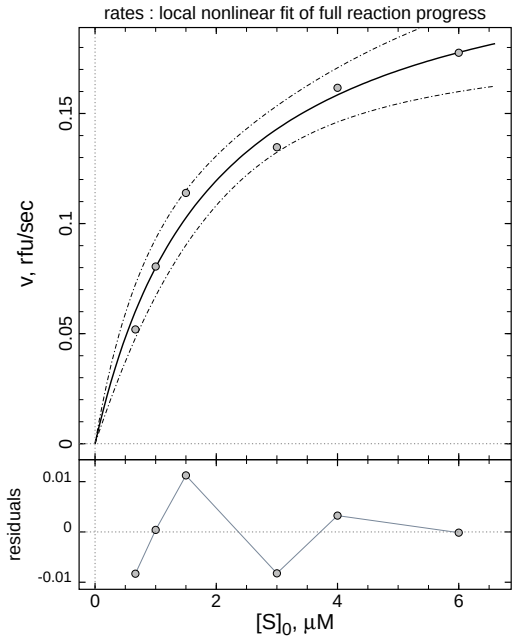


Figure 3: Results of least-squares fit of automatically generated initial rates. For details see text.

4. Summary and conclusions

1. Using only a *single* progress curve we can obtain a well-defined estimate of K_M and k_{cat}.
2. The best-fit values are indistinguishable from the results obtained by the standard initial rate method.
3. A necessary prerequisite is that the substrate concentration must be at least twice as high as the K_M.

All experimental data utilized in this report, plus all DynaFit input (script) files that were used to produce the report, are available for download from http://www.biokin.com/TN/2016/03.

Bibliographic Information

How to Cite this Publication:

Kuzmič, P. *Integrated Michaelis-Menten equation in DynaFit. 3. Application to HIV protease*, BioKin Technical Note TN-2016-03, BioKin Ltd., Watertown MA, [Online] www.biokin.com/TN/2016/03 (2016-)

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Appendix

A. DynaFit scripts

A.1. "Local" fit of individual progress curves to Eqn (3)

The DynaFit [6, 7] script listed below was utilized to determine K_M and $k_S \equiv k_{cat}/K_M$ values by "local" fit of individual enzymatic progress curves, obtained at various substrate concentrations. The experimental data are contained in the comma separated value (CSV) file d01.csv following the DynaFit keyword `sheet` (see below). The data file d01.csv contains the reaction time in seconds in the first column. Subsequent columns nol 2 through 7 contain the readings of tyrosine fluorescence [3] at substrate concentrations ranging from 0.666 μM to 6.0 μM, as indicated in the script file.

```
[task]
   task = fit
   data = generic
   code = built-in
[equation]
   MichaelisMentenProgressKmKs
[parameters]
   Eo = 0.01
[data]
   variable t
   directory ./proj/IMM/HIVprot/data
   sheet     d01.csv
graph local fit
   column   2 | param Fo = 0 ? (-10000 .. 10000), So = 0.67 | label 0.67
              param rP = 2 ?, kS = 5 ?, Km = 2 ??
   column   3 | param Fo = 0 ? (-10000 .. 10000), So = 1.00 | label 1.00
              param rP = 2 ?, kS = 5 ?, Km = 2 ??
   column   4 | param Fo = 0 ? (-10000 .. 10000), So = 1.50 | label 1.50
              param rP = 2 ?, kS = 5 ?, Km = 2 ??
   column   5 | param Fo = 0 ? (-10000 .. 10000), So = 3.00 | label 3.00
              param rP = 2 ?, kS = 5 ?, Km = 2 ??
   column   6 | param Fo = 0 ? (-10000 .. 10000), So = 4.00 | label 4.00
              param rP = 2 ?, kS = 5 ?, Km = 2 ??
   column   7 | param Fo = 0 ? (-10000 .. 10000), So = 6.00 | label 6.00
              param rP = 2 ?, kS = 5 ?, Km = 2 ??
[output]
   directory ./proj/IMM/HIVprot/output/fit-progress-local
   rate-file ./proj/IMM/HIVprot/data/rates-local.txt
[settings]
{Filter}
   ZeroBaselineSignal = y
   ReadEveryNthPoint = 5
{Output}
   XAxisLabel = t, sec
   YAxisLabel = {/Symbol D}F, rfu
   WriteTeX = y
```

```
{ConfidenceIntervals}
   SquaresIncreasePercent = 5
[end]
```

A.2. Global fit of combined progress curves to Eqn (3)

The DynaFit [6, 7] script listed below was utilized to determine K_M and k_S values by global fit [4] of combined enzymatic progress curves, obtained at various substrate concentrations. Note that unlike in the immediately preceding script listing, the parameters `Km` and `kS` are listed in the `[parameters]` section, as opposed being associated with any particular column in the data file (see above in section A.1. Placing any parameter name and initial estimate into the `[parameters]` section of the script indicates that this particular parameter is to be *globally* optimized.

```
[task]
   task = fit
   data = generic
   code = built-in
[equation]
   MichaelisMentenProgressKmKs
[parameters]
   Eo = 0.01
   rP = 2.39     ; fixed!
   kS = 5 ??
   Km = 2 ??
[data]
   variable t
   directory ./proj/IMM/HIVprot/data
   sheet     d01.csv
graph local fit
   column   2 | param Fo = 0 ? (-10000 .. 10000), So = 0.67 ? | label 0.67
   column   3 | param Fo = 0 ? (-10000 .. 10000), So = 1.00 ? | label 1.00
   column   4 | param Fo = 0 ? (-10000 .. 10000), So = 1.50 ? | label 1.50
   column   5 | param Fo = 0 ? (-10000 .. 10000), So = 3.00 ? | label 3.00
   column   6 | param Fo = 0 ? (-10000 .. 10000), So = 4.00 ? | label 4.00
   column   7 | param Fo = 0 ? (-10000 .. 10000), So = 6.00 ? | label 6.00
[output]
   directory ./proj/IMM/HIVprot/output/fit-progress-global
[settings]
{Filter}
   ZeroBaselineSignal = y
   ReadEveryNthPoint = 5
{Output}
   XAxisLabel = t, sec
   YAxisLabel = {/Symbol D}F, rfu
   WriteTeX = y
{ConfidenceIntervals}
   SquaresIncreasePercent = 5
[end]
```

A.3. Fit of initial reaction rates to the Michaelis-Menten model

The DynaFit [6, 7] script listed below was utilized to determine K_M and k_{cat} values by the least-squares fit of initial reaction rates automatically determined in section A.1 above. Note that, in this specific example, the symbolic formalism utilized below (`E + S <==> E.S`, etc.) is exactly equivalent to specifying the standard Michaelis-Menten rate law, $v = [E]_0\, k_{cat}[S]_0/([S]_0 + K_M)$. Experience shows that many practicing enzymologists, especially those without strong mathematical education, prefer to utilize the symbolic notation over of DynaFit over algebraic formulas, even in those cases where the two notational systems are functionally equivalent.

```
[task]
   task = fit
   data = rates
   approximation = rapid-equilibrium
[mechanism]
   E + S <==> E.S     :   Km    dissoc
   E.S ---> E + P     :   kcat
[constants]
   Km = 2 ??
   kcat = 1 ??
[concentrations]
   E = 0.01
[responses]
   P = 2.39
[data]
   variable S
   directory ./proj/IMM/HIVprot/data
graph rates : local nonlinear fit of full reaction progress
   sheet rates-local.csv
   column 2
[output]
   directory ./proj/IMM/HIVprot/output/fit-rates-local
[settings]
{Output}
   XAxisLabel = [S]_0, {/Symbol m}M
   YAxisLabel = v, rfu/sec
   ConfidenceBands = y
[end]
```