

# Determination of substrate kinetic parameters from progress curve data

A DynaFit Tutorial

**BioKin Technical Note TN-2015-05**

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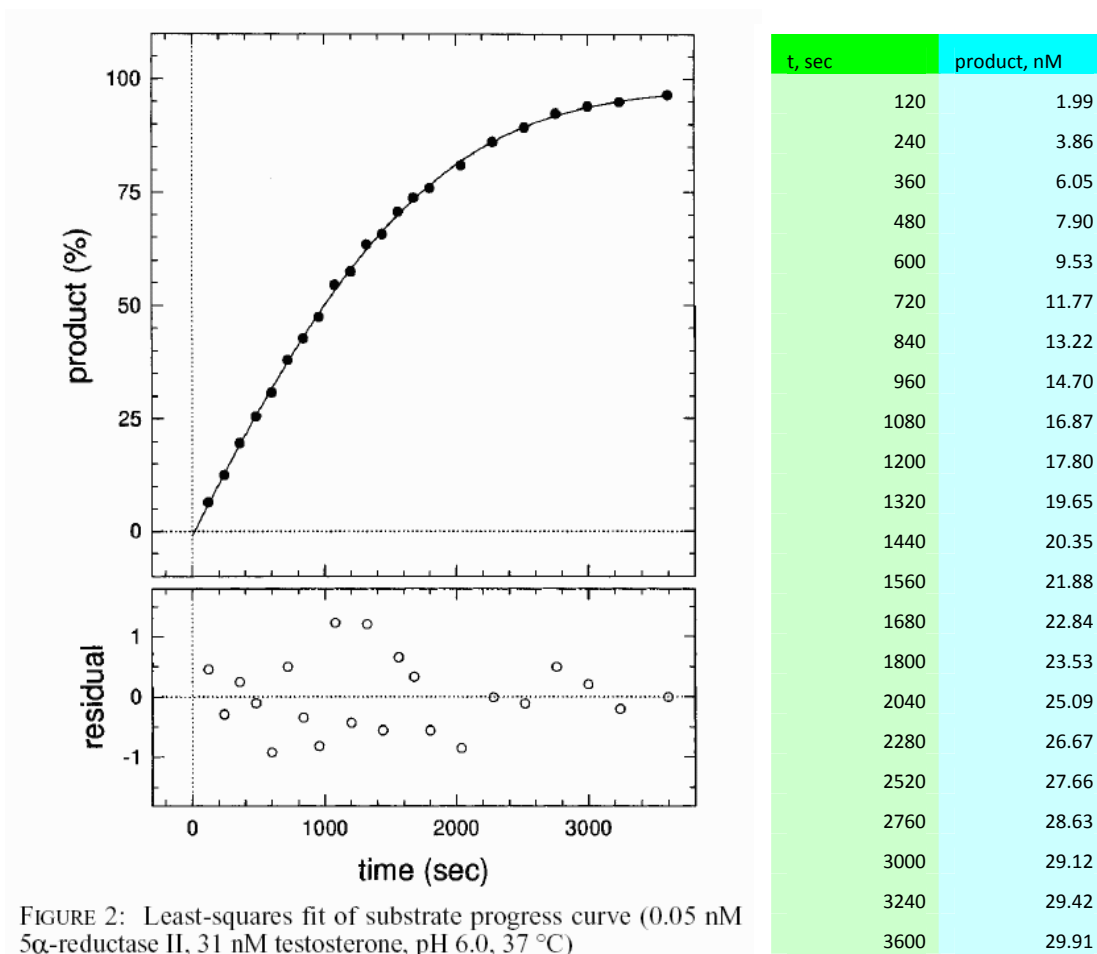
**BioKin Ltd.**

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## Introduction

The purpose of this DynaFit [1, 2] tutorial is to present detailed, step-by-step method to determine the substrate kinetic constants from a single well chosen reaction progress curve, which is purposely allowed to achieve full conversion of substrate into product. The experimental data are taken from ref. [3], Figure 2. The originally published data (percent product formation) were converted to absolute product concentrations, as shown in the Table below:



LEFT:

### Figure 1

Time course of testosterone reduction to dihydrotestosterone, catalyzed by 5 $\alpha$ -reductase, in the presence of NADPH. See ref. [3], Figure 2.

RIGHT

### Table 1

Data from Figure 1 converted from percent conversion to absolute concentrations of product (dihydrotestosterone, nM).

## Experimental Methods

An Eppendorf tube was prepared containing 31 nM [1,2,6,7-<sup>3</sup>H(N)]testosterone in 1.04 mL of the reaction buffer (17.6 mM imidazole, 17.6 mM diethanolamine, 0.3 M KCl, 13.2 mM succinic acid, 1 mM DTT at pH 7.0, 37 °C). To this tube 60 µL of enzyme (50 µM final concentration) was added to initiate the reaction. Aliquots were removed at various reaction times and quenched with excess ethanol. Product formation was monitored by HPLC (separating tritiated testosterone, T, and tritiated dihydrotestosterone, DHT) with radiometric detection, as described in ref. [4]. The T/DHT peak area ratios were utilized to compute the percent conversion of substrate to product at each reaction time. The raw data were subsequently converted to absolute product concentrations, in nM units (see Table immediately above). For additional details see ref. [3].

## DynaFit: Step-by-step tutorial

### 1. Export raw data in ASCII (text) format

Experimental data for DynaFit processing needs to be converted to the ASCII (plain text) format, either tab-delimited, space-delimited, or comma-delimited.

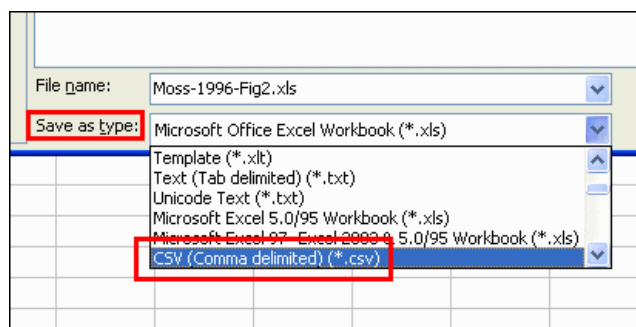
In this step we will export an Excel data sheet to a comma-delimited .CSV ("Comma Separated Values") plain text file, which will be subsequently analyzed by DynaFit.

#### PROCEDURE

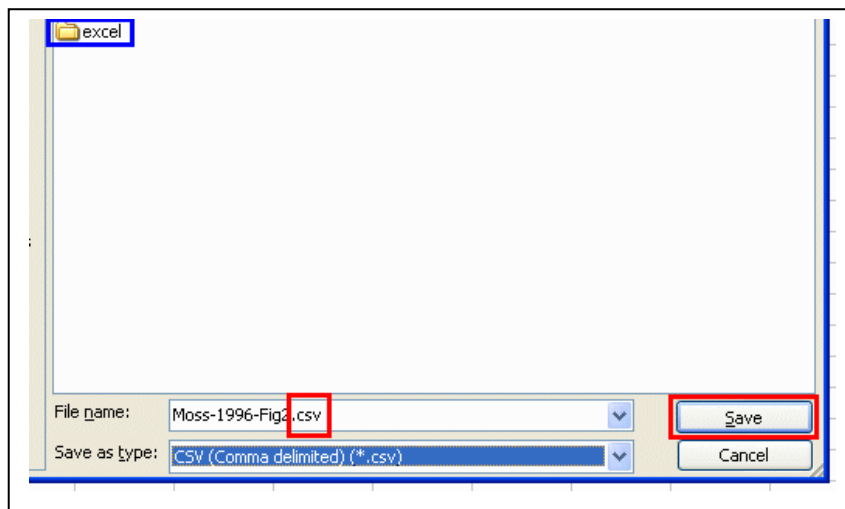
1. Navigate to the main DynaFit installation directory [**DynaFit4**]. From now on this directory will be abbreviated as **"/**".
2. Navigate to subdirectory **./technotes/2015/05/data/excel**
3. Open file **Moss-1996-Fig2.xls**
4. Go to sheet **"data"**
5. Select menu **"File ... Save As"**
6. Navigate to subdirectory **./technotes/2015/05/data**

This directory is one level up from the subdirectory **excel**.

7. Click on **"Save as Type"** drop down menu
8. Select **CSV (Comma delimited) (\*.csv)**



9. Save the file as **Moss-1996-Fig2.csv** (the default file extension).



## 2. Create DynaFit script

DynaFit scripts can be created in several different ways:

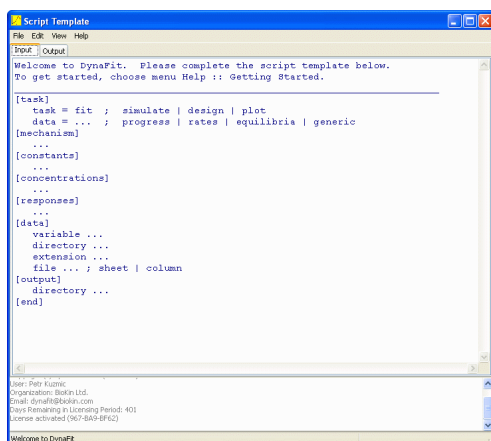
- From scratch (by modifying the built-in standard template).
- By modifying one of distributed template fragments (subdirectory **templates**).
- By modifying one of the complete example scripts.

In this step we will create a DynaFit script file by reusing one of the distributed templates. The script file will be subsequently used to determine substrate kinetic parameters from progress curve data.

## Insert a distributed template script

### PROCEDURE

1. Start DynaFit. This will display the text editor window containing the start-up minimal template:



2. Press "**Ctrl+N**" or equivalently select menu "**File ... New**".
3. Press **N** or equivalently click on the [No] (do not save changes) button.
4. Press "**Ctrl+S**" or equivalently select menu "**File ... Save**".
5. Navigate to directory **./technotes/2015/05**, where "." is the main DynaFit installation directory.
6. Type **fit** into the **File name** field. The file name extension **.txt** will be added automatically. Click [**Save**] button.
7. Press "**Alt+E ... I ... F ... F [Enter]**" or equivalently select menu "**Edit ... Insert ... File...**".
8. Navigate to directory **./templates/enzyme-kinetics/substrate/progress**.
9. Double-click to select file **michaelis-menten.txt**.
10. Press "**Ctrl+S**" to save the modified script file.

Our goal in the next several steps is to modify (edit) the default template text, which was just inserted into the DynaFit text editor.

## Modify the default script template

### *Modify the [concentrations] section*

The purpose of this section is to assign numerical values of initial concentrations.

#### **ORIGINAL**

```
[concentrations]
S = ...
E = ...
```

#### **MODIFIED**

```
[concentrations]
S = 31
E = 0.05
```

#### **NOTES**

- The concentration unit is **nanomolar**. Thus the above notation represents the initial concentrations  $[E]_0 = 50 \text{ pM}$ ,  $[S]_0 = 31 \text{ nM}$ .
- The concentration units must be identical throughout the entire DynaFit script and must correspond to the concentrations units employed in the experimental data files.

**Modify the [responses] section**

The purpose of this section is to assign numerical values of physical constants that linearly transform reacting species concentrations to the observed experimental signal.

**ORIGINAL**

```
[responses]
...
```

**MODIFIED**

```
[responses]
P = 1 ?
```

**NOTES**

- The raw experimental data file in this particular case contains nanomolar concentrations of the reaction product, as determined by HPLC. Therefore, the proportionality constant linking the data values to concentration values is simply unity.
- The question mark means that we wish to determine from the experimental data any possible non-unit value of the instrument scaling factor (i.e., essentially a systematic error of the HPLC peak integrator).

**Modify the [data] section**

This section specifies the location of experimental data file(s) located on the disk.

**ORIGINAL**

```
[data]
sheet ...
monitor E, E.S
offset auto ?
column ...
```

**MODIFIED**

```
[data]
sheet ./technotes/2015/05/data/Moss-1996-Fig2.csv
monitor E, E.S
offset auto ?
column 2
```

**PROCEDURE**

1. Delete the place-holder "..." following the keyword **sheet**.
2. Press "**Alt+E ... I ... F [Enter]**" or equivalently select menu "**Edit ... Insert ... File Name...**"
3. Navigate to directory **./technotes/2015/05/data**.

4. Select **CSV files (\*.csv)** from the **File types** drop-down menu.
5. Double-click on file **Moss-1996-Fig2.csv** to select it.
6. Delete the place-holder "... " following the keyword **column**.
7. Type **2** to specify the column number.

## NOTES

- The "offset" keyword represents the possible baseline shift of the measuring instrument (in this case the HPLC peak integrator).
- The question mark means that we wish to determine any possible nonzero value of the baseline shift from the experimental data.

## ***Modify the [output] section***

This section specifies the location of the output files to be generated by DynaFit.

### ORIGINAL

```
[output]
  directory ...
```

### MODIFIED

```
[output]
  directory ./technotes/2015/05/output/fit
```

### PRODEDURE

1. Delete the placeholder notation ("...") following the keyword **directory**.
2. Type "**Alt+E ... I ... D ... D ... [Enter]**" or equivalently select menu "**Edit ... Insert ... Directory name**"
3. Navigate to subdirectory **./technotes/2015/05** [OK]
4. Type the following at the end of the inserted line:  
**/output/fit**

### ALTERNATE PROCEDURE

1. Copy the text **./technotes/2015/05** from the [data] section.
2. Paste it to replace the placeholder ("...") following the keyword **directory**.
3. Type the following at the end of the inserted line:  
**/output/fit**

## **Modify the comment header**

### **ORIGINAL**

Enzyme substrate kinetics (units: uM, sec).  
Fit reaction progress to the Michaelis-Menten mechanism.

### **MODIFIED**

Fit data from Moss et al. (1996) Biochemistry 35, 3457, Fig. 2  
to the Michaelis-Menten kinetic model.

### **NOTES**

- Any text preceding the first **[task]** section and following the **[end]** line is ignored by DynaFit.
- This portion of the DynaFit script is well suited for placing brief comments summarizing various important items, such as:
  - origin of the data being analyzed (notebook pages or literature references);
  - goal of the analysis (why are we doing this);
  - summary of results (what did we learn).

**Save ("Ctrl+S") the final edited version of the script file.**

### **Final form of the modified template text**

After all modifications described above, the DynaFit script file **fit-001.txt** should read as follows:

```
Fit data from Moss et al. (1996) Biochemistry 35, 3457, Fig. 2
to the Michaelis-Menten kinetic model.
;
[task]
  data = progress
  task = fit
[mechanism]
  E + S <=> E.S      :   ka.S   kd.S
  E.S ---> E + P     :   kd.P
[constants]
  ka.S = 1
  kd.S = {0.0001, 0.001, 0.01, 0.1, 1, 10} ?
  kd.P = {0.0001, 0.001, 0.01, 0.1, 1, 10} ?
[concentrations]
  S = 31
  E = 0.05
[responses]
  P = 1 ?
[data]
  sheet ./technotes/2015/05/data/Moss-1996-Fig2.csv
  monitor E, E.S
  offset auto ?
  column 2
```



```
[output]
  directory ./technotes/2015/05/output/fit
[end]
;
;
```

---



---

### 3. Perform the least-squares fit

#### PROCEDURE

1. Press **Ctrl+U** or equivalently select menu "**File ... Run**".
2. Press **Ctrl+J** or select menu "**View ... Results in External Browser**".
3. Navigate in the various parts of the auto-generated output

#### NOTES

- Upon execution, DynaFit automatically writes a collection of various output files (HTML, GIF, EPS, TXT) into a newly created directory named in the **[output]** section.
- The main output file is named **index.html**. It is automatically displayed in the DynaFit main output windows.
- The output files can be navigated using a rudimentary HTML web browser built into DynaFit. However, the DynaFit web browser is *not* equipped to display output in highly stylized form. Instead, it is advantageous to view results of fit using the default web browser installed on the user's computer.
- It is advantageous to use the following shortcuts either in DynaFit or in the external web browser:

**Alt+Left Arrow**      go to the previous page  
**Alt+Right Arrow**    go to the next page

### 4. Examine the final results of fit

This section assumes that steps #1 through #3 above were performed as described.

#### Theoretical model derived by DynaFit

#### PROCEDURE

1. Click on the "**Model**" link shown on the main output page
2. Examine the fitting model automatically derived by DynaFit:

**DYNAFIT OUTPUT****Model equation**

The fitting function (model equation) for each individual data set is

$$S(t) = S_0 + \sum_{i=1}^n r_i c_i(t)$$

where

$S(t)$  the experimental signal observed at time  $t$

$S_0$  offset on the signal axis (a property of the instrument)

$n$  number of unique molecular species participating in the reaction mechanism

$c_i(t)$  the concentration of the  $i$ th species at time  $t$

$r_i$  the molar response coefficient of the  $i$ th species

In this case,  $n = 4$ . The molecular species participating in the mechanism are: **E**, **S**, **E.S**, **P**.

The concentrations of these molecular species at time  $t$  are computed from their initial concentrations (at time zero,  $t = 0$ ) by solving an **initial-value problem** defined by a system of simultaneous first-order Ordinary Differential Equations (**ODEs**) listed below.

**ODE system**

*Initial value problem:* Given the concentrations of molecular species at  $t = 0$ , compute the concentrations at an arbitrary later time  $t > 0$  by solving the following system of ODEs. The solution is obtained by *numerical integration*.

$$d[E]/dt = -k_{a,s}[E][S] + k_{d,s}[E.S] + k_{cat}[E.S]$$

$$d[S]/dt = -k_{a,s}[E][S] + k_{d,s}[E.S]$$

$$d[E.S]/dt = +k_{a,s}[E][S] - k_{d,s}[E.S] - k_{cat}[E.S]$$

$$d[P]/dt = +k_{cat}[E.S]$$

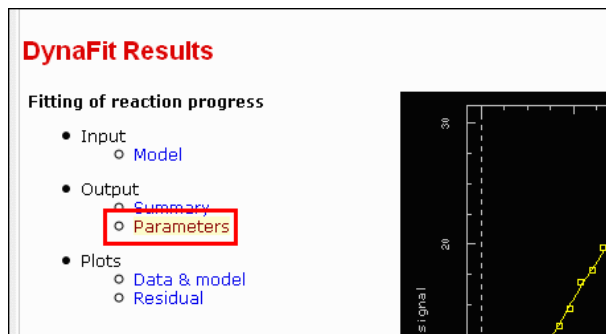
## NOTES

- Many DynaFit users are frequently asking (perhaps because they were asked by the reviewers of manuscripts where DynaFit was used to analyze the data): **"What was, in fact, the fitting equation?"**
- In the case of fitting the reaction progress (`data = progress`) the proper answer is shown in the **highlighted** mathematical notation above.
- In plain English, there is never just *one* fitting equation (in particular an *algebraic* equation) but rather a complete *system of differential* equations that is be "solved numerically" (i.e., iteratively).
- This differential equation formalism is nothing novel, somehow freshly "invented" for use in DynaFit. In fact, the ODE formalism has been used for many decades, in literally thousands of published reports, to analyze the time course biochemical reactions [5-9].
- When asked to provide "the fitting equation", copy and paste the mathematical code highlighted above, including the accompanying word definitions.

## Best-fit parameter values

### PROCEDURE

1. Click on the "Parameters" link shown on the main output page



2. Copy and past the Optimized Parameters table into a report, such as a Microsoft Word file:

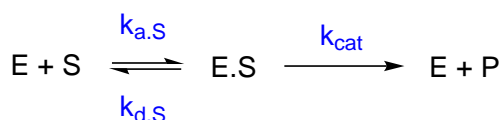
No.	Par#Set	Initial	Final	Std. Error	CV (%)	Note
#1	kd.S	100	19.9	3.3	16.7	
#2	k.cat	1	0.611	0.048	7.9	
#3	r(P)	1	0.996	0.013	1.3	
#4	offset#1	0	-0.32	0.19	58.4	

#### Optimized Parameters

No.	Par#Set	Initial	Final	Std. Error	CV (%)	Note
#1	kd.S	100	19.9	3.3	16.7	
#2	k.cat	1	0.611	0.048	7.9	
#3	r(P)	1	0.996	0.013	1.3	
#4	offset#1	0	-0.32	0.19	58.4	

### Units of rate constants

The reaction mechanisms assumed throughout this tutorial is as follows:



In the DynaFit script listed above, we assumed that the concentrations are in *nanomolar* units and the reaction time is in *seconds*. We also assumed that the association rate constant  $k_{a,S}$  is fixed at the diffusion-limited value  $10^9 \text{ M}^{-1} \text{ s}^{-1}$  ("rapid equilibrium"). Therefore, the microscopic rate constants listed in the scheme above can be summarized as follows:

Constant	Unit	Value	Standard error	Note
$k_{a,S}$	$10^9 \text{ M}^{-1} \text{ s}^{-1}$	1		Fixed parameter
$k_{d,S}$	$\text{s}^{-1}$	19.9	3.3	
$k_{cat}$	$\text{s}^{-1}$	0.611	0.048	

In a separate Technical Note, these values will be used as fixed model parameters in subsequent analyses of time-dependent inhibition data [3].

### Systematic error of the HPLC detection method

The best fit value of the product response function,  $r_p = (1.00 \pm 0.01)$ , suggests that in this case the *scaling* of the HPLC detection method was free of any systematic errors.

However, there is a hint that the *baseline* of the HPLC detection method might be biased toward a slightly negative value, **offset = (-0.3 ± 0.2) nM**. Given that the initial concentration of testosterone as substrate was 31 nM, a bias of -0.3 nM corresponds to at most one percent systematic error, which is negligibly small given the random experimental noise (approximately one percent, as see in the distribution of residuals).

### Steady-state kinetic constants, $K_m$ and $k_{cat}$

Although the primary use of substrate rate constants determined in this tutorial is to use them as fixed parameters in the analysis of *time-dependent inhibition* data (see separate BioKin Technical Note TN-2015-06), it might be of interest to compute from the best-fit values of microscopic rate constants the corresponding steady-state kinetic constants,  $K_m$  and  $k_{cat}$ . For the reaction mechanism shown above, the definition of the Michaelis constant is given by Eqn (1):

$$K_m = \frac{k_{dS} + k_{cat}}{k_{aS}} \quad (1)$$

Thus we can calculate

$$K_m = \frac{19.9 + 0.6}{1} = 20.5 \text{ nM}$$

The steady-state turnover number,  $k_{cat}$ , is simply the value listed in the parameter table above.

If we wished to compute also the standard error of  $K_m$  from the standard errors of the microscopic rate constants, we could utilize the standard statistical *error propagation theory*, as explained for example by Bevington [10]. Accordingly the standard error of  $K_m$  (denoted as  $\delta K_m$  below) will be computed as the standard error of a *sum* of two terms, each of which is affected by the standard error  $\Delta$ :

$$\delta K_m = K_m \sqrt{\left(\frac{\delta k_{dS}}{k_{dS}}\right)^2 + \left(\frac{\delta k_{cat}}{k_{cat}}\right)^2} = 19.9 \sqrt{\left(\frac{3.3}{19.9}\right)^2 + \left(\frac{0.048}{0.611}\right)^2} = 3.7$$

Thus, the final values of steady-state parameters, including the associated formal standard errors, are:

- $K_m = (21 \pm 4) \text{ nM}$  **this work**
- $k_{cat} = (0.61 \pm 0.05) \text{ s}^{-1}$

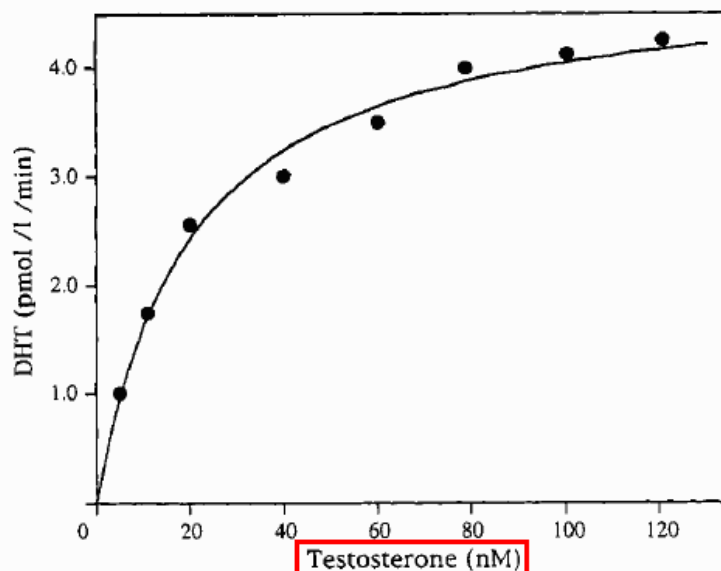
It is instructive to compare these results with the published values obtained under exactly identical experimental conditions, while utilizing the classic "initial rate" method. Faller *et al.* [11] reported the value

- $K_m = (20 \pm 3) \text{ nM}$  **reported**

as illustrated in the Figure below copied from ref. [11]:

*Biochemistry, Vol. 32, No. 21, 1993*

Faller et al.



**FIGURE 1:** Determination of the  $K_m$  value of human prostatic 5-AR for testosterone. Reactions were initiated by the addition of enzyme (25  $\mu\text{g}/\text{mL}$  final concentration) to a buffered solution containing 0.4 mM NADPH and [ $^3\text{H}$ ]testosterone (5–120 nM). The solid line represents the best fit to the Michaelis–Menten equation. A  $K_m$  value of  $20 \pm 3$  nM is calculated from nonlinear regression analysis of the experimental data.

## Publication-quality plot in PostScript (EPS) format

To produce a publication quality plot in EPS format follow these steps:

### PROCEDURE:

1. Append the following text at the end of the script file **fit-001.txt**:

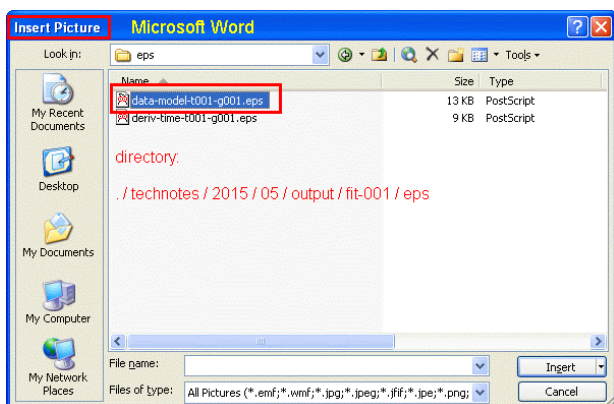
```
[settings]
{Output}
  XAxisLabel = time, sec
  YAxisLabel = [DHT], nM
[end]
```

DHT is dihydrotestosterone, the reaction product.

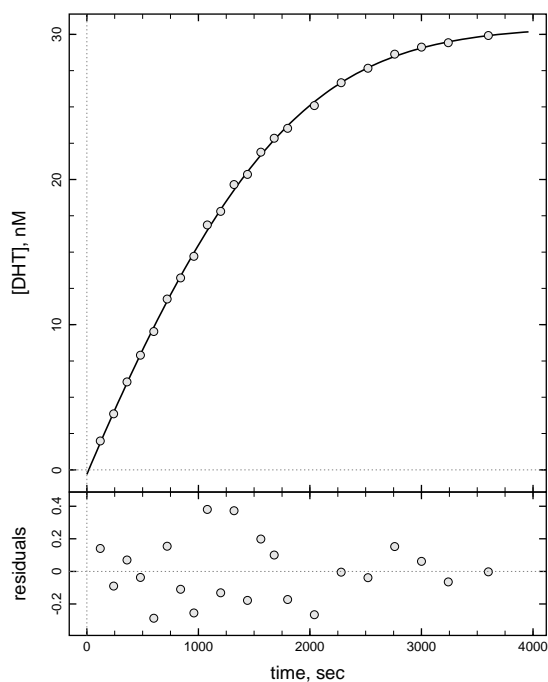
2. Select menu "**File ... Run**" again, or press **Ctrl-U**
3. Open a suitable report file, such as Microsoft Word or PowerPoint.
4. In Microsoft Word, select menu "Insert ... Picture ... From File"
5. Navigate to subdirectory **./technotes/2015/05/output/fit-001/eps**
6. Select file **data-model-t001-g001.eps** [OK]

This will insert the DynaFit-generated EPS file into your document, as shown below:

SELECTING AN EPS FILE:



EPS FILE AFTER INSERTION:



## Discussion

A variety of reliable numerical methods can be used profitably to determine steady-state kinetic constants ( $K_m$  and  $k_{cat}$ ) from a *single* well chosen enzyme progress curve. Over the last approximately four decades, starting in the late 1970's, several data processing methods in this category have been developed by Duggleby [12, 13]. Similar methods have been devised by Boeker [14, 15] at approximately the same time.

Both Duggleby's and Boeker's methods rely on *analytic (closed-form)* integration of the requisite differential rate equation, for example the Michaelis-Menten equation:

$$\frac{d[S]}{dt} = -[E]_0 k_{cat} \frac{[S]/K_m}{1 + [S]/K_m}$$

In this DynaFit tutorial we have performed *numerical (iterative)* integration of an equivalent system of simultaneous differential equations, using an initial-value ODE solver [16] embedded in the DynaFit software package. However, regardless of the integration method (analytic or numerical) the fundamental, well tested approach is the same.

The results show that the  $K_m$  values obtained either by the classical initial rate method,  $K_m = (20 \pm 3)$  nM as reported in ref. [11], or by the numerical integration method reported here,  $K_m = (20 \pm 4)$  nM, are identical.<sup>1</sup> The advantage of the present method is that it requires only a single kinetic experiment (i.e., a single well chosen substrate concentration), whereas the classic initial rate method relies on multiple experiments performed at a variety of substrate concentrations.

Very important prerequisites for successful application of the single progress curve method are that:

1. The enzyme reaction is allowed to proceed essentially to completion (complete or nearly complete conversion of substrate to product).
2. The enzyme does not undergo denaturation in the course of the assay.<sup>2</sup>
3. The substrate concentration is chosen such that it is at least as high as the Michaelis constant, but optimally it should be approximately two-fold higher.

However, the main purpose of the data analysis presented in this tutorial was to obtain reliable estimates of the *microscopic* rate constants ( $k_{aS}$ ,  $k_{dS}$ , and  $k_{cat}$ ), rather than the steady-state parameters such as  $K_m$ . These micro-constants will be subsequently used to properly analyze the time course of time-dependent ("slow, tight") enzyme inhibition as originally described in ref. [3]

---

<sup>1</sup> The original report by Faller et al. [11] did not report a  $k_{cat}$  value, because those authors worked with microsomal fraction containing the enzyme, rather than with a cloned and purified enzyme.

<sup>2</sup> Fortunately, if the enzyme does lose activity over time, this would manifest clearly in the lack of fit to the simple Michaelis-Menten kinetics mechanism. In this particular regard the present method is robust, in the sense that we could easily tell whether it is applicable or not in any given situation.



## References

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15. Boeker, E. A., Integrated rate equations for irreversible enzyme-catalysed first-order and second-order reactions, *Biochem. J.* 226 (1985) 29-35.
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