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Binding and Kinetics for Experimental Biologists

Lecture 6

Enzyme kinetics: Analysis of reaction progress

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY OP Vzdělávání pro konkurenceschopnost UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

BioKin L t d.

Lecture outline

- Goal:

  Demonstrate K_M determination from progress curves using numerical methods.
  Use a "real-world" problem arising from the instructor's consulting practice.

- Topics:

  The Michaelis constant: theory and practice.
  The illusion of linearity in enzyme assays.
  Global vs. local fit of multiple data sets: Mechanistic implications.
  The choice of proper time interval to measure the "initial" rates.
  Assessing the randomness of residual plots.
  Enzyme deactivation mechanisms.

- Implementation:

  The DynaFit software package.

Determination of "K_M" – a Case Study

or

A Day in the Life of an Enzymology Consultant

The setup: FDA put a "hold" on a drug application

FDA = UNITED STATES **FOOD AND DRUG ADMINISTRATION** AGENCY

DEPARTMENT OF HEALTH & HUMAN SERVICES — Public Health Service

Food and Drug Administration
Rockville, MD 20857

FULL CLINICAL HOLD

your proposed study has been placed on clinical hold.

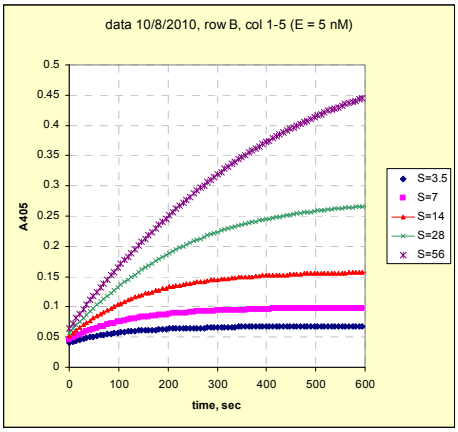
We have the following comments, to be addressed prior to submission of a license application:

1. A potency assay that measures the kinetic parameters (K_M and k_{cat}) and uses a physiologically relevant substrate should be incorporated in release testing and stability programs for drug substance and product prior to initiation of the Phase 3 clinical trial.

- The drug candidate is an enzyme.
- This is a small biotech company – no kineticist on staff.
- The solution: **Hire a consultant** to
  - design a biochemical assay; and
  - propose a method of data analysis.

The raw data

- vary **[Substrate]** from 3.5 to 56 μM
- add **[Enzyme]** at 5 nM
- observe UV/Vis absorbance at 405 nM



➡ K_M ?

Michaelis constant: Mechanistic definition

"K_M" HAS **TWO DIFFERENT MEANINGS**, EVEN FOR THE SIMPLEST POSSIBLE ENZYME MECHANISM

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

$$K_M = \frac{k_2 + k_3}{k_1}$$

$k_{cat} = k_3$

equilibrium dissociation constant

$K_d = k_2 / k_1$

"strength of enzyme/substrate binding"

special case: when dissociation (k_2) is much faster than chemical reaction (k_3)

$k_2 >> k_3$ ➡ $k_2 + k_3 \approx k_2$ ➡ $K_M = (k_2 + k_3) / k_1 \approx k_2 / k_1 = K_d$

K_M ..... **substrate binding**

k_{cat} ..... **reactivity to form product**

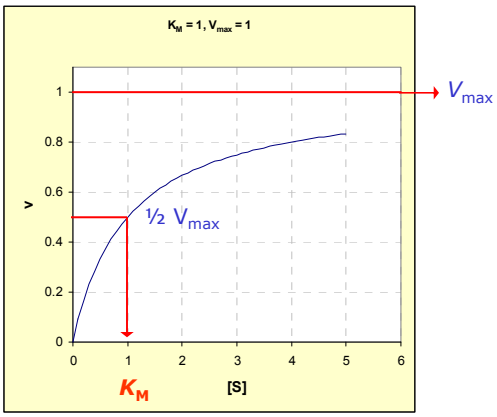
Michaelis constant: Experimental definition

IF THE DOSE-RESPONSE CURVE IS HYPERBOLIC, THEN "K_M" HAS ONLY **ONE MEANING**

Michaelis-Menten equation:

$$v = k_{cat}\,[E]\,\frac{[S]}{[S] + K_M}$$

$$V_{max} = k_{cat}\,[E]$$



Michaelis constant: The classic recipe

THE SIMPLEST POSSIBLE APPROACH ("BIOCHEM-101 STUDENT LAB")

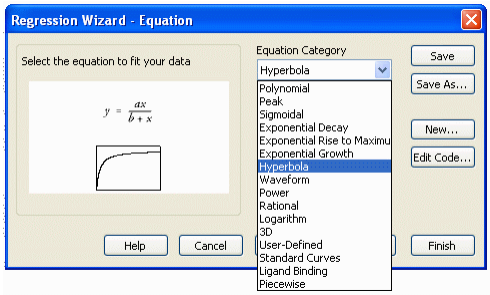
1. Perform a number of assays at constant [E] and varied [S]
2. Fit each progress curve (or "initial portion") to a **straight line**
3. Consider the slope of each best-fit line as the "initial rate" (***v***)
4. Start up a computer program for nonlinear regression
5. In the list of built-in nonlinear models, find the "rectangular hyperbola"

$$\mathbf{y} = a\,\mathbf{x} / (b + \mathbf{x})$$

where

$\mathbf{x}$ = [S]
$\mathbf{y}$ = ***v***
a = V_{max}
b = K_M

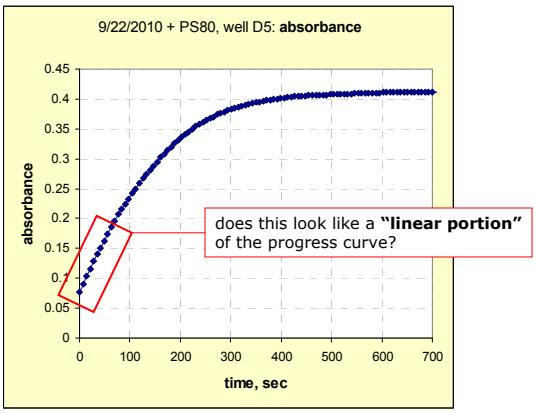
model parameters



6. Click the [OK] button. Paste K_M and V_{max} into your final Report.
7. If k_{cat} is also required, compute it as V_{max} / [E]

The human eye is very bad at seeing nonlinearity

WE **EVOLVED** TO SEE **STRAIGHT LINES** - EVEN WHEN THERE ARE NONE!

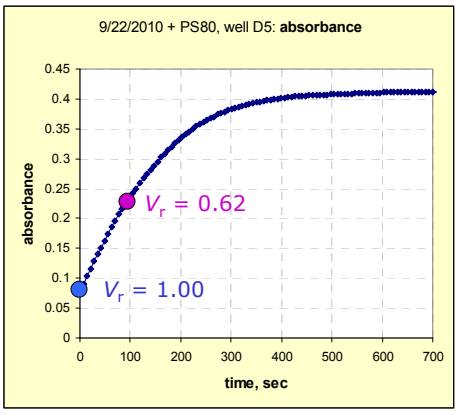


If this *were* a **"linear portion"**, our problems would be over:
We could get the initial rate by fitting a **straight line** to the first **100 seconds**.

Derivatives approximated as "finite differences"

CHECKING LINEARITY BY SIMPLE COMPUTATIONS

rate ≈ Δ[Signal] / Δtime



D3 =1000*(C3-C2)/(B3-B2)

	A	B	C	D	E
1	#	time	A_{405}	1000 x V	V / V_0
2	0	0	0.077		
3	1	7	0.090	1.8571	1.00
4	2	14	0.103	1.8571	1.00
5	3	21	0.116	1.8571	1.00
6	4	28	0.128	1.7143	0.92
7	5	35	0.140	1.7143	0.92
8	6	42	0.151	1.5714	0.85
9	7	49	0.163	1.7143	0.92
10	8	56	0.174	1.5714	0.85
11	9	63	0.185	1.5714	0.85
12	10	70	0.196	1.5714	0.85
13	11	77	0.207	1.5714	0.85
14	12	84	0.216	1.2857	0.69
15	13	91	0.225	1.2857	0.69
16	14	98	0.233	1.1429	0.62

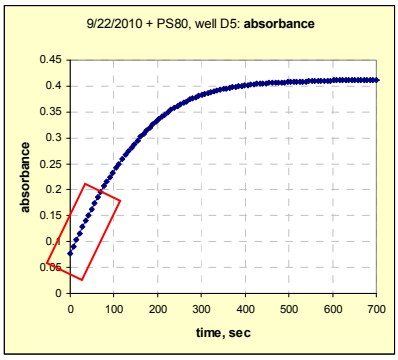
relative rate

By the time the assay reached 100 seconds,
the reaction rate decreased by almost one half!

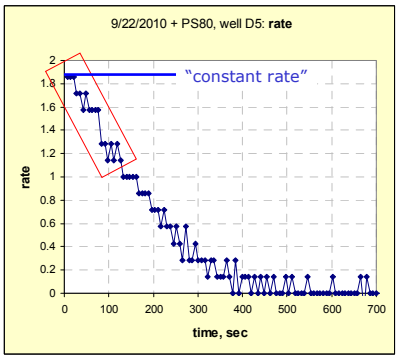
The plot of instantaneous rates

IF THERE WERE A "LINEAR PORTION", THE **RATE** PLOT SHOULD BE **STRICTLY HORIZONTAL**

signal



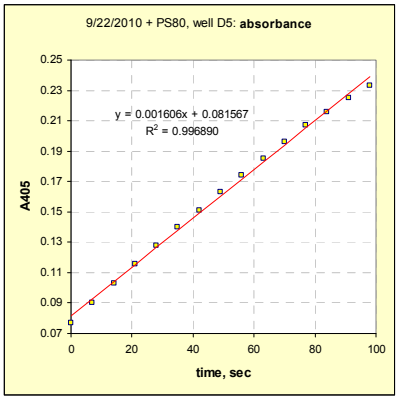
rate ≈ Δ [Signal] / Δ time

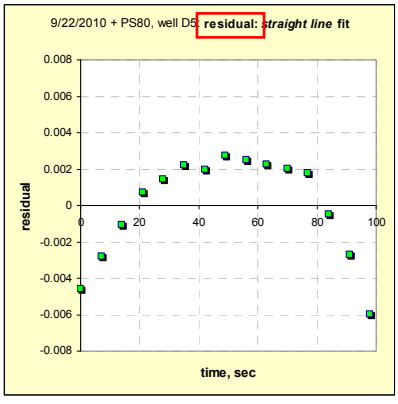


By the time the assay reached 100 seconds,
the reaction rate decreased by almost one half!

Trying the straight-line fit anyway

HOW BAD COULD IT BE, REALLY?



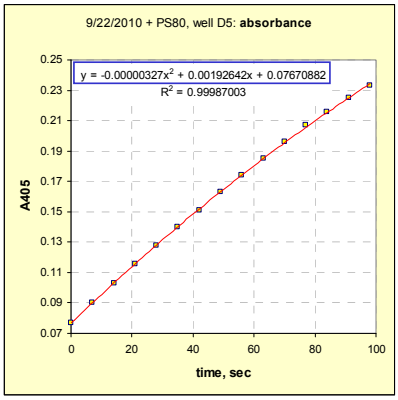


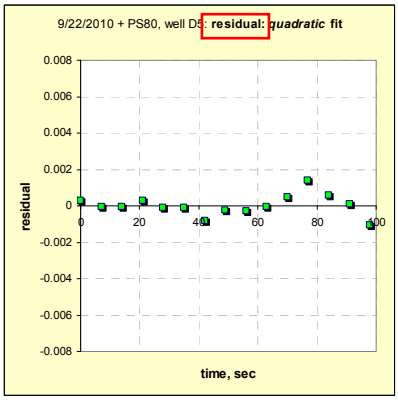
quite bad, actually:
this plot should look completely "random"

What about a quadratic polynomial?

CAREFUL HERE: POLYNOMIALS ARE **UNSUITABLE FOR EXTRAPOLATIONS!**

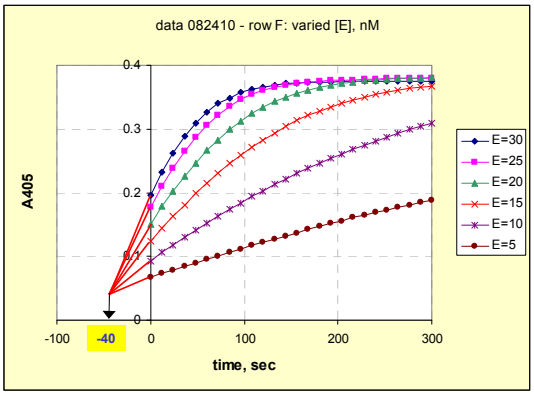
Why would we need to ***extrapolate***?





Mixing delay time

IN MOST KINETIC EXPERIMENTS WE NEVER "SEE" THE VERY INITIAL TIME POINT



enzyme is UV/Vis transparent
substrate concentration is the same

absorbance at time zero should be identical...

... unless a lot of product was formed already at "t = 0"

actual start of the assay: "*time* = **-40** sec"

The devil in the details: *Initial* reaction rates

A FEW INNOCENT-SOUNDING CHALLENGES IN ANALYZING THE **PROGRESS OF ENZYME ASSAYS**:

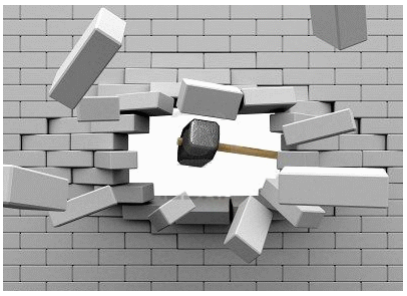
1. The time-course of enzyme reaction is *nonlinear.*
   It can be considered "almost linear" only in special cases.
2. There is a finite *mixing delay* requiring *extrapolation.*
   In our experiments we always "miss" the *initial* rate.
3. Extrapolation is dangerous - unless we use a *mechanistic* model.
   Generic mathematical models such as polynomials are "out".

All these problems would disappear if/when
the progress of enzyme assays *can* be treated as **linear**.

This is ***almost never*** the case.

linear, polynomial

We hit the brick wall with algebraic models...



... so why not do it the right way?

Mechanistic mathematical model

MECHANISTIC MODELS ARE WELL SUITED FOR **EXTRAPOLATIONS**

Michaelis-Menten mechanism:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

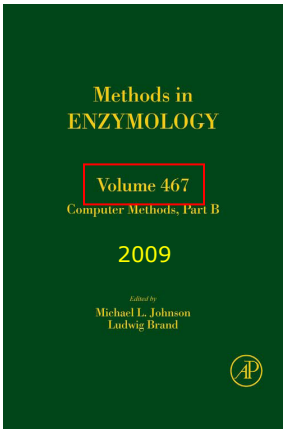
Mathematical model for reaction progress:

$$d[E]/dt = -k_1[E][S] + k_2[ES] + k_3[ES]$$
$$d[S]/dt = -k_1[E][S] + k_2[ES]$$
$$d[ES]/dt = +k_1[E][S] - k_2[ES] - k_3[ES]$$
$$d[P]/dt = +k_3[ES]$$

requires numerical (iterative) solution using a specialized complex algorithm

Specialized numerical software: *DynaFit*

MORE THAN **600 PAPERS** PUBLISHED WITH IT (1996 – 2009)



CHAPTER TEN

DYNAFIT—A SOFTWARE PACKAGE FOR ENZYMOLOGY

Petr Kuzmič

DOWNLOAD http://www.biokin.com/dynafit

REFERENCES

1. Kuzmic (1996) *Anal. Biochem.* **237**, 260-273
2. Kuzmic (2009) *Meth. Enzymol.* **467**, 247-280

The Van Slyke – Cullen mechanism

JUST AS GOOD AS MICHAELIS-MENTEN

Van Slyke-Cullen mechanism:

$$\mathbf{E + S} \xrightarrow{k_1} \mathbf{ES} \xrightarrow{k_3} \mathbf{E + P}$$

Mathematical model for reaction progress:

$$d[E]/dt = -k_1[E][S] + k_3[ES]$$
$$d[S]/dt = -k_1[E][S]$$
$$d[ES]/dt = +k_1[E][S] - k_3[ES]$$
$$d[P]/dt = +k_3[ES]$$

DynaFit input:

```
[task]

   task = fit
   data = progress

[mechanism]

   E + S ---> E.S     :     k1
   E.S ---> E + P     :     k3

...
```

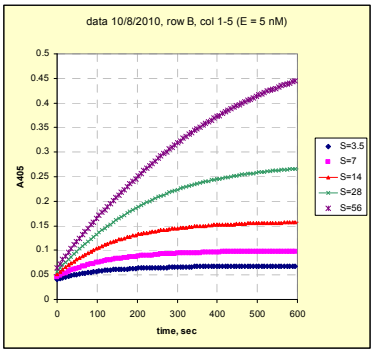
Application of the Van Slyke–Cullen irreversible mechanism in the analysis of enzymatic progress curves
Kuzmic (2009) *Anal. Biochem.* **394**, 287-289

Global vs. "local" analysis

A VERY IMPORTANT CONCEPT

[2] Global Analysis of Biochemical and Biophysical Data
By JOSEPH M. BEECHEM

Meth. Enzymol. **210**, 37-54 (1992)



global analysis:
analyze **all five** curves together
as a single data set

local analysis:
analyze each curve **individually**
as five separate data sets

Local analysis to determine initial reaction rates

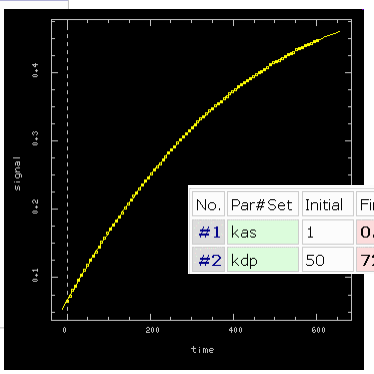
WE NEED INITIAL RATES FOR THE MICHAELIS-MENTEN EQUATION

DynaFit : row-B-local-003.txt

File Edit View Help

Input Output

```
Fit one progress curve locally, to determine init
(more precisely: reaction velocity at t = 1 sec).

;_____________________________________________

[task]

   task = fit
   data = progress

[mechanism]

   E + S ---> E.S     :     kas
   E.S ---> E + P     :     kdp

[constants]

   kas = 1 ?
   kdp = 50 ?

[concentrations]

   E = 0.005
```



No.	Par#Set	Initial	Final
#1	kas	1	0.721467
#2	kdp	50	72.6279

Reading off the initial rates

"INITIAL RATE" IS COMPUTED AT A SPECIFIC REACTION TIME (E.G. 1 SEC)

Input Output

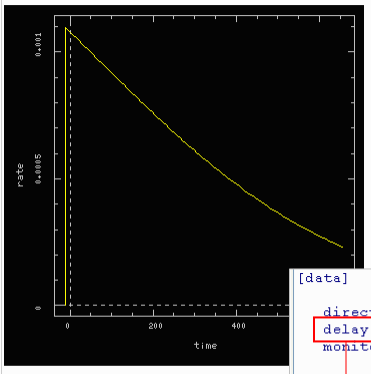
DynaFit Results

- Input
  - Script
  - Settings

Fitting of reaction progress

- Input
  - Model
- Output
  - Summary
  - Parameters
- Plots
  - Data & model
  - State variables
  - Residual

Derivatives (reaction rates)



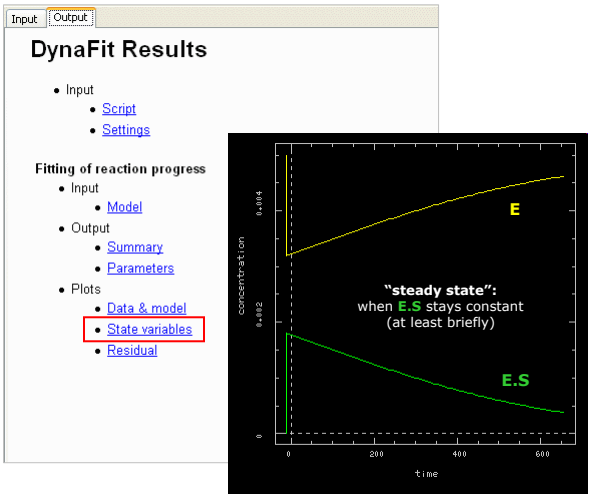

```
[data]

   directory ./courses/BKEB/
   delay 12
   monitor E, E.S

   file B05.txt  |  offset =
```

Initial reaction rates

No.	Dataset	Time (sec)	Rate
1	./../courses/BKEB/lec-6/km/data-10min/B05.txt	-11	0.00109813

Plot of state variables (species concentrations)

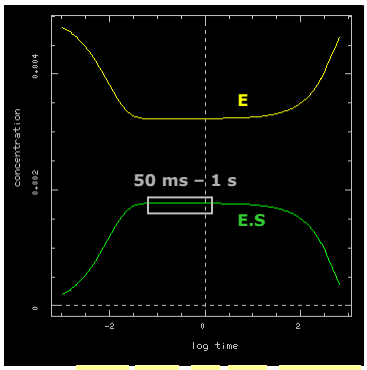
ALWAYS EXTREMELY HELPFUL




```
[data]

   directory ./courses/BKEB/
   delay 12
   monitor E, E.S

   file B05.txt  |  offset =
```


Logarithmic plot: Identify the steady-state range

WHEN A BIOCHEMICAL ASSAY REACHES THE **STEADY-STATE** IS SYSTEM-SPECIFIC



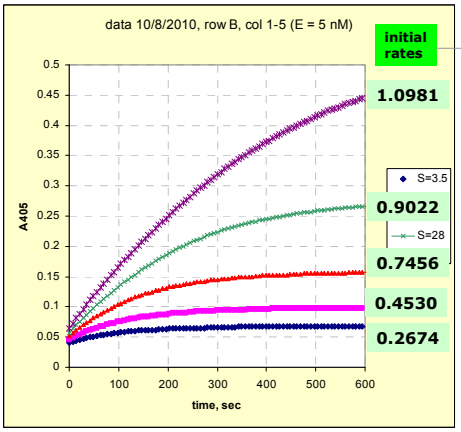

```
[settings]

(Filter)
   TimeInitialRate = 1
```

tell DynaFit at what time we want to read-off the **"initial" reaction rate**

```
[data]

   directory ./courses/BKEB/
   plot      logarithmic
   monitor   E, E.S
```

Initial rates from local fit of progress curves

FINALLY READY TO COMPUTE THE K_M



K_M ?

Fit initial rates – initial estimate

DynaFit : rates-001.txt

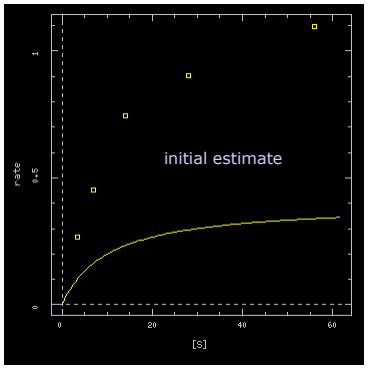
File Edit View Help

Input Output

```
[task]
   task = fit
   data = rates
   approximation = rapid-equilibrium

[mechanism]

   E + S <==> E.S    :    Km    dissoc
   E.S ---> E + P    :    kcat

[constants]

   Km   = 10 ??
   kcat = 10 ??

[responses]

   P = 8

[concentrations]

   E = 0.005

[data]

   variable S
   set rates
```

```
[set:rates]

S        rate

3.5      0.2674
7        0.4520
14       0.7457
28       0.9022
56       1.0981

[end]
```

"File .. Try"



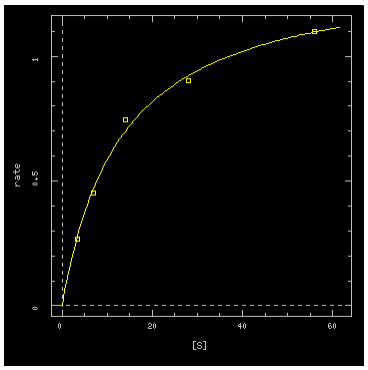
Fit initial rates – results

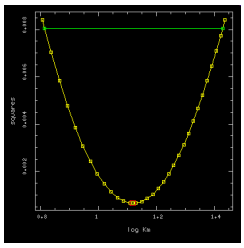
REMEMBER: THE STANDARD ERROR (± **VALUE** FREQUENTLY REPORTED) IS **MEANINGLESS**

standard error

K_M = (13.1 ± 1.6) [6.5 .. 26.8] μM

confidence interval (99% probability





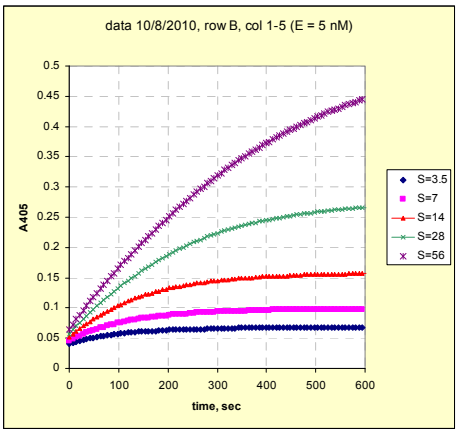
sum of squares

log K_M

No.	Par#Set	Initial	Final	Std. Error	CV (%)	Low	High
#1	Km	10	13.1442	1.57495	11.98	6.52297	26.8159
#2	kcat	10	33.8915	1.5029	4.43	26.649	45.2416

The Michaelis constant from initial rates

ALMOST DONE .... *MAYBE*



K_M = **13.1 μM**

are we done yet?

A closer look:

what else we can learn from the same data?

Global fit of all five progress curves

VAN-SLYKE CULLEN MECHANISM (SAME AS BEFORE, IN THE LOCAL FIT)

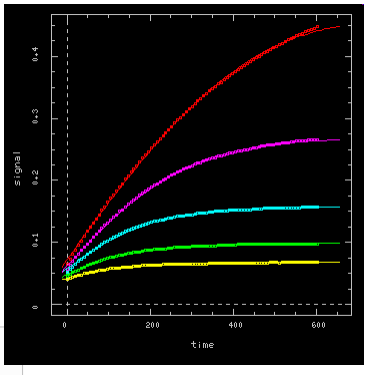
DynaFit : row-B-global-001.txt

File Edit View Help

Input Output

```
Global fit of all five progress curves.
;
[task]

   task = fit
   data = progress

[mechanism]

   E + S ---> E.S    :    kas
   E.S ---> E + P    :    kdp

[constants]

[data]

   directory ./courses/BKEB/lec-6/km/data-10min
   delay 12

   file B01.txt |  offset = auto ? | conc S = 3.5 ?
   file B02.txt |  offset = auto ? | conc S =   7 ?
   file B03.txt |  offset = auto ? | conc S =  14 ?
   file B04.txt |  offset = auto ? | conc S =  28 ?
   file B05.txt |  offset = auto ? | conc S =  56 ?
```

File .. Run

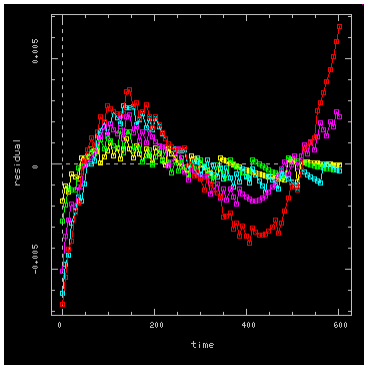


looks "reasonably good"

or does it ?!

Global fit – residual plot

DOES NOT LOOK GOOD AT ALL



If you see a residual plot as **bad** as this one,

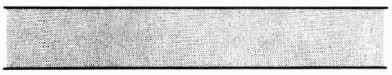
completely ignore any other result from DynaFit.

Always look at residual plots.

"Good" and "bad" residual plots

ASSESSING RESIDUAL PLOTS IS ONE OF THE **MOST IMPORTANT** SKILLS OF A DATA ANALYST

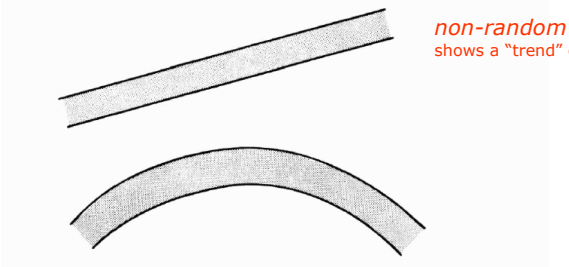
GOOD:



A satisfactory residuals plot should give this overall impression.

random sequence
no "trend" or "pattern"

BAD:



Examples of characteristics shown by unsatisfactory residuals behavior.

non-random sequence
shows a "trend" or a "pattern"

Draper & Smith (1981) *Applied Regression Analysis*, p. 146

Global fit – model parameters

DOES NOT LOOK GOOD AT ALL, EITHER

No.	Par#Set	Initial	Final
#1	kas	1	1.74027
#2	kdp	50	31.7959
#3	[S]#1	3.5	3.31032
#4	offset#1	0.041	0.0360932
#5	[S]#2	7	6.43236
#6	offset#2	0.046	0.0362807
#7	[S]#3	14	12.6996
#8	offset#3	0.052	0.0351632
#9	[S]#4	28	25.5942
#10	offset#4	0.057	0.0195611
#11	[S]#5	56	48.0148
#12	offset#5	0.065	-0.00229371

```
[mechanism]

   E + S ---> E.S     :     kas
   E.S ---> E + P     :     kdp
```

$K_M = k_{dp}/k_{as}$ = 18.2 µM

13.1 µM from initial rates

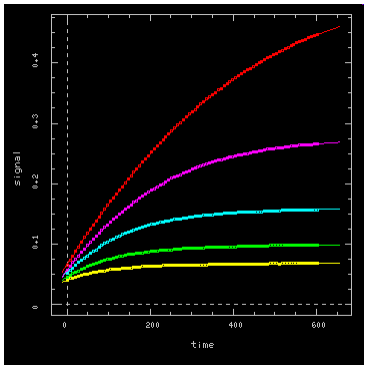
These "best fit" concentrations are highly improbable:

It has been established that the actual **titration error** is much smaller than this.

Modified global model: *Enzyme deactivation*

ENZYMES DO "DIE" IN THE COURSE OF MANY ASSAYS

```
[mechanism]

   E + S ---> E.S     :     kas
   E.S ---> E + P     :     kdp
   E     ---> E*      :     kie
   E.S ---> E.S*      :     kies
            denatured
```

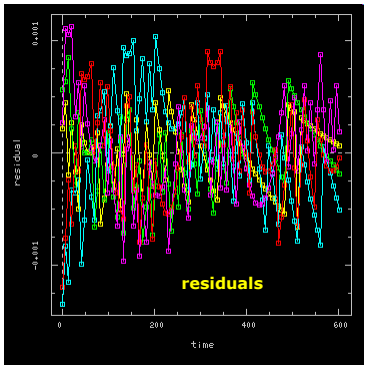


Mechanical Effects on the Kinetics of the HIV Proteinase Deactivation
Kuzmic *et al.* (1996) *Biochem. Biophys. Res. Commun.* **221**, 313-317

Sadana (1991) *Biocatalysis - Fundamentals of Enzyme Deactivation Kinetics*
Prentice-Hall, Englewood Cliffs, NJ.

Enzyme deactivation model: Results

IT LOOKS GOOD EVEN UP CLOSE (RESIDUALS AND PARAMETERS)



No.	Par#Set	Initial	Final
#1	kas	1	2.37068
#2	kdp	50	32.4275
#3	kie	0.01	0.00256721
#4	kies	0.001	0.000874611
#5	[S]#1	3.5	3.73601
#6	offset#1	0.041	0.0326275
#7	[S]#2	7	7.15989
#8	offset#2	0.046	0.0307653
#9	[S]#3	14	13.9672
#10	offset#3	0.052	0.0271767
#11	[S]#4	28	28.3765
#12	offset#4	0.057	0.00900732
#13	[S]#5	56	59.6727
#14	offset#5	0.065	-0.0245337

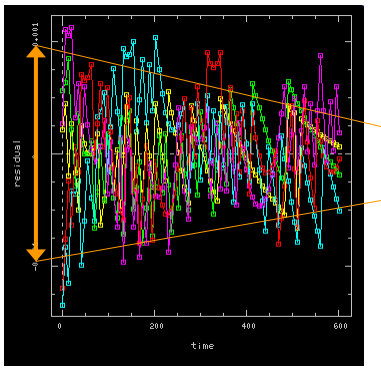
K_M = 13.6 μM

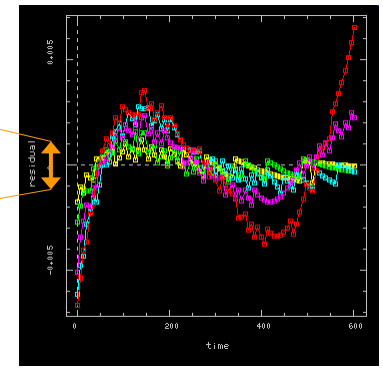
13.1 μM
initial rates

These concentration look OK.

Residual plots: A "good" *vs.* "bad" comparison

ASSESSING RESIDUAL PLOTS IS ONE OF THE **MOST IMPORTANT** SKILLS OF A DATA ANALYST





with enzyme deactivation

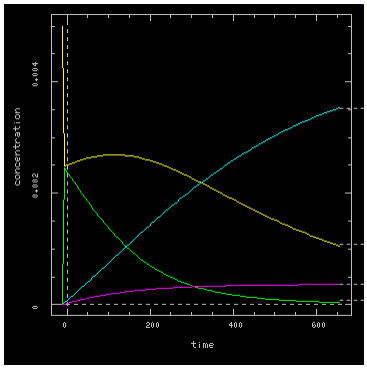
no recognizable pattern:
random

without enzyme deactivation

a clear "zig-zag" pattern
non-random

Concentration plot: How much deactivation?

example assay: **[S] = 14 µM**



E* — **denatured enzyme** is the main molecular form at the end of the assay

E

E.S*

E.S

K_M determination: Summary

1. The progress curves must be treated as nonlinear

   There is no "linear" portion, even if it seems that there is one.

2. Michaelis constant, K_M, was determined by two independent methods

   A. local fit of progress curves → fit of initial rates (K_M = 13.1 µM)
   B. global fit of progress curves (K_M = 13.6 µM)

   Both methods gave the same results.

3. Global fit revealed that the enzyme undergoes deactivation.

   Deactivation (denaturation) is almost complete after 15 minutes.

4. Deactivation does not interfere with K_M determination.

   If we use the initial rate method; *or*
   if we take deactivation into account in the global fit.

5. Overall: *This substrate is very suitable for a K_M assay.*

   If we use an appropriate (nonlinear) method of data analysis.

What about k_{cat} ?

THE **FDA** WANTS US TO DO TWO THINGS: K_M **AND** k_{cat}

We have the following comments, to be addressed prior to submission of a license application:

1. A potency assay that measures the kinetic parameters (K_M and k_{cat}) and uses a physiologically relevant substrate should be incorporated in release testing and stability programs for drug substance and product prior to initiation of the Phase 3 clinical trial.

Michaelis-Menten equation:

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

$$V_{max} = k_{cat}\,[E]$$

The problem:

Even with the differential-equation model, we can (essentially) **measure only** K_M and V_{max}.

$$k_{cat} = V_{max} / [E]$$

The solution:

At some point we must determine the concentration of **active enzyme [E]** (active site concentration).

The only other possibility:

1. Determine **"apparent k_{cat}"**, from [E] as total protein, for a **reference sample** of enzyme.
2. Relate every other "apparent k_{cat}" value to the reference sample in the future.

Summary and conclusions

1. Even a "simple task" such as K_M determination can be quite tricky if it is to be done correctly and rigorously.
2. DynaFit can help in dealing with the nonlinearity of reaction progress.
3. Local fit of individual progress curves is not sufficiently powerful to reveal the true kinetic mechanism.
4. Global fit of multiple curve is much preferred for mechanistic studies.
5. Residual plots are crucially important for model validation.
6. DynaFit is not a "silver bullet": You must still use your brain a lot.