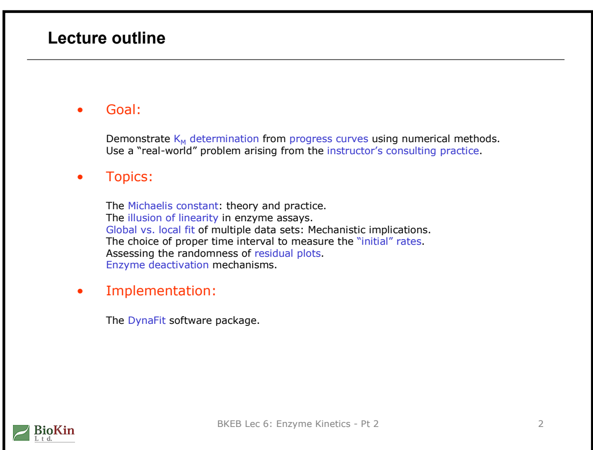
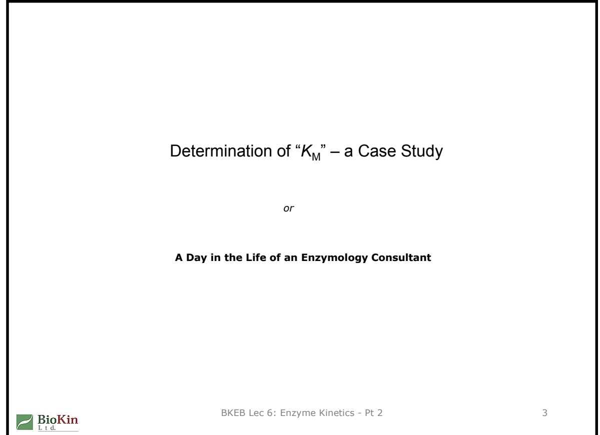
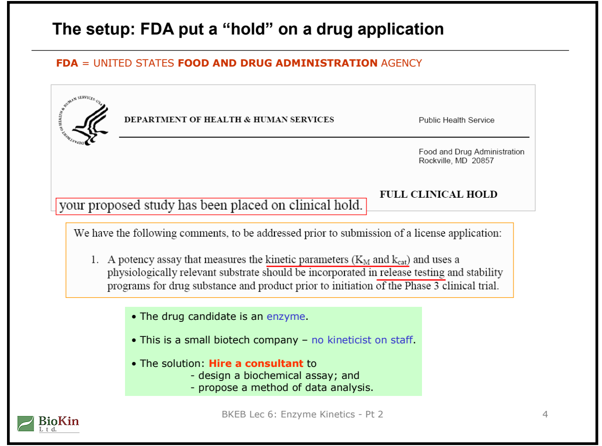
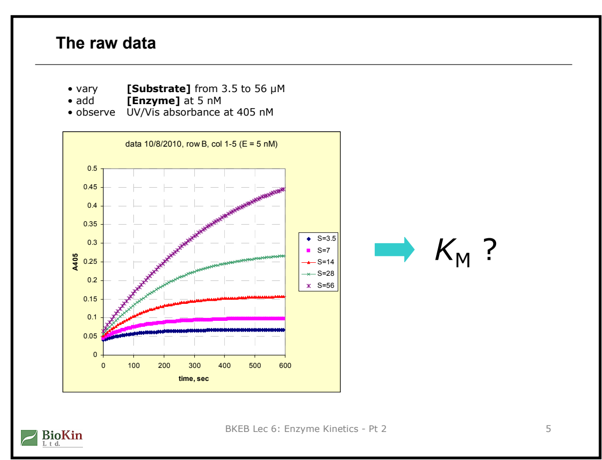
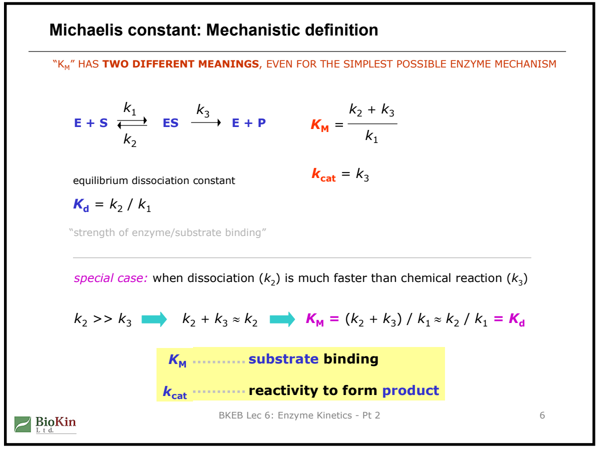
MASARYKOVA UNIVERZITA
www.muni.cz
Binding and Kinetics for Experimental Biologists
Lecture 6
Enzyme kinetics: Analysis of reaction progress
Petr Kuzmič, Ph.D.
BioKin, Ltd.
WATERTOWN, MASSACHUSETTS, U.S.A.
Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.
INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ
BioKin

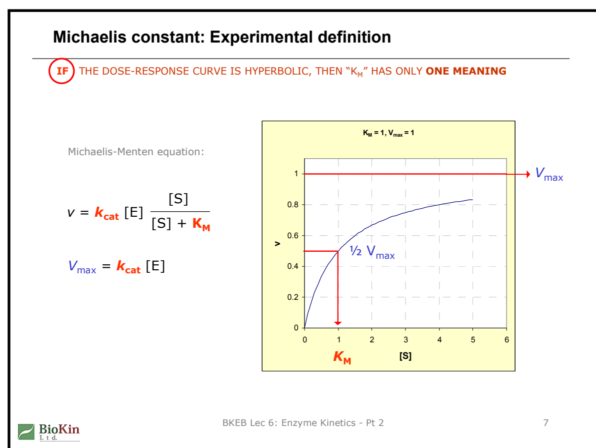

Lecture outline
Goal:
Demonstrate K_M determination from progress curves using numerical methods.
Use a "real-world" problem arising from the instructor's consulting practice.
Topics:
The Michaelis constant: theory and practice.
The illusion of linearity in enzyme assays.
Global vs. local fit of multiple 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.
BKEB Lec 6: Enzyme Kinetics - Pt 2
2

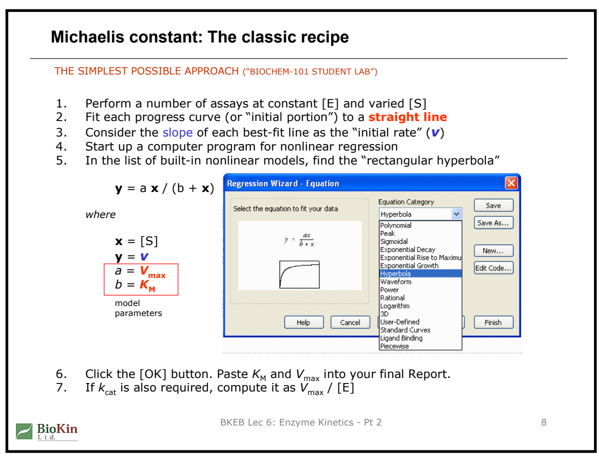

Determination of "K_M" – a Case Study
or
A Day in the Life of an Enzymology Consultant
BKEB Lec 6: Enzyme Kinetics - Pt 2
3

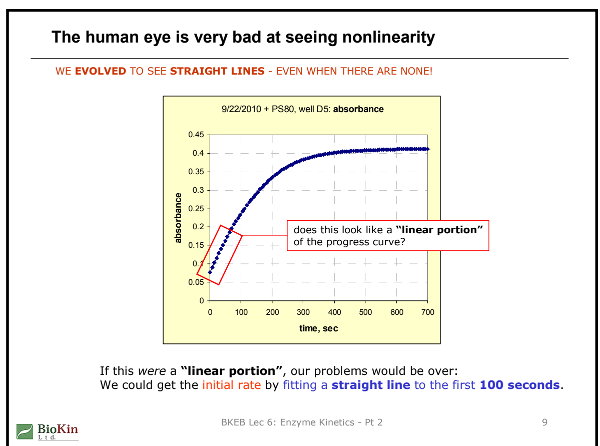

The setup: FDA put a "hold" on a drug application
FDA = UNITED STATES FOOD AND DRUG ADMINISTRATION AGENCY
DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service
Food and Drug Administration
Rockville, MD 20857
FULL CLINICAL HOLD
your proposed study has been placed on clinical hold.
We have the following 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.
BKEB Lec 6: Enzyme Kinetics - Pt 2
4

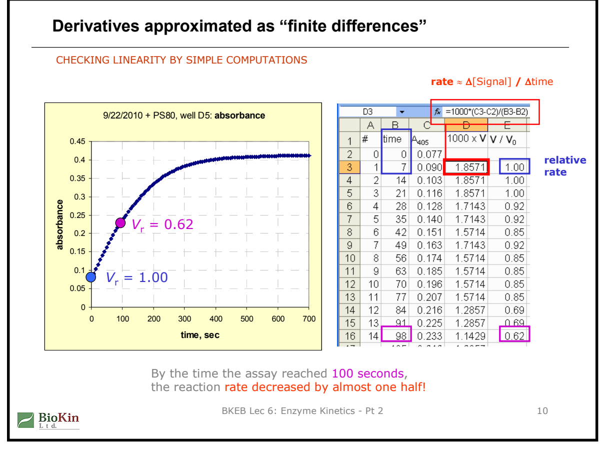

The raw data
• vary [Substrate] from 3.5 to 56 µM
• add [Enzyme] at 5 nM
• observe UV/Vis absorbance at 405 nM
data 10/8/2010, row B, col 1-5 (E = 5 nM)
A405
time, sec
S=3.5
S=7
S=14
S=28
S=56
K_M ?
BKEB Lec 6: Enzyme Kinetics - Pt 2
5


Michaelis constant: Mechanistic definition
"K_M" HAS TWO DIFFERENT MEANINGS, EVEN FOR THE SIMPLEST POSSIBLE ENZYME MECHANISM
E + S ⇌ ES → E + P
K_M = (k2 + k3) / k1
k_cat = k3
equilibrium dissociation constant
K_d = k2 / k1
"strength of enzyme/substrate binding"
special case: when dissociation (k2) is much faster than chemical reaction (k3)
k2 >> k3 → k2 + k3 ≈ k2 → K_M = (k2 + k3) / k1 ≈ k2 / k1 = K_d
K_M .... substrate binding
k_cat .... reactivity to form product
BKEB Lec 6: Enzyme Kinetics - Pt 2
6

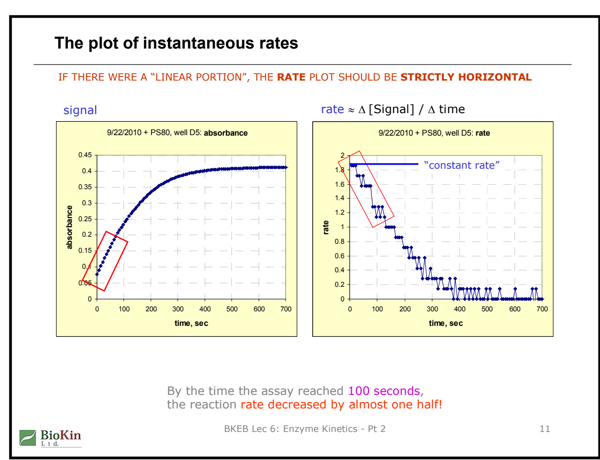


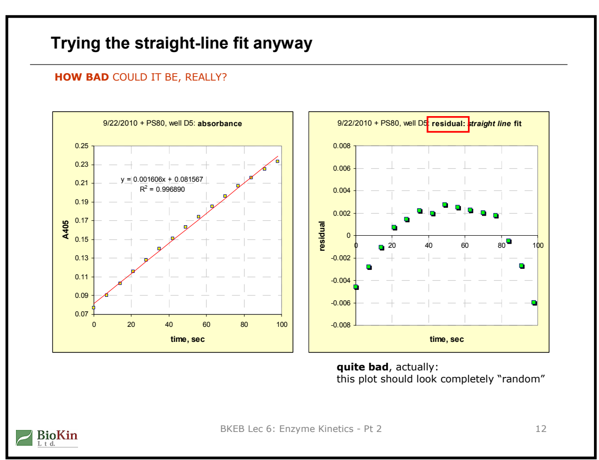


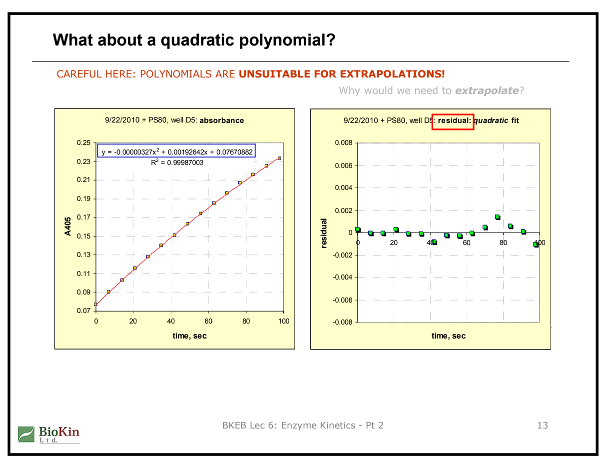


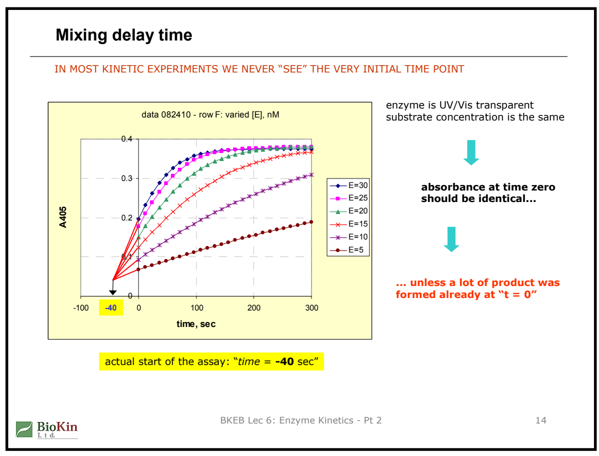


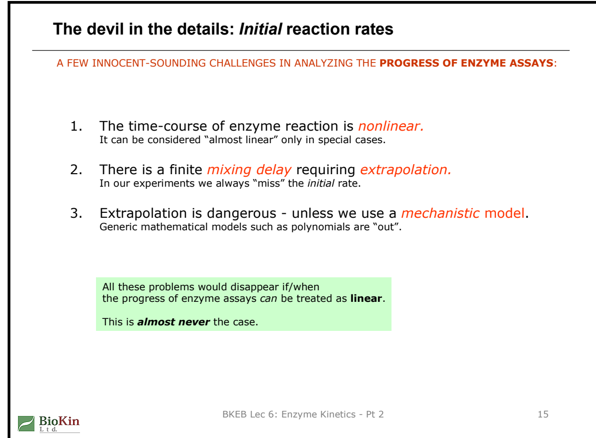
	A	B	C	D	E
1	#	time	A_{405}	1000 x V	V / V_0
2	0	0	0.077		
3	1	7	0.090	1.8571	1.00
4	2	14	0.103	1.8571	1.00
5	3	21	0.116	1.8571	1.00
6	4	28	0.128	1.7143	0.92
7	5	35	0.140	1.7143	0.92
8	6	42	0.151	1.5714	0.85
9	7	49	0.163	1.7143	0.92
10	8	56	0.174	1.5714	0.85
11	9	63	0.185	1.5714	0.85
12	10	70	0.196	1.5714	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

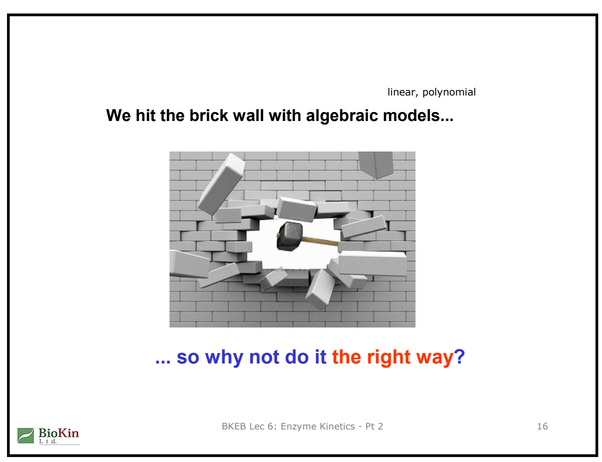


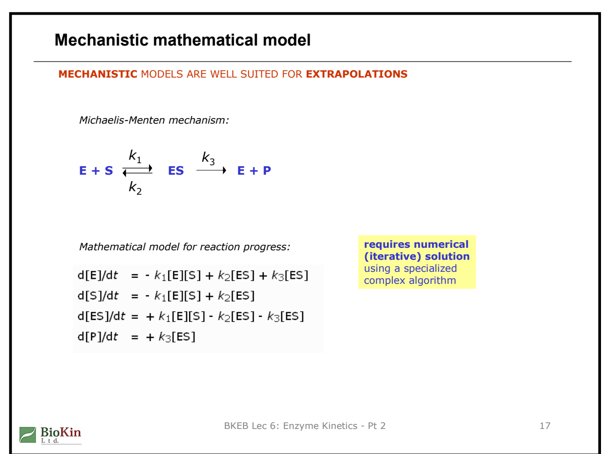


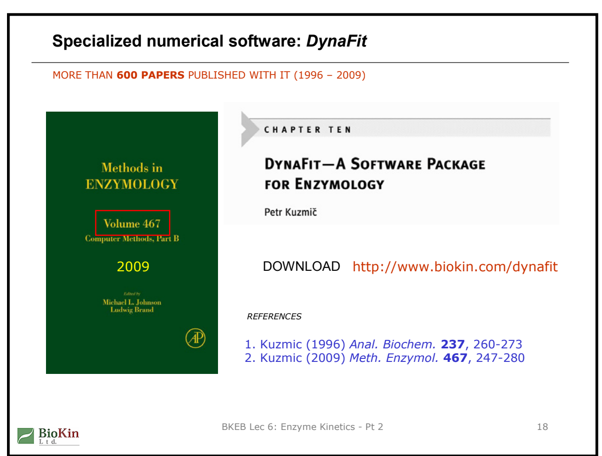

What about a quadratic polynomial?
CAREFUL HERE: POLYNOMIALS ARE UNSUITABLE FOR EXTRAPOLATIONS!
Why would we need to extrapolate?
9/22/2010 + PS80, well D5: absorbance
y = -0.00000327x² + 0.00192642x + 0.07670882
R² = 0.99987003
A405
time, sec
9/22/2010 + PS80, well D5: residual: quadratic fit
residual
time, sec
BKEB Lec 6: Enzyme Kinetics - Pt 2
13


Mixing delay time
IN MOST KINETIC EXPERIMENTS WE NEVER "SEE" THE VERY INITIAL TIME POINT
data 082410 - row F: varied [E], nM
A405
time, sec
E=30
E=25
E=20
E=15
E=10
E=5
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"
BKEB Lec 6: Enzyme Kinetics - Pt 2
14


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.
BKEB Lec 6: Enzyme Kinetics - Pt 2
15


linear, polynomial
We hit the brick wall with algebraic models...
... so why not do it the right way?
BKEB Lec 6: Enzyme Kinetics - Pt 2
16


Mechanistic mathematical model
MECHANISTIC MODELS ARE WELL SUITED FOR EXTRAPOLATIONS
Michaelis-Menten mechanism:
E + S ⇄ ES → E + P (k1, k2, k3)
Mathematical model for reaction progress:
d[E]/dt = - k1[E][S] + k2[ES] + k3[ES]
d[S]/dt = - k1[E][S] + k2[ES]
d[ES]/dt = + k1[E][S] - k2[ES] - k3[ES]
d[P]/dt = + k3[ES]
requires numerical (iterative) solution
using a specialized complex algorithm
BKEB Lec 6: Enzyme Kinetics - Pt 2
17


Specialized numerical software: DynaFit
MORE THAN 600 PAPERS PUBLISHED WITH IT (1996 – 2009)
Methods in ENZYMOLOGY
Volume 467
Computer Methods, Part B
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
BKEB Lec 6: Enzyme Kinetics - Pt 2
18

The Van Slyke – Cullen mechanism

JUST AS GOOD AS MICHAELIS-MENTEN

Van Slyke-Cullen mechanism:

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

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:

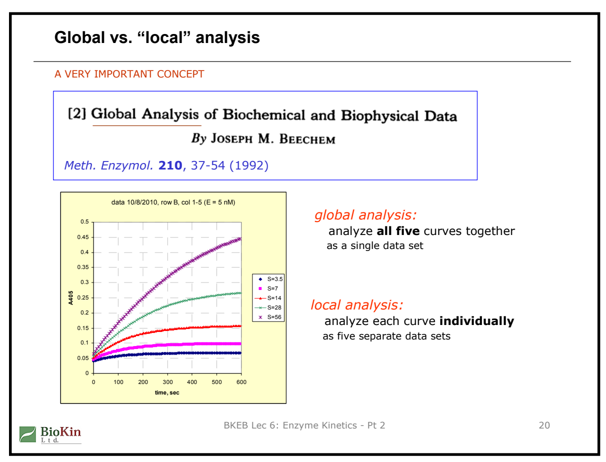
```
[task]

   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)

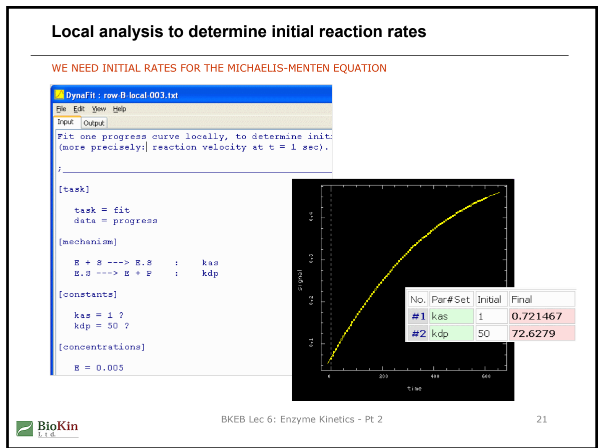


global analysis:
analyze **all five** curves together
as a single data set

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

Local analysis to determine initial reaction rates

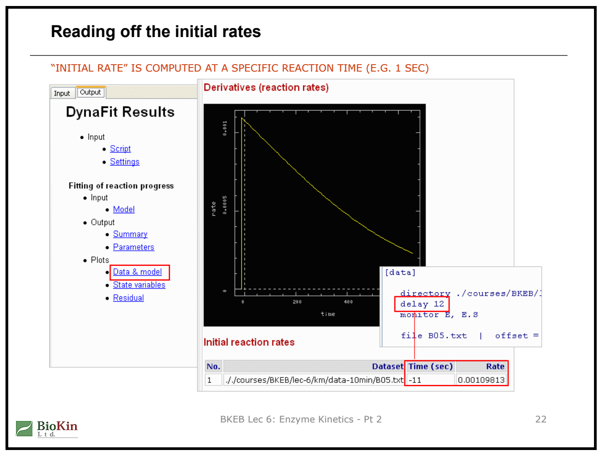
WE NEED INITIAL RATES FOR THE MICHAELIS-MENTEN EQUATION



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

Reading off the initial rates

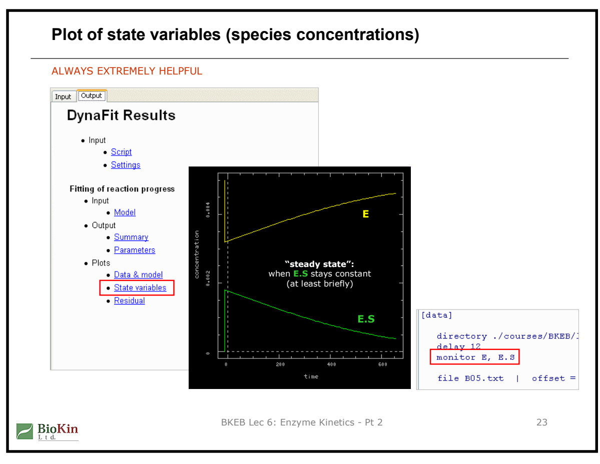
"INITIAL RATE" IS COMPUTED AT A SPECIFIC REACTION TIME (E.G. 1 SEC)



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

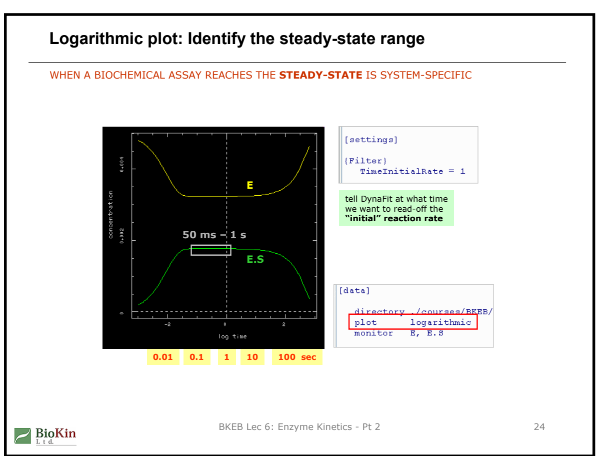
Plot of state variables (species concentrations)

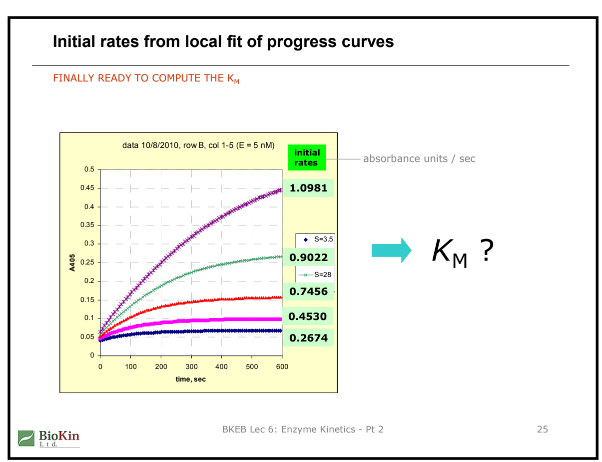
ALWAYS EXTREMELY HELPFUL

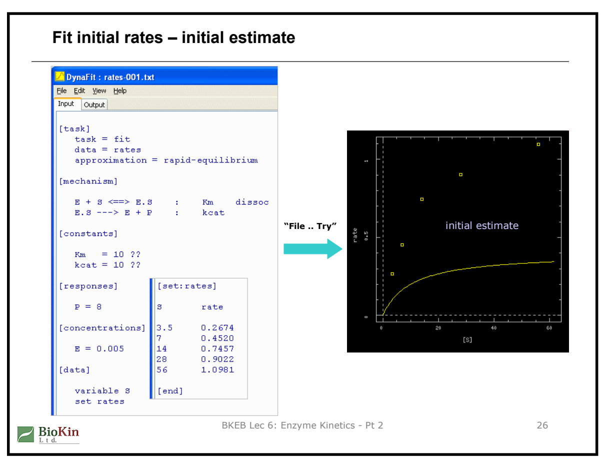


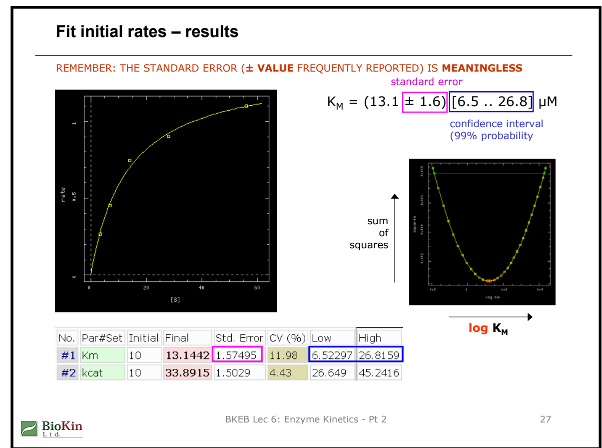
Logarithmic plot: Identify the steady-state range

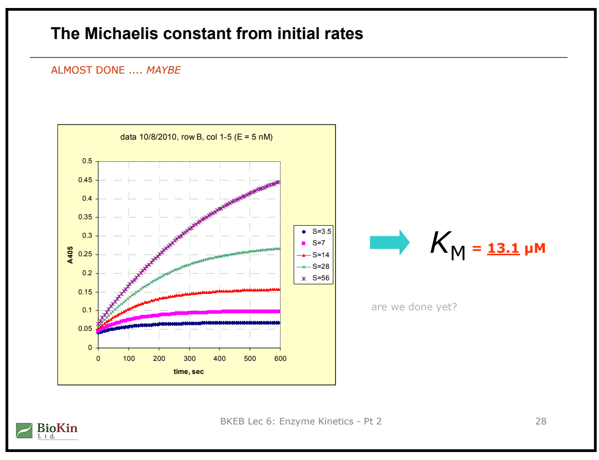
WHEN A BIOCHEMICAL ASSAY REACHES THE **STEADY-STATE** IS SYSTEM-SPECIFIC

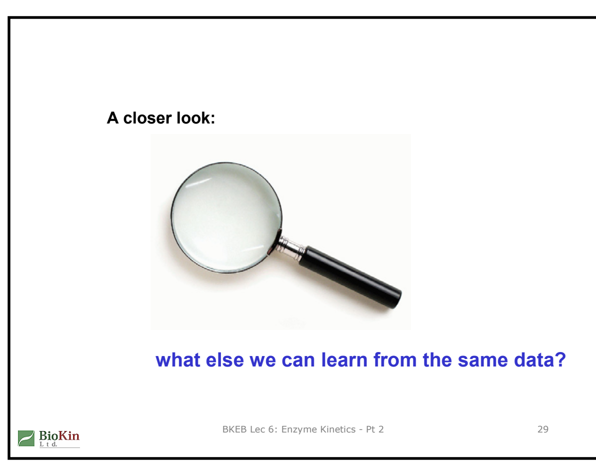


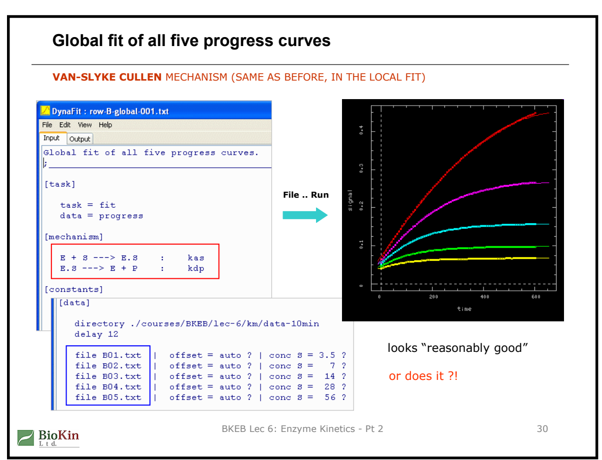

Initial rates from local fit of progress curves
FINALLY READY TO COMPUTE THE K_M
data 10/8/2010, row B, col 1-5 (E = 5 nM)
initial rates
absorbance units / sec
1.0981
0.9022
0.7456
0.4530
0.2674
A405
time, sec
K_M ?
BioKin
BKEB Lec 6: Enzyme Kinetics - Pt 2
25

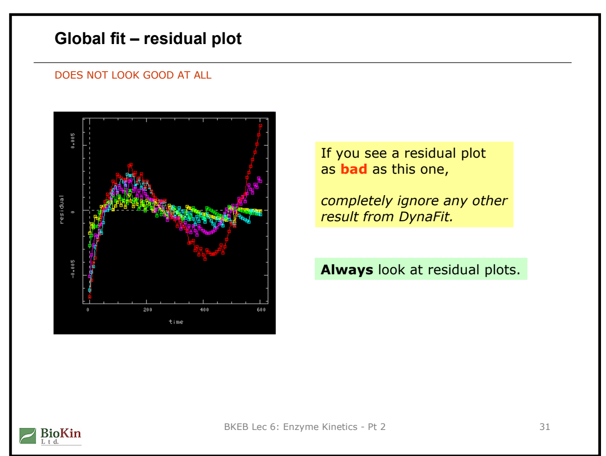

Fit initial rates – initial estimate
DynaFit : rates-001.txt
[task]
task = fit
data = rates
approximation = rapid-equilibrium
[mechanism]
E + S <==> E.S : Km dissoc
E.S ---> E + P : kcat
[constants]
Km = 10 ??
kcat = 10 ??
[responses]
P = 8
[concentrations]
E = 0.005
[data]
variable S
set rates
[set:rates]
S rate
3.5 0.2674
7 0.4520
14 0.7457
28 0.9022
56 1.0981
[end]
"File .. Try"
initial estimate
BioKin
BKEB Lec 6: Enzyme Kinetics - Pt 2
26

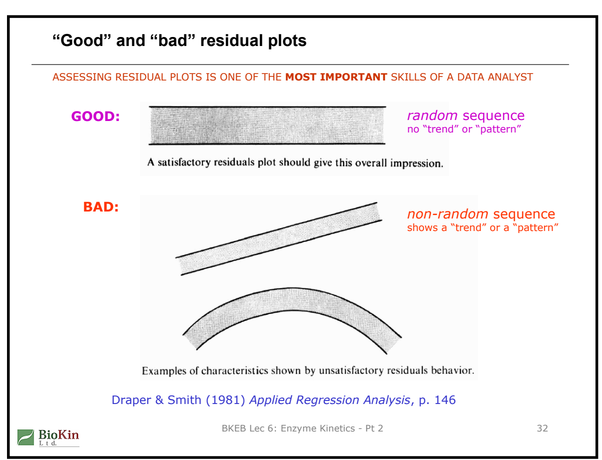

Fit initial rates – results
REMEMBER: THE STANDARD ERROR (± VALUE FREQUENTLY REPORTED) IS MEANINGLESS
standard error
K_M = (13.1 ± 1.6) [6.5 .. 26.8] μM
confidence interval
(99% probability
sum of squares
log K_M
No. Par#Set Initial Final Std. Error CV (%) Low High
#1 Km 10 13.1442 1.57495 11.98 6.52297 26.8159
#2 kcat 10 33.8915 1.5029 4.43 26.649 45.2416
BioKin
BKEB Lec 6: Enzyme Kinetics - Pt 2
27

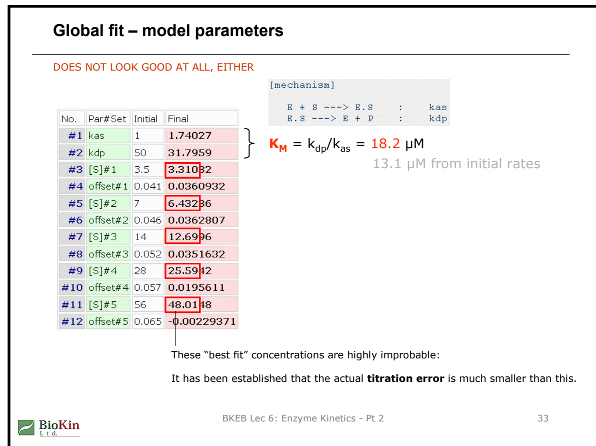

The Michaelis constant from initial rates
ALMOST DONE .... MAYBE
data 10/8/2010, row B, col 1-5 (E = 5 nM)
A405
time, sec
K_M = 13.1 μM
are we done yet?
BioKin
BKEB Lec 6: Enzyme Kinetics - Pt 2
28


A closer look:
what else we can learn from the same data?
BioKin
BKEB Lec 6: Enzyme Kinetics - Pt 2
29

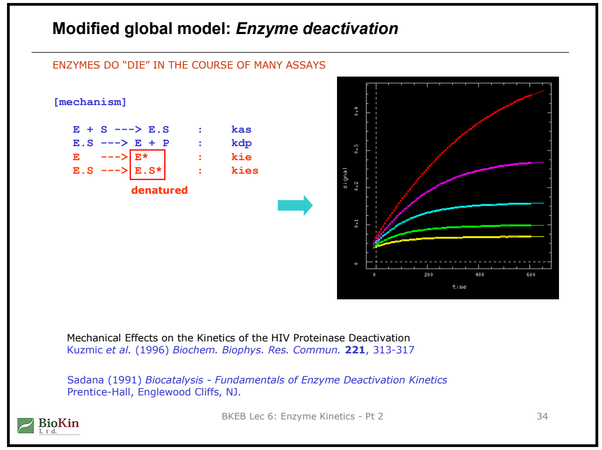

Global fit of all five progress curves
VAN-SLYKE CULLEN MECHANISM (SAME AS BEFORE, IN THE LOCAL FIT)
DynaFit : row-B-global-001.txt
Global fit of all five progress curves.
[task]
task = fit
data = progress
[mechanism]
E + S ---> E.S : kas
E.S ---> E + P : kdp
[constants]
[data]
directory ./courses/BKEB/lec-6/km/data-10min
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 ?!
BioKin
BKEB Lec 6: Enzyme Kinetics - Pt 2
30

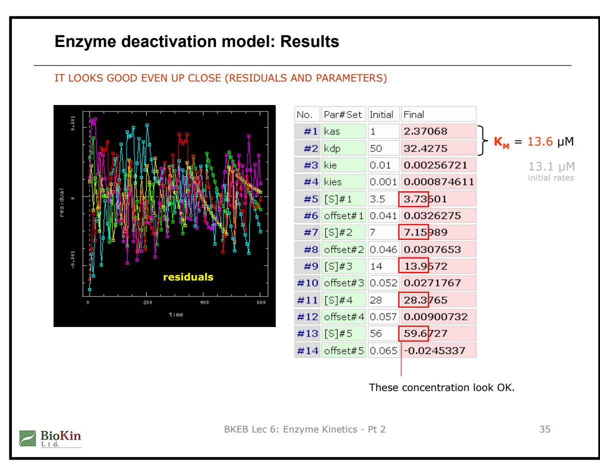




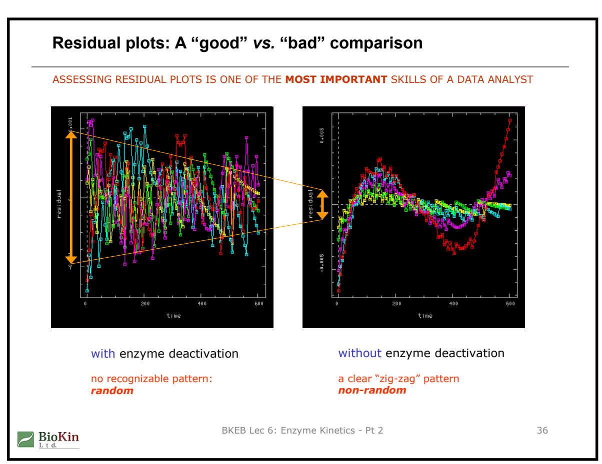


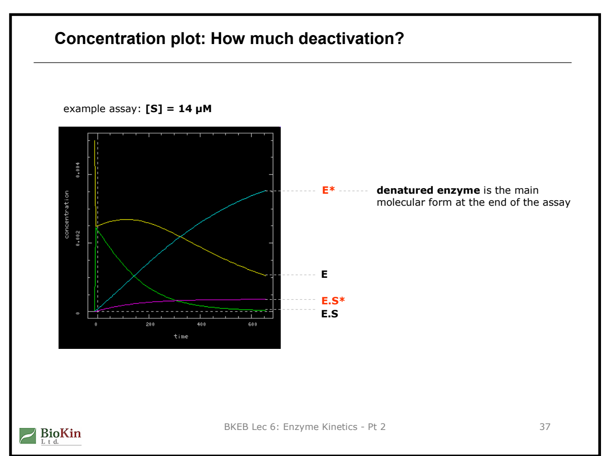
No.	Par#Set	Initial	Final
#1	kas	1	1.74027
#2	kdp	50	31.7959
#3	[S]#1	3.5	3.31032
#4	offset#1	0.041	0.0360932
#5	[S]#2	7	6.43236
#6	offset#2	0.046	0.0362807
#7	[S]#3	14	12.6996
#8	offset#3	0.052	0.0351632
#9	[S]#4	28	25.5942
#10	offset#4	0.057	0.0195611
#11	[S]#5	56	48.0148
#12	offset#5	0.065	-0.00229371

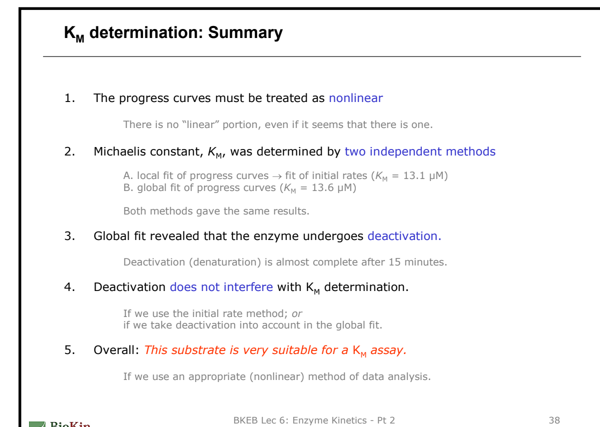


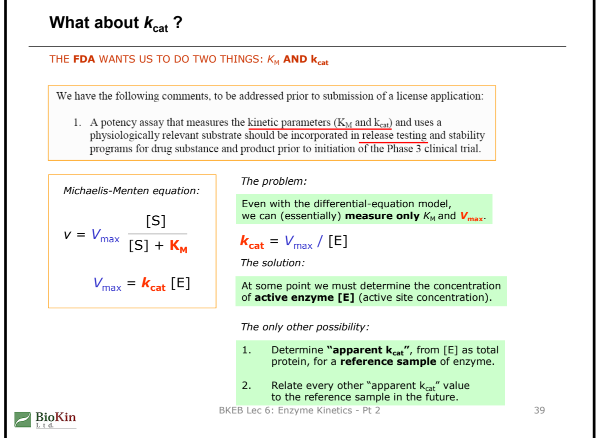


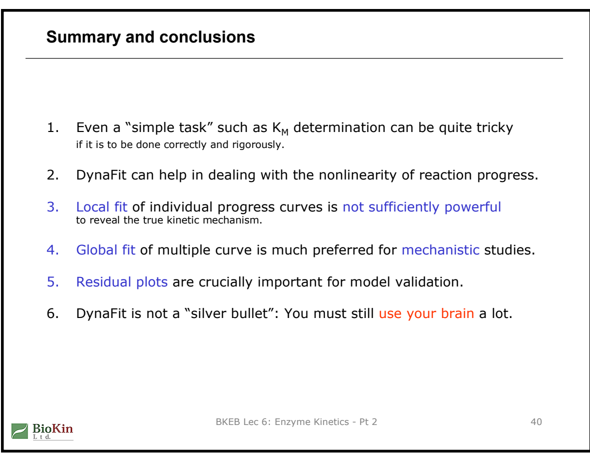
No.	Par#Set	Initial	Final
#1	kas	1	2.37068
#2	kdp	50	32.4275
#3	kie	0.01	0.00256721
#4	kies	0.001	0.000874611
#5	[S]#1	3.5	3.73601
#6	offset#1	0.041	0.0326275
#7	[S]#2	7	7.15989
#8	offset#2	0.046	0.0307653
#9	[S]#3	14	13.9672
#10	offset#3	0.052	0.0271767
#11	[S]#4	28	28.3765
#12	offset#4	0.057	0.00900732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




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
BKEB Lec 6: Enzyme Kinetics - Pt 2
37


KM 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, KM, was determined by two independent methods
A. local fit of progress curves → fit of initial rates (KM = 13.1 µM)
B. global fit of progress curves (KM = 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 KM 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 KM assay.
If we use an appropriate (nonlinear) method of data analysis.
BKEB Lec 6: Enzyme Kinetics - Pt 2
38


What about kcat ?
THE FDA WANTS US TO DO TWO THINGS: KM AND kcat
We have the following comments, to be addressed prior to submission of a license application:
1. A potency assay that measures the kinetic parameters (KM and kcat) 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 = Vmax [S] / ([S] + KM)
Vmax = kcat [E]
The problem:
Even with the differential-equation model, we can (essentially) measure only KM and Vmax.
kcat = Vmax / [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 kcat", from [E] as total protein, for a reference sample of enzyme.
2. Relate every other "apparent kcat" value to the reference sample in the future.
BKEB Lec 6: Enzyme Kinetics - Pt 2
39


Summary and conclusions
1. Even a "simple task" such as KM 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.
BKEB Lec 6: Enzyme Kinetics - Pt 2
40