Bio/Chemical Kinetics Made Easier

A Numerical Approach

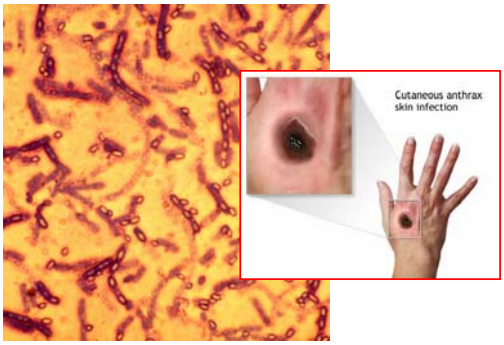
Petr Kuzmič, Ph.D.
BioKin, Ltd.

1. Case study: Inhibition of LF protease from *B. anthracis*
2. Method: **Numerical Enzyme Kinetics**

BioKin

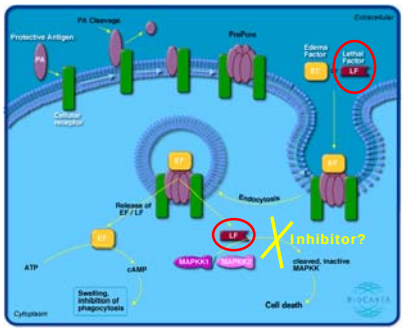
Anthrax bacillus

CUTANEOUS AND INHALATION ANTHRAX DISEASE



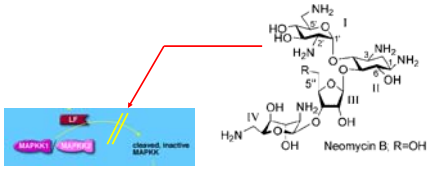
Lethal Factor (LF) protease from *B. anthracis*

CLEAVES MITOGEN ACTIVATED PROTEIN KINASE KINASE (**MAPKK**)



Neomycin B: an aminoglycoside inhibitor

PRESUMABLY A "COMPETITIVE" INHIBITOR OF **LF** PROTEASE

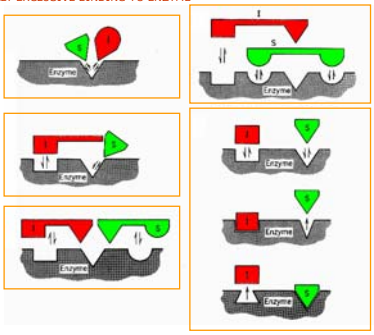


> "All the compounds tested were found to be competitive inhibitors."

Fridman *et al.* (2004) *Angew. Chem. Int. Ed. Eng.* **44**, 447-452

Competitive inhibition - Possible mechanisms

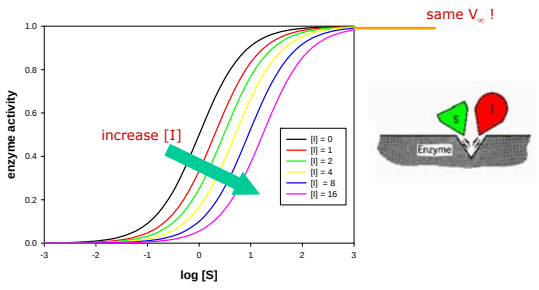
MUTUALLY EXCLUSIVE BINDING TO ENZYME



Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 102

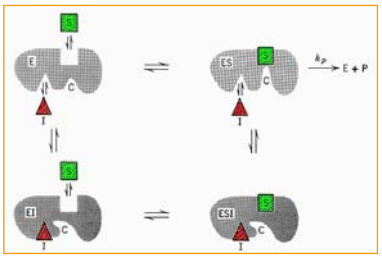
Competitive inhibition - Kinetics

AT VERY HIGH [SUBSTRATE], ANZYME ACTIVITY IS COMPLETELY RESTORED



Non-competitive inhibition - A possible mechanism

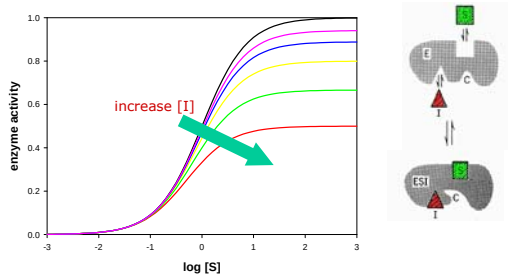
NON-EXCLUSIVE BINDING, BUT TERNARY COMPLEX HAS NO CATALYTIC ACTIVITY



Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 126

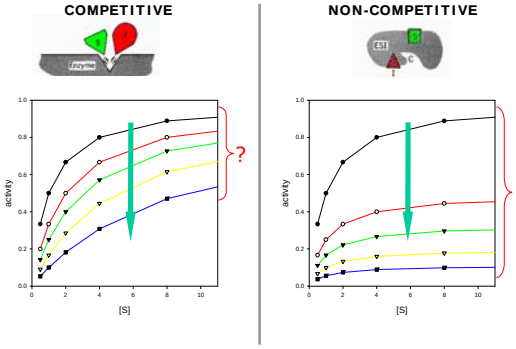
Non-competitive inhibition - Kinetics

EVEN AT VERY HIGH [SUBSTRATE], ANZYME ACTIVITY IS NEVER FULLY RESTORED



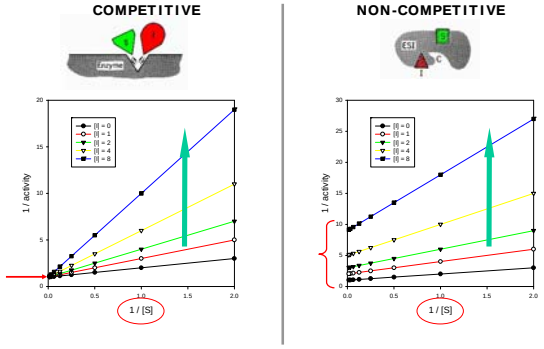
Compare saturation curves

DIAGNOSIS OF MECHANISMS: SAME OR DIFFERENT RATE AT VERY LARGE [S]?



Compare "double-reciprocal" plots

DIAGNOSIS OF MECHANISMS: STRAIGHT LINES INTERCEPT ON VERTICAL AXIS?



Traditional plan to determine inhibition mechanism

THE TRADITIONAL APPROACH

1. Measure enzyme activity at increasing [S]

   Collect multiple substrate-saturation curves at varied [I]

2. Convert [S] *vs.* activity data to **double-reciprocal** coordinates
3. Perform a **linear fit** of transformed (double-reciprocal) data
4. Check if resulting straight lines **intersect** on the vertical axis

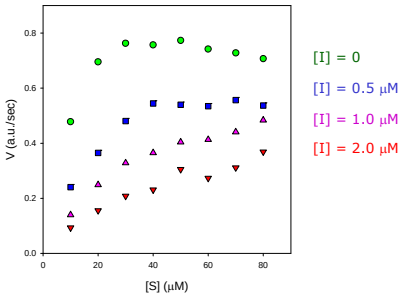
   If yes, declare the inhibition mechanism **competitive**

> of all other compounds were 0, 165, 330, and 550 nM). The K_i values were estimated from double-reciprocal plots of initial velocities as a function of substrate concentration. [b] High-salt conditions: potassium HEPES

Fridman *et al.* (2004) *Angew. Chem. Int. Ed. Eng.* **44**, 447-452

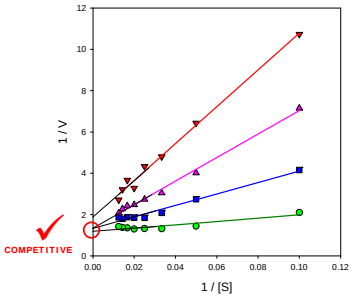
Collect experimental data at varied [S] and [I]

THE RAW DATA



Check for intersection of double-reciprocal plots

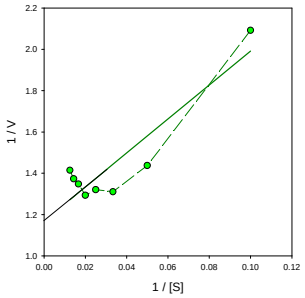
DO LINEWEAVER-BURK PLOTS INTERSECT?



[I] = 0
[I] = 0.5 μM
[I] = 1.0 μM
[I] = 2.0 μM

Doubts begin to appear...

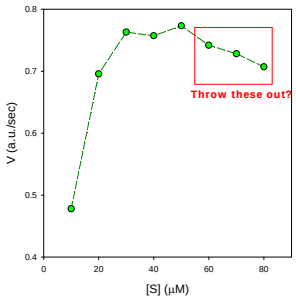
IS THIS A STRAIGHT LINE?



[I] = 0

Mysterious substrate saturation data

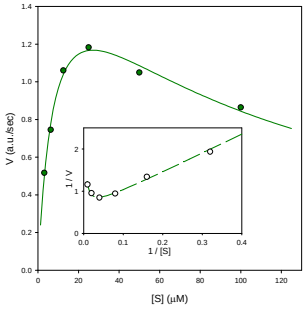
MICHAELIS-MENTEN KINETICS IS NOT SUPPOSED TO SHOW A MAXIMUM !



[I] = 0

Repeat substrate experiment at higher [S]

SEE IF MAXIMUM HOLDS UP AT HIGHER [S]



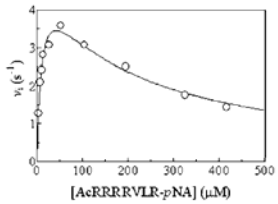
[I] = 0

Substrate inhibition in LF protease is real

HAS ANYONE ELSE SEEN IT?

The Metalloproteolytic Activity of the Anthrax Lethal Factor Is Substrate-inhibited*

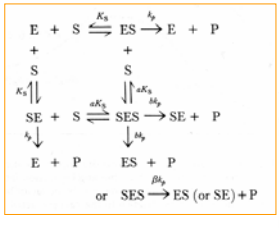
$$v_i = (k_{cat} \times [E] \times [S])/(K_m + [S] + ([S]^2/K_i)) \quad \text{(Eq. 1)}$$



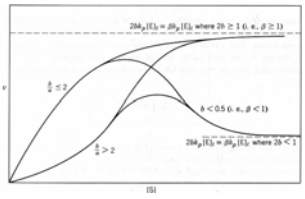
Tonello *et al.* (2003) *J. Biol. Chem.* **278**, 40075-78.

Rate equation for inhibition by substrate

WHAT DOES THE "BIG BLUE BOOK" SAY?



$$\frac{v}{[E]_t} = \frac{k_p \frac{2[S]}{K_S} + 2bk_p \frac{[S]^2}{aK_S^2}}{1 + \frac{2[S]}{K_S} + \frac{[S]^2}{aK_S^2}}$$



Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 126

Rate equation for inhibition by substrate + inhibitor

WHAT DOES THE "BIG BLUE BOOK" SAY?

?

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A Numerical Approach

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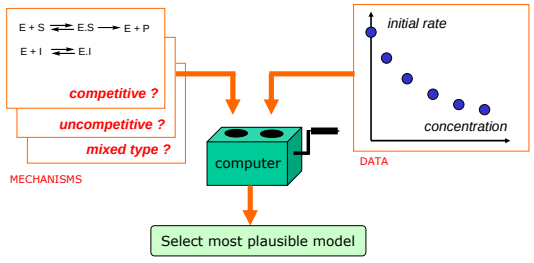
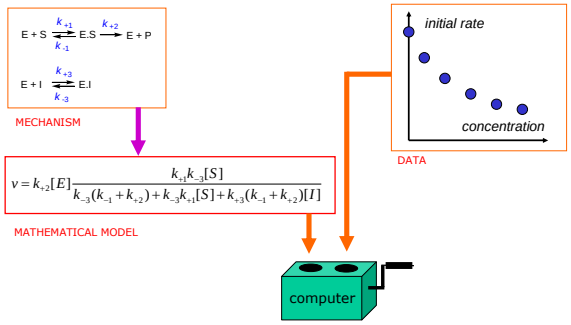
The task of mechanistic enzyme kinetics

SELECT AMONG MULTIPLE CANDIDATE MECHANISMS



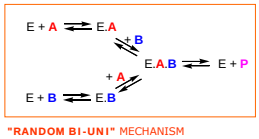
From mechanistic to mathematical models

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL IDEAS



Problem: Simple mechanisms ...

MERELY FIVE REACTIONS ...



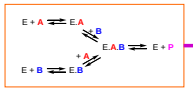
"RANDOM BI-UNI" MECHANISM

- 2 reactants (A, B)
- 1 product (P)
- 5 reversible reactions
- 10 rate constant

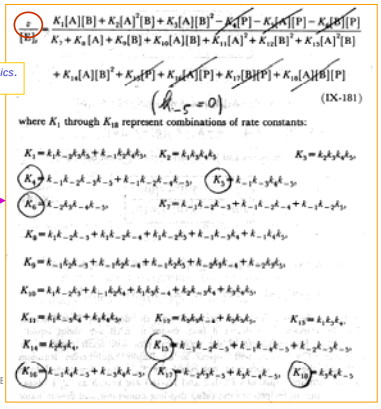
... lead to complex algebraic models

MERELY FIVE REACTIONS ...

Segel, I. (1975) *Enzyme Kinetics*. John Wiley, New York, p. 646.



"RANDOM BI-UNI" MECHANISM



A solution: Forget about algebra

POSSIBLE STRATEGY FOR MECHANISTIC MODEL BUILDING

- Do not even try to derive complex algebraic equations
- Instead, derive *systems* of *simple*, *simultaneous* equations
- Solve these systems using numerical methods

Theoretical foundations: *Mass Action Law*

RATE IS PROPORTIONAL TO CONCENTRATION(S)

"rate" ... "derivative"

MONOMOLECULAR REACTIONS

A ⟶ products

rate is proportional to **[A]**

$-\,d[A]/dt = k[A]$

monomolecular rate constant
1 / time

BIMOLECULAR REACTIONS

A + B ⟶ products

rate is proportional to **[A] × [B]**

$-\,d[A]/dt = -\,d[B]/dt = k[A] \times [B]$

bimolecular rate constant
1 / (concentration × time)

Theoretical foundations: *Mass Conservation Law*

PRODUCTS ARE FORMED WITH THE SAME RATE AS REACTANTS DISAPPEAR

EXAMPLE

A ⟶ P + Q

$-\,d[A]/dt = +\,d[P]/dt = +\,d[Q]/dt$

COMPOSITION RULE ADDITIVITY OF TERMS FROM SEPARATE REACTIONS

mechanism:

A $\xrightarrow{k_1}$ B

B $\xrightarrow{k_2}$ C

$d[B]/dt = +k_1[A] - k_2[B]$

Composition Rule: Example

EXAMPLE MECHANISM

E + A $\underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}}$ EA

EA + B $\underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}}$ EAB

E + B $\underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}}$ EB

EB + A $\underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}}$ EAB

EAB $\xrightarrow{k_{+5}}$ E + P + Q

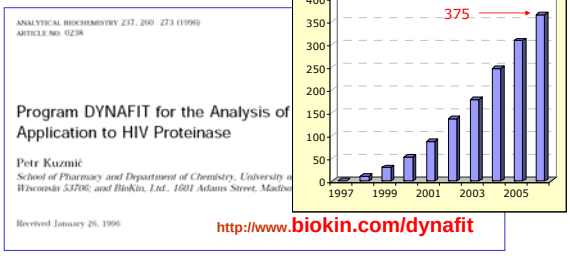
RATE EQUATIONS

$d[P]/dt = +k_{+5}[EAB]$

$d[EAB]/dt = +k_{+2}[EA]\times[B] - k_{-2}[EAB] + k_{+4}[EB]\times[A] - k_{-4}[EAB] - k_{+5}[EAB]$

Similarly for other species...

Program DYNAFIT (1996)


ANALYTICAL BIOCHEMISTRY 237, 260–273 (1996)
ARTICLE NO. 0238

Program DYNAFIT for the Analysis of
Application to HIV Proteinase

Petr Kuzmič
School of Pharmacy and Department of Chemistry, University o
Wisconsin 53706; and BioKin, Ltd., 1601 Adams Street, Madiso

Received January 26, 1996

http://www.biokin.com/dynafit

Kuzmic P. (1996) *Anal. Biochem.* **237**, 260-273.

A "Kinetic Compiler"

HOW DYNAFIT PROCESSES YOUR BIOCHEMICAL EQUATIONS

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

Input (plain text file):	Rate terms:	Rate equations:
E + S ---> ES : k1	$k_1 \times [E] \times [S]$	$d[E]/dt = -k_1 \times [E] \times [S] + k_2 \times [ES] + k_3 \times [ES]$
ES ---> E + S : k2	$k_2 \times [ES]$	$d[ES]/dt = +k_1 \times [E] \times [S] - k_2 \times [ES] - k_3 \times [ES]$
ES ---> E + P : k3	$k_3 \times [ES]$	

Similarly for other species...

System of Simple, Simultaneous Equations

HOW DYNAFIT PROCESSES YOUR BIOCHEMICAL EQUATIONS

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

"The LEGO method" of deriving rate equations

Input (plain text file):

```
E + S ---> ES  :  k1
ES ---> E + S  :  k2
ES ---> E + P  :  k3
```

Rate terms:

$k_1 \times [E] \times [S]$

$k_2 \times [ES]$

$k_3 \times [ES]$

Rate equations:

Initial rate kinetics

TWO BASIC APPROXIMATIONS

1. **Rapid-Equilibrium** Approximation

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

assumed very much **slower** than k_1, k_2

2. **Steady-State** Approximation

New in DynaFit

- *no assumptions* made about relative magnitude of k_1, k_2, k_3
- concentrations of enzyme forms are *unchanging*

Initial rate kinetics - Traditional approach

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL IDEAS

$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E.S \xrightarrow{k_{+2}} E + P$

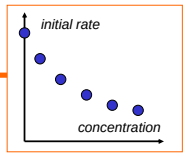
$E + I \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E.I$

MECHANISM

Think!

$$v = k_{+2}[E]\frac{k_{+1}k_{-3}[S]}{k_{-3}(k_{-1}+k_{+2}) + k_{-3}k_{+1}[S] + k_{+3}(k_{-1}+k_{+2})[I]}$$

MATHEMATICAL MODEL



DATA

computer

Initial rate kinetics in DynaFit

GOOD NEWS: MODEL DERIVATION CAN BE FULLY AUTOMATED!

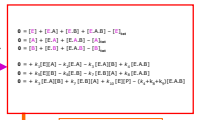
DynaFit input file

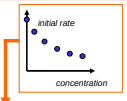
```
[task]
   task = fit
   data = rates
   approximation = Steady-State

[mechanism]

   E + A   <==> E.A    :  k1   k2
   E.A + B <==> E.A.B  :  k3   k4
   E + B   <==> E.B    :  k5   k6
   E.B + A <==> E.A.B  :  k7   k8
   E.A.B   <==> E + P  :  k9   k10

[constants]
   ...
```

MECHANISM

CRANK!

MATHEMATICAL MODEL



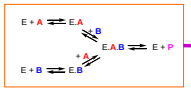


DATA

computer

Initial rate kinetics in DynaFit *vs.* traditional method

WHICH DO YOU LIKE BETTER?



```
[task]
   task = fit
   data = rates
   approximation = Steady-State

[reaction]
   A + B --> P

[mechanism]

   E + A   <==> E.A    :  k1   k2
   E.A + B <==> E.A.B  :  k3   k4
   E + B   <==> E.B    :  k5   k6
   E.B + A <==> E.A.B  :  k7   k8
   E.A.B   <==> E + P  :  k9   k10

[constants]
   ...

[concentrations]
   ...
```

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BioKin Ltd.

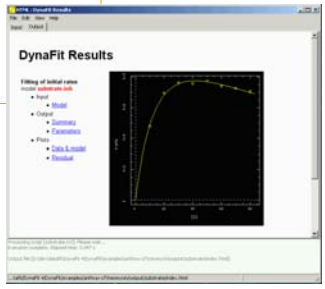
DynaFit model for inhibition by substrate

ENZYME KINETICS MADE **EASIER**

```
[reaction]    |  S ---> P
[enzyme]      |  E
[modifiers]   |  I

[mechanism]

   E + S <===> E.S      :  Ks
   E.S + S <===> E.S.S  :  Ks2
   E.S ---> E + P       :  kcat
...
```



DynaFit model for inhibition by substrate + inhibitor

ENZYME KINETICS MADE **EASIER**

```
[reaction]    |  S ---> P
[enzyme]      |  E
[modifiers]   |  I

[mechanism]

   E + S <===> E.S      :  Ks    dissoc
   E.S + S <===> E.S.S  :  Ks2   dissoc
   E.S ---> E + P       :  kcat
   E + I <===> E.I      :  Ki    dissoc
   E.S + I <===> E.S.I  :  Kis   dissoc

[constants]

   Ks = 1 ?, Ks2 = 1 ?, kcat = 1 ?
   Ki = 1 ?, Kis = 1 ?

...
...
```

optimization flag

initial estimate

How do we know which mechanism is "best"?

COMPARE ANY NUMBER OF MODELS IN A SINGLE RUN

```
[task]
   task = fit | data = rates
   model = mixed-type ?

[reaction]    |  S ---> P
[enzyme]      |  E
[modifiers]   |  I

...

[task]

   task = fit | data = rates
   model = competitive ?

...

[task]

   task = fit | data = rates
   model = uncompetitive ?

...
```

Akaike Information Criterion

Review: Burnham & Anderson (2004)

The best model: mixed-type noncompetitive

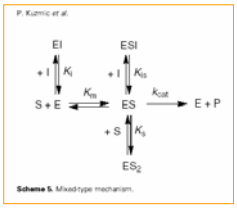
NEOMYCIN B IS **NOT** A COMPETITIVE INHBITOR OF LETHAL FACTOR PROTEASE

FEBS Journal

Mixed-type noncompetitive inhibition of anthrax lethal factor protease by aminoglycosides

Petr Kuzmic[1], Lynne Cregar[2], Sherri Z. Millis[2] and Mark Goldman[2,*]

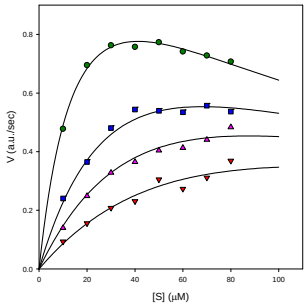
1 BioKin Ltd, Pullman, WA, USA
2 Hawaii Biotech Inc., Aiea, HI, USA



Scheme 5. Mixed-type mechanism.

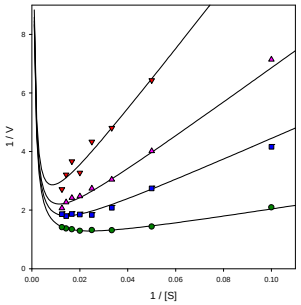
Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Direct plot: maximum on dose-response curves



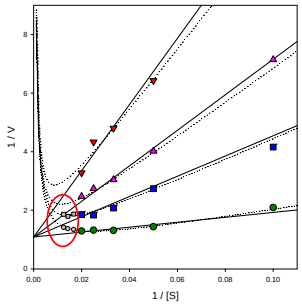
Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Double-reciprocal plot is nonlinear



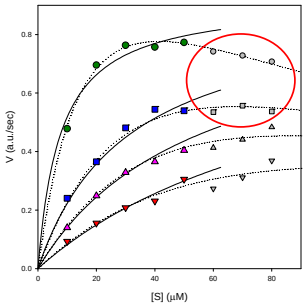
Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

DR plot obscures deviations from the model



Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Direct plot makes model departures more visible



Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Summary: Enzyme kinetics made (almost) easy

HOW DO I BUILD A MATHEMATICAL MODEL FOR AN ENZYME MECHANISM?

- Let the **computer** derive your model - don't bother with algebra.
- For many important mechanisms, algebraic models don't exist anyway.
- The theoretical foundation is simple and well understood:
  - **mass action law**
  - **mass conservation law**
- The same set of LEGO-like rules apply to all types of kinetic models:
  - *reaction progress curves*
  - *initial reaction rates*

Acknowledgements: Lethal Factor protease work