Biochemical Kinetics Made Easier

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BioKin, Ltd.

1. **Theory**: differential equations
   - **DYNAFIT** software
2. **Example I**: Initial rate experiment
   - **p56^{lck}** kinase / **"ATP analog"** inhibitor
3. **Example II**: Time course experiment
   - **p38α** kinase / **desatinib** / competitive ligand displacement

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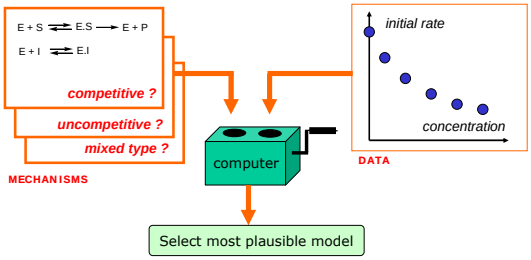
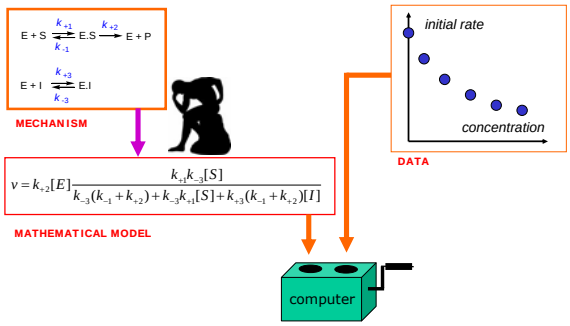
The task of mechanistic enzyme kinetics

SELECT AMONG MULTIPLE CANDIDATE MECHANISMS



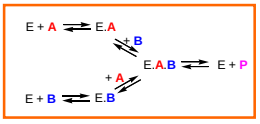
From mechanistic to mathematical models

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL IDEAS



Problem: Simple mechanisms ...

MERELY FIVE REACTIONS ...



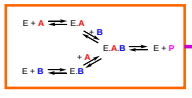
"RANDOM BI-UNI" MECHANISM

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

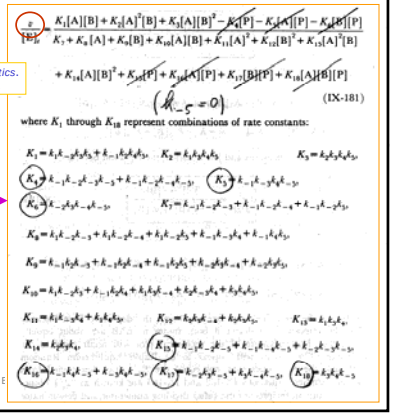
... lead to complex algebraic models

MERELY FIVE REACTIONS ...

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



"RANDOM BI-UNI" MECHANISM



New approach: Numerical Enzyme Kinetics

NO MORE ALGEBRA: LET THE COMPUTER DEAL WITH IT !

Theoretical foundations: *Mass Action Law*

RATE IS PROPORTIONAL TO CONCENTRATION(S)

MONOMOLECULAR REACTIONS

A ⟶ products

rate is proportional to **[A]**

$- d[A] / d t = (k)[A]$

BIMOLECULAR REACTIONS

A + B ⟶ products

rate is proportional to **[A] × [B]**

$- d[A] / d t = - d[B] / d t = (k)[A] \times [B]$

Theoretical foundations: *Mass Conservation Law*

PRODUCTS ARE FORMED WITH THE SAME RATE AS REACTANTS DISAPPEAR

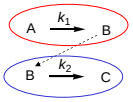
EXAMPLE

A ⟶ P + Q

$- d[\mathbf{A}]/dt = + d[\mathbf{P}]/dt = + d[\mathbf{Q}]/dt$

COMPOSITION RULE ADDITIVITY OF TERMS FROM SEPARATE REACTIONS

mechanism:



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

Program DYNAFIT

REFERENCES

1. Kuzmic P. (1996) *Anal. Biochem.* **237**, 260-273. "Program DYNAFIT for the analysis of enzyme kinetic data"
2. Kuzmic P. (2009) *Methods in Enzymology*, **in press** "DYNAFIT – A software package for enzymology"

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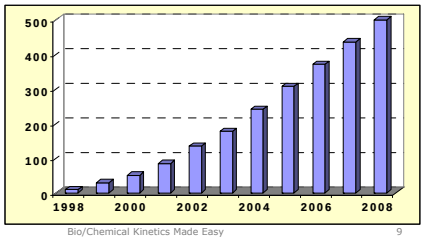
Ref. [1] – total citations:



Initial rate kinetics

TWO BASIC APPROXIMATIONS

1. **Rapid-Equilibrium** Approximation

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

2. **Steady-State** Approximation

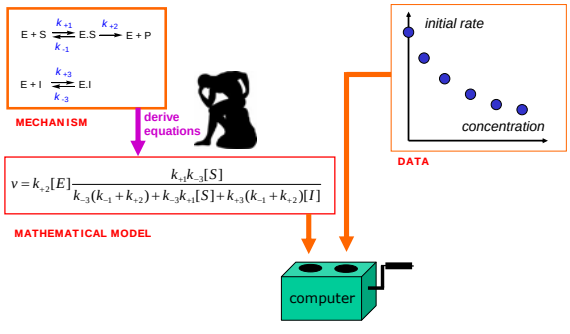
New in DynaFit

Mathematical details in ***BBA – Proteins & Proteomics***, submitted

- *no assumptions* made about relative magnitude of k_1, k_2, k_3

Initial rate kinetics - Traditional approach

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL IDEAS



Initial rate kinetics in DynaFit

GOOD NEWS: MODEL DERIVATION CAN BE FULLY AUTOMATED!

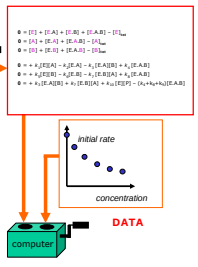
DynaFit input file

```
[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

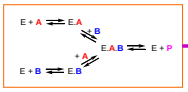
push button

MATHEMATICAL MODEL



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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DynaFit applications to protein kinases

Case study #1: **INITIAL RATES OF ENZYME REACTIONS**

inhibition constants and kinetic mechanism

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WIN-61651: Presumably an ATP analog?

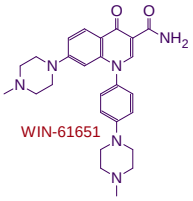
TRADITIONAL STUDY: KINASE INHIBITOR 'WIN-61651' IS COMPETITIVE WITH ATP

Faltynek *et al.* (1995) *J. Enz. Inhib.* **9**, 111-122.

INHIBITION OF T LYMPHOCYTE ACTIVATION BY A NOVEL p56^{lck} TYROSINE KINASE INHIBITOR

CONNIE R. FALTYNEK,[1]* SU WANG,[1] DEBORAH MILLER,[1] PATRICIA MAUVAIS,[1] BRUCE GAUVIN,[2] JOHN REID,[2] WEN XIE,[3] SUSAN HOEKSTRA,[3] PAUL JUNIEWICZ,[3] JAY SARUP,[3] RUTH LEHR,[4] DAVID G. SAWUTZ[2] and DENNIS MURPHY[2]

Departments of [1]Immunology, [2]Biochemistry, [3]Oncopharmacology, [4]Molecular Biology, Sterling Winthrop Pharmaceuticals Research Division, Collegeville, PA 19426, USA

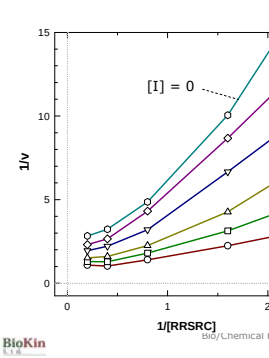


A new p56^{lck} tyrosine kinase inhibitor WIN 61651 [1,4-dihydro-7-(4-methyl-1-piperizinyl)-1-(4-(4-methyl-1-piperizinyl))phenyl-4-oxo-3-quinolinecarboxamide] is described. WIN 61651, which is competitive with ATP, demonstrates selectivity for the lymphoid restricted tyrosine kinase p56^{lck} over serine/threonine kinases, such as protein kinase C and protein kinase A, and over some other tyrosine kinases, including

Lineweaver-Burk plots for WIN-61651

LINEWEAVER-BURK PLOTS AT VARIED [PEPTIDE] AND FIXED [ATP] ARE NONLINEAR

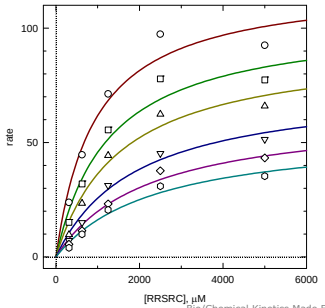
Faltynek *et al.* (1995) *J. Enz. Inhib.* **9**, 111-122.



Direct plot for WIN-61651: Initial rate vs. [peptide]

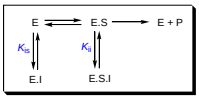
MIXED-TYPE INHIBITION MECHANISM: WHICH IS SMALLER, K_{is} or K_i?

Faltynek *et al.* (1995) *J. Enz. Inhib.* **9**, 111-122. – **FIGURE 1B**



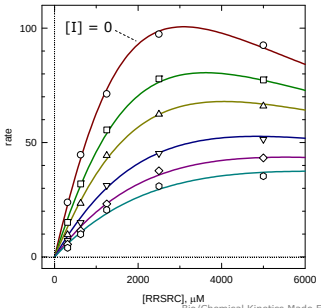

```
[mechanism]
   E + S <===> ES
   ES ---> E + P

   E + I <===> EI
   ES + I <===> ESI
```

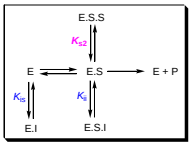


Adding a substrate inhibition term improves fit

GLOBAL NUMERICAL FIT IS BOTH MORE PRECISE AND MORE ACCURATE




```
[mechanism]
   E + S <===> ES
   ES ---> E + P
   ES + S <===> ES2
   E + I <===> EI
   ES + I <===> ESI
```



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)

WIN-61651 summary: Comparison of methods

WIN-61651 IS A MIXED-TYPE INHIBITOR, NOT COMPETITIVE WITH ATP

	parameter (mM)	*DynaFit*	Faltynek *et al.* (1995)
	K_s	9100 ± 3700	990 ± 140
	K_{s2}	1100 ± 450	—
competitive:	K_{is}	28 ± 2	**18** ± 4
uncompetitive:	K_{ii}	**14** ± 5	67 ± 18
	residual squares	***2.1***	***19.5***

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DynaFit applications to protein kinases

Case study #2: **REACTION PROGRESS**

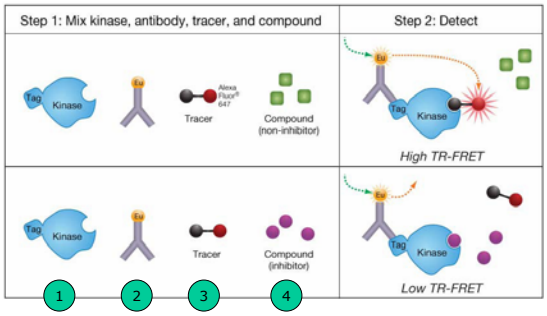
rate constants for kinase-inhibitor interactions
competitive ligand displacement FRET assay

Preliminary experimental data: **Bryan Marks**, *Invitrogen (life Technologies)*

BioKin Ltd. invitrogen

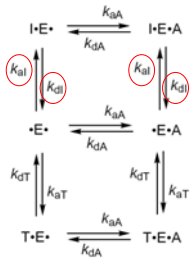
Kinase – Antibody – Tracer – Inhibitor assay

A **FOUR-COMPONENT** MIXTURE



Kinase – Antibody – Tracer – Inhibitor: mechanism

PURPOSE: OBTAIN **RATE CONSTANTS** FOR INHIBITOR ASSOCIATION & DISSOCIATION



E … enzyme
A … antibody (FRET donor)
T … tracer (FRET acceptor)
I … inhibitor

- **four** components
- **five** complexes (3 binary, 2 ternary)
- **six** *unique* rate constants

Rate constants and receptor-ligand residence time

IS IT WORTH CHASING AFTER **RATE** CONSTANTS?

Mbalaviele *et al.* (2009) *J. Pharm. Exp. Ther.* **329**, 14-25

"PHA-408 is an ATP competitive inhibitor, which binds **IKK-2** tightly with a relatively slow **off rate**."

Puttini *et al.* (2008) *haematologica* **93**, 653-61

"The present results suggest a slower **off-rate** (dissociation rate) of [a novel **Abl kinase** inhibitor] compared to **imatinib** as an explanation for the increased cellular activity of the former."

Tummino & Copeland (2008) *Biochemistry* **47**, 5481-92

"... the extent and duration of responses to receptor-ligand interactions depend greatly on the **time period** over which the ligand is in **residence** on its receptor."

Kinase - Antibody - Tracer - Inhibitor: data

KINASE: p38α | ANTIBODY: anti-GST | TRACER: Invitrogen "Tracer-199" | **INHIBITOR: desatinib**

Data: **Bryan Marks**, *Invitrogen*

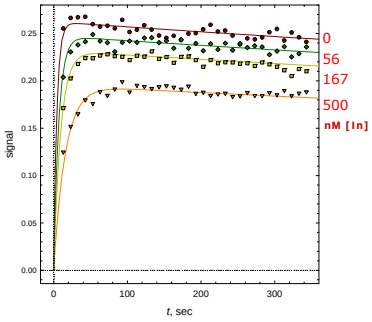
EXPERIMENT:

1. incubate
   [E] = 4 nM
   [Ab] = 40 nM
   [In] = *varied*

   30 minutes

2. dilute 1:20 with Tracer
   final concentrations

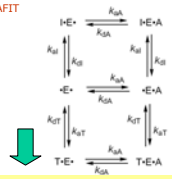
   [E] = 0.2 nM
   [Ab] = 2 nM
   [Tr] = 100 nM
   [In] = *varied*



Kinase - Antibody - Tracer - Inhibitor: fitting model

AUTOMATICALLY DERIVED BY DYNAFIT

system of simultaneous
ordinary
differential equations



[mechanism] ***DynaFit Input***

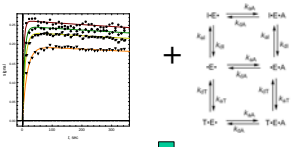
```
E + In <===> E.In      :  kaI  kdI
E + Tr <===> E.Tr      :  kaT  kdT
E + Ab <===> E.Ab      :  kaA  kdA

E.In + Ab <===> E.In.Ab  :  kaA  kdA
E.Ab + In <===> E.In.Ab  :  kaI  kdI
E.Tr + Ab <===> E.Tr.Ab  :  kaA  kdA
E.Ab + Tr <===> E.Tr.Ab  :  kaT  kdT
```


Kinase - Antibody - Tracer - Inhibitor: rate constants

ASSUMPTION: **INDEPDENT** BINDING SITES – ONLY **TWO ADDITIONAL** RATE CONSTANTS

DATA + **MODEL**



LEAST-SQUARES FIT

k_{aI} = 2.1 × 10^{9} $M^{-1}.s^{-1}$
k_{dI} = 19 s^{-1}

PARAMETERS

"RESIDENCE TIME"

τ = 0.05 sec

Kinase - Antibody - Tracer - Inhibitor: state variables

EVOLUTION OF SPECIES CONCENTRATIONS DURING THE KINETIC EXPERIMENT

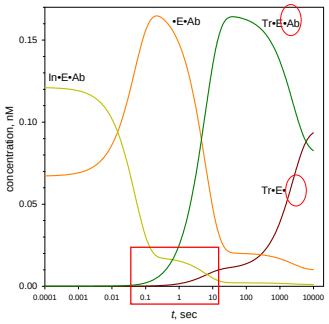
EXPERIMENT:

1. incubate
   [E] = 4 nM
   [Ab] = 40 nM
   [In] = 370 nM
   30 minutes

2. dilute 1:20 with Tracer
   final concentrations

   [E] = 0.2 nM
   [Ab] = 2 nM
   [Tr] = 100 nM
   [In] = 18.5 nM

optimize design!



Acknowledgments

ACADEMIC COLLABORATION:

- **Bryan Marks**: all kinase experiments
  Invitrogen, a.k.a. Life Technologies, Madison, Wisconsin
- **Steve Riddle**: project management
  Invitrogen, a.k.a. Life Technologies, Madison, Wisconsin

INVITATION TO PRESENT:

- **IPK2009 organizers**, **Jan Antosiewicz** (IBB)

Questions ?

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