# **DynaFit** in the Analysis of **Enzyme Progress Curves**

*Irreversible enzyme inhibition*

Petr Kuzmic, Ph.D.
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WATERTOWN, MASSACHUSETTS, U.S.A.

*TOPICS*

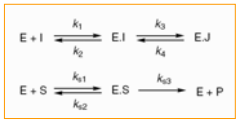
1. Numerical integration vs. algebraic models: DYNAFIT
2. Case study: Caliper assay of irreversible inhibitors
3. Devil is in the details: Problems to be aware of

BioKin Ltd.

## Algebraic solution for time course of enzyme assays

ONLY THE **SIMPLEST** REACTION MECHANISMS CAN BE TREATED IN THIS WAY

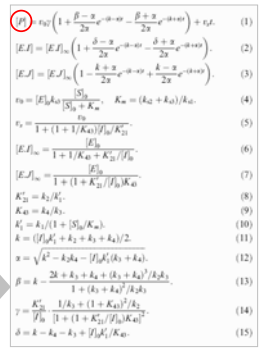
**EXAMPLE**: "slow binding" inhibition




**TASK:** compute **[P]** over time

**SIMPLIFYING ASSUMPTIONS**:

1. No substrate depletion
2. No "tight" binding



$$[P] = v_{ss}\left(1 + \frac{\beta - \alpha}{2\alpha}e^{-(\delta-\alpha)t} - \frac{\beta + \alpha}{2\alpha}e^{-(\delta+\alpha)t}\right) + v_s t. \quad (1)$$

$$[E.I] = [E.I]_\infty\left(1 + \frac{\delta - \alpha}{2\alpha}e^{-(\delta-\alpha)t} - \frac{\delta + \alpha}{2\alpha}e^{-(\delta+\alpha)t}\right). \quad (2)$$

$$[E.J] = [E.J]_\infty\left(1 - \frac{k + \alpha}{2\alpha}e^{-(\delta-\alpha)t} + \frac{k - \alpha}{2\alpha}e^{-(\delta+\alpha)t}\right). \quad (3)$$

$$v_0 = [E]_0 k_{c3}\frac{[S]_0}{[S]_0 + K_m}, \quad K_m = (k_{c2} + k_{c3})/k_{c1}. \quad (4)$$

$$v_s = \frac{v_0}{1 + (1 + 1/K_{e3})/[I]_0/K'_{21}}. \quad (5)$$

$$[E.I]_\infty = \frac{[E]_0}{1 + 1/K_{e3} + K'_{21}/[I]_0}. \quad (6)$$

$$[E.J]_\infty = \frac{[E]_0}{1 + (1 + K'_{21}/[I]_0)K_{e3}}. \quad (7)$$

$$K'_{21} = k_2/k'_1. \quad (8)$$

$$K_{e3} = k_4/k_3. \quad (9)$$

$$k'_1 = k_1/(1 + [S]_0/K_m). \quad (10)$$

$$k = ([I]_0 k'_1 + k_2 + k_3 + k_4)/2. \quad (11)$$

$$\alpha = \sqrt{k^2 - k_3 k_4 - [I]_0 k'_1(k_3 + k_4)}. \quad (12)$$

$$\beta = k - \frac{2k + k_3 + k_4 + (k_3 + k_4)^3/k_3 k_4}{1 + (k_3 + k_4)^2/k_3 k_4}. \quad (13)$$

$$\gamma = \frac{K'_{21}}{[I]_0}\cdot\frac{1/k_3 + (1 + K_{e3})^2/k_3}{[1 + (1 + K'_{21}/[I]_0)K_{e3}]^2}. \quad (14)$$

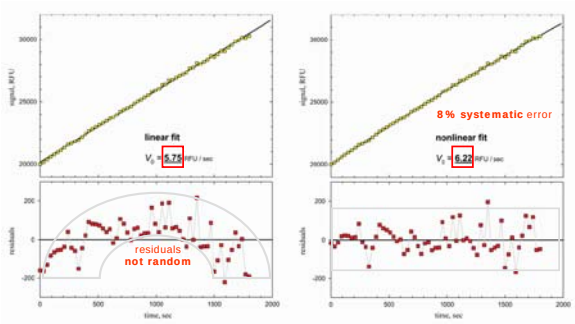
$$\delta = k - k_4 - k_3 + [I]_0 k'_1/K_{e3}. \quad (15)$$

Kuzmic (2008) *Anal. Biochem.* **380**, 5-12



## Enzyme kinetics in the "real world"

**SUBSTRATE DEPLETION** USUALLY CANNOT BE NEGLECTED




Sexton, Kuzmic, *et al.* (2009) *Biochem. J.* **422**, 383-392

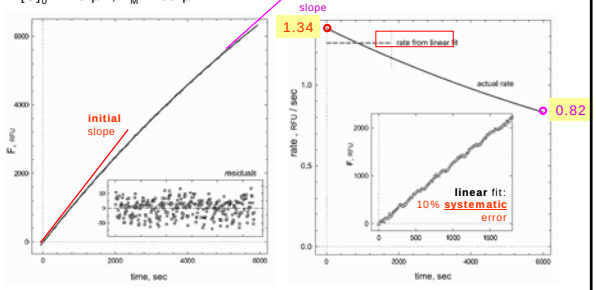


## Progress curvature at low initial [substrate]

**SUBSTRATE DEPLETION** IS MOST IMPORTANT AT $[S]_0 << K_M$

$[S]_0$ = 10 μM, $K_M$ = 90 μM

**~40%** decrease in rate




Kuzmic, Sexton, Martik (2010) *Anal. Biochem.*, **submitted**



## Problems with *algebraic* models in enzyme kinetics

THERE ARE MANY SERIOUS PROBLEMS AND LIMITATIONS

- Can be derived for only a limited number of simplest mechanisms
- Based on many restrictive assumptions:
  - no substrate depletion
  - weak inhibition only (no "tight binding")
- Quite complicated when they do exist

## The solution: *numerical* models

- Can be derived for an arbitrary mechanism
- No restrictions on the experiment (e.g., no excess of inhibitor over enzyme)
- No restrictions on the system itself ("tight binding", "slow binding", etc.)
- Very simple to derive



## Numerical solution of ODE systems: Euler method

COMPLETE REACTION PROGRESS IS COMPUTED IN TINY **LINEAR** INCREMENTS

mechanism: $A \xrightarrow{k} B$

differential rate equation

practically useful methods are much more complex!

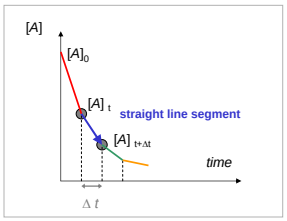
$$\mathbf{d}\,[A] / \mathbf{d}\,t = -k\,[A]$$

$$\Delta\,[A] / \Delta\,t = -k\,[A]$$

$$\Delta\,[A] = -k\,[A]\,\Delta\,t$$

$$[A]_{t+\Delta t} - [A]_t = -k\,[A]_t\,\Delta\,t$$

$$[A]_{t+\Delta t} = [A]_t - k\,[A]_t\,\Delta\,t$$



## Automatic derivation of differential equations

IT IS SO SIMPLE THAT EVEN A "DUMB" MACHINE (THE COMPUTER) CAN DO IT

| Example input (plain text file): | Rate terms: | Rate equations: |
|---|---|---|
| k1<br>E + S ---> ES<br>k2<br>ES ---> E + S<br>k3<br>ES ---> E + P | $k_1 \times [E] \times [S]$<br>$k_2 \times [ES]$<br>$k_3 \times [ES]$ | "E disappears"<br>$d[E]/dt = \ominus k_1 \times [E] \times [S]$<br>"E is formed" $\oplus k_2 \times [ES]$<br>$\oplus k_3 \times [ES]$ |

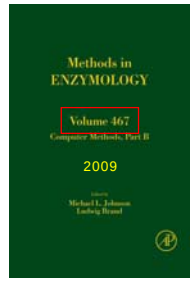
"multiply [E] × [S]"

similarly for other species (**S**, **ES**, and **P**)



## Software DYNAFIT (1996 - 2010)

PRACTICAL IMPLEMENTATION OF "NUMERICAL ENZYME KINETICS"



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

Kuzmic (2009) *Meth. Enzymol.*, **467**, 247-280



## DYNAFIT: What can you do with it?

ANALYZE/SIMULATE MANY TYPES OF EXPERIMENTAL DATA ARISING IN BIOCHEMICAL LABORATORIES

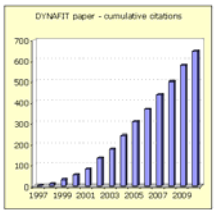
- **Basic tasks:**
  - simulate artificial data (assay design and optimization)
  - fit experimental data (determine inhibition constants)
  - design optimal experiments (in preparation)
- **Experiment types:**
  - time course of enzyme assays
  - initial rates in enzyme kinetics
  - equilibrium binding assays (pharmacology)
- **Advanced features:**
  - confidence intervals for kinetic constants
    - *Monte-Carlo intervals*
    - *profile-t method (Bates & Watts)*
  - goodness of fit - residual analysis (Runs-of-Signs Test)
  - model discrimination analysis (Akaike Information Criterion)
  - robust initial estimates (Differential Evolution)
  - robust regression estimates (Huber's Mini-Max)



## DYNAFIT applications: mostly biochemical kinetics

BUT NOT NECESSARILY: ANY SYSTEM THAT CAN BE DESCRIBED BY A FIRST-ORDER ODEs

| | |
|---|---|
| 141 | *Biochemistry* |
| 78 | *J. Biol. Chem.* |
| 30 | *J. Am. Chem. Soc.* |
| 19 | *J. Org. Chem.* |
| 19 | *J. Mol. Biol.* |
| 15 | *Proc. Natl. Acad. Sci. U. S. A.* |
| 12 | *Anal. Biochem.* |
| 11 | *Biochem. J.* |




~ **650** journal articles total



## DynaFit in the Analysis of Enzyme Progress Curves

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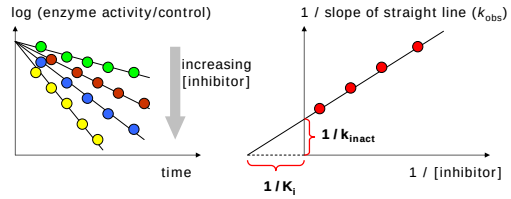
## Traditional analysis of irreversible inhibition

**BEFORE 1981** (IBM-PC) ALL LABORATORY DATA MUST BE CONVERTED TO **STRAIGHT LINES**

1962

Kitz-Wilson plot

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$$




Kitz & Wilson (1962) J. Biol. Chem. **237**, 3245-3249

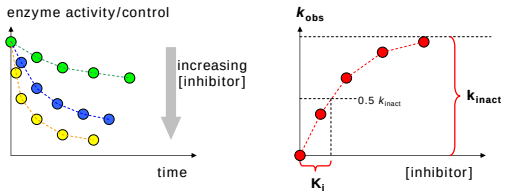
## Traditional analysis – "Take 2": nonlinear

**AFTER 1981** STRAIGHT LINES ARE NO LONGER NECESSARY (**"NONLINEAR REGRESSION"**)

1981

IBM-PC (Intel 8086)

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$$




$A = A_0 \exp(-k_{obs}\, t)$

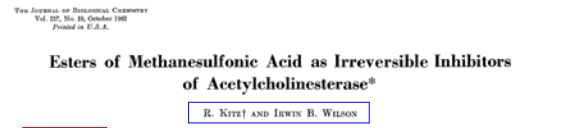
$k_{obs} = k_{inact} / (1 + K_i / [I])$



## Traditional analysis: Three assumptions (part 1)

"LINEAR" OR "NONLINEAR" ANALYSIS – THE SAME ASSUMPTIONS APPLY

1. Inhibitor **binds only *weakly*** to the enzyme


THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 237, No. 10, October 1962
Printed in U.S.A.

Esters of Methanesulfonic Acid as Irreversible Inhibitors of Acetylcholinesterase*

R. KITZ† AND IRWIN B. WILSON

For $(I) \gg E^0$, let

$$k_{app} = \frac{k_3}{1 + K_I/(I)}$$

no "tight binding"

[I], $K_i$ must not be comparable with [E]



## Traditional analysis: Three assumptions (part 2)

"LINEAR" OR "NONLINEAR" ANALYSIS – THE SAME ASSUMPTIONS APPLY

2. **Enzyme activity** over time is measured **"directly"**
In a ***substrate*** assay, plot of **product [P]** vs. time must be a **straight line** at **[I] = 0**

ASSUMED MECHANISM:

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$$

ACTUAL MECHANISM IN MANY CASES:

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$$

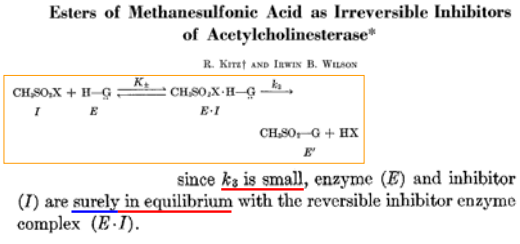
$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E{\bullet}S \xrightarrow{k_{cat}} E + P$$



## Traditional analysis: Three assumptions (part 3)

"LINEAR" OR "NONLINEAR" ANALYSIS – THE SAME ASSUMPTIONS APPLY

3. Initial **binding / dissociation** is much **faster** than inactivation
**("rapid equilibrium approximation")**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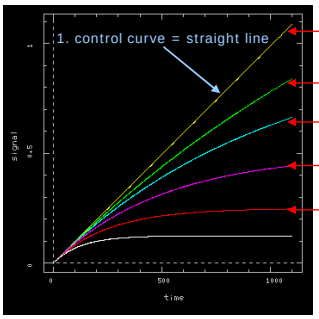
$$\underset{I}{CH_3SO_2X} + \underset{E}{H{-}\underset{\cdot\cdot}{G}} \underset{}{\overset{K_b}{\rightleftharpoons}} \underset{E\cdot I}{CH_3SO_2X\cdot H{-}\underset{\cdot\cdot}{G}} \xrightarrow{k_3} \underset{E'}{CH_3SO_2{-}G} + HX$$

since $k_3$ is small, enzyme $(E)$ and inhibitor $(I)$ are surely in equilibrium with the reversible inhibitor enzyme complex $(E \cdot I)$.



## Simplifying assumptions: Requirements for data

**HOW MUST OUR DATA LOOK** SO THAT WE CAN ANALYZE IT BY THE **TRADITIONAL** METHOD ?




[I] = 0

[I] = 100 nM

[I] = 200 nM

[I] = 400 nM

[I] = 800 nM

SIMULATED:

[E] = 1 nM

[S] = 10 μM

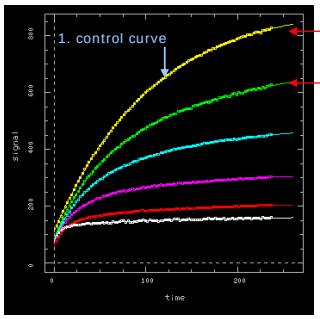
$K_m$ = 1 μM

2. inhibitor concentrations must be much higher than enzyme concentration



## Actual experimental data (COURTESY OF Art Wittwer, Pfizer)

**NEITHER** OF THE TWO MAJOR SIMPLIFYING ASSUMPTION ARE SATISFIED !




[I] = 0

[I] = 2.5 nM

[E] = 0.3 nM

2. inhibitor concentrations within the same order of magnitude

## Numerical model for Caliper assay data

**NO ASSUMPTIONS** ARE MADE ABOUT EXPERIMENTAL CONDITIONS

DynaFit input:

```
[mechanism]
   E + S ---> E + P     :     kdp
   E + I <===> EI       :     kai   kdi
   EI ---> X            :     kx
```

Automatically generated fitting model:

$$d[E]/dt = -k_{dp}[E][S] + k_{dp}[E][S] - k_{ai}[E][I] + k_{di}[EI]$$
$$d[S]/dt = -k_{dp}[E][S]$$
$$d[P]/dt = +k_{dp}[E][S]$$
$$d[I]/dt = -k_{ai}[E][I] + k_{di}[EI]$$
$$d[EI]/dt = +k_{ai}[E][I] - k_{di}[EI] - k_{x}[EI]$$
$$d[X]/dt = +k_{x}[EI]$$

Back to Index...

Program **DynaFit** ver. **4.04.040**



## Caliper assay: Results of fit – optimized $[E]_0$

THE ACTUAL **ENZYME CONCENTRATION** SEEMS **HIGHER** THAN THE NOMINAL VALUE

Optimized Parameters *units:* μM, minutes

| No. | Par#Set | Initial | Final | Std. Error | CV (%) |
|---|---|---|---|---|---|
| #1 | kdp | 30 | 9.25349 | 1.27026 | 13.73 |
| #2 | kai | 1 | 4.24578 | 0.107625 | 2.53 |
| #3 | kdi | 0.01 | 0.00420401 | 0.000254043 | 6.04 |
| #4 | kx | 0.01 | 0.00818624 | 0.000497752 | 6.08 |
| #5 | [E] | 0.0003 | 0.00102678 | 0.000141023 | 13.73 |

Ki ~ $[E]_0$
"tight binding"

$k_{off}$ ~ $k_{inact}$
**not** "rapid equilibrium"

$K_i$ = $k_{off}/k_{on}$
= **1 nM**

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$$

$$E + S \xrightarrow{k_{cat}/K_m} E + P$$

| | | |
|---|---|---|
| $k_{on}$ | $7.1 \times 10^4$ | $M^{-1}.sec^{-1}$ |
| $k_{off}$ | 0.00007 | $sec^{-1}$ |
| $k_{inact}$ | 0.00014 | $sec^{-1}$ |
| $k_{cat}/K_m$ | $1.5 \times 10^5$ | $M^{-1}.sec^{-1}$ |
| $[E]_0$ | 1.0 | nM |



## Caliper assay violates assumptions of classic analysis

**ALL THREE ASSUMPTIONS** OF THE TRADITIONAL ANALYSIS WOULD BE VIOLATED

1. Enzyme concentration is *not* much lower than $[I]_0$ or $K_i$
2. The dissociation of the E•I complex is *not* much faster than inactivation
3. The control progress curve ([I] = 0) is *not* a straight line
   (Substrate **depletion** is significant)

If we used the traditional algebraic analysis, the results ($K_i$, $k_{inact}$) would likely be **incorrect**.



## Numerical model more informative than algebraic

**MORE INFORMATION** EXTRACTED FROM THE **SAME DATA**

| Traditional model (Kitz & Wilson, 1962) | General numerical model |
|---|---|
| $E + I \underset{}{\overset{K_i}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$ | $E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$ |
| very fast / very slow | no assumptions ! |
| **Two** model parameters | **Three** model parameters |

Add another dimension (à la "residence time") to the QSAR ?



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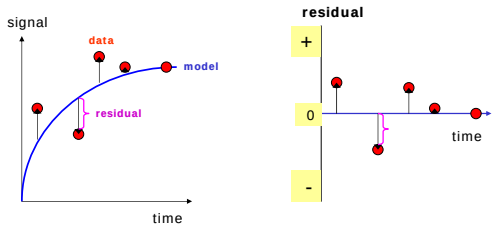
## Numerical modeling looks simple, but...

A **RANDOM** SELECTION OF **A FEW** TRAPS AND PITFALLS

- Residual plots
  we must always look at them
- Adjustable concentrations
  we must always "float" some concentrations in a global fit
- Initial estimates: the "false minimum" problem
  nonlinear regression requires us to **guess** the solution beforehand
- Model discrimination: Use your judgment
  the theory of model discrimination is far from perfect

## Residual plots

RESIDUAL PLOTS SHOWS THE **DIFFERENCE** BETWEEN THE **DATA** AND THE "BEST-FIT" **MODEL**

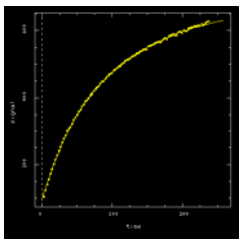





## Direct plots of data: Example 1

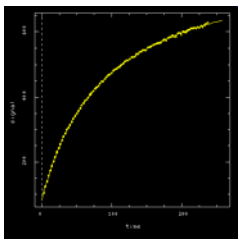
THESE TWO PLOTS LOOK "**INDISTINGUISHABLE**", DO THEY NOT ?

**One-step** inhibition



$E + I \xrightarrow{k_{inact}} X$

**Two-step** inhibition



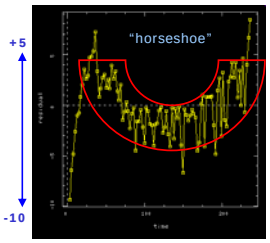
$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$



## Residual plots: Example 1

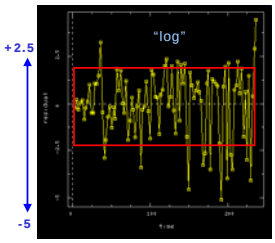
THESE TWO PLOTS LOOK "**VERY DIFFERENT**", DO THEY NOT ?

**One-step** inhibition




$E + I \xrightarrow{k_{inact}} X$

**Two-step** inhibition



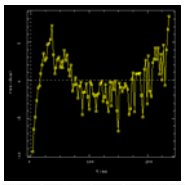

$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} X$



## Residual plots: Runs-of-signs test

WE DON'T HAVE TO RELY ON VISUALS ("LOG" VS. "HORSESHOE")

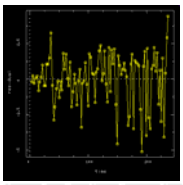
**One-step** inhibition



| points | positive | runs | expect | st.dev. | p |
|---|---|---|---|---|---|
| 100 | 44 | 18 | 50.28 | 4.90232 | 0 |

probability 0%

**Two-step** inhibition



| points | positive | runs | expect | st.dev. | p |
|---|---|---|---|---|---|
| 100 | 57 | 46 | 50.02 | 4.87618 | 0.307292 |

probability 31%

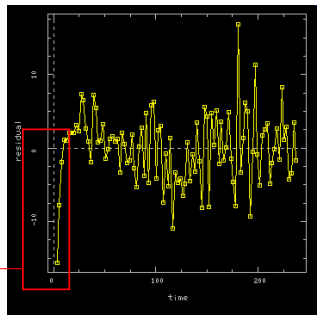
passes $p > 0.05$ test



## Residual plots: Example 2

SOMETIMES IT'S O.K. TO HAVE "OUTLIERS" – ***USE YOUR JUDGMENT***

It's not always easy to judge "just how good" the residuals are:



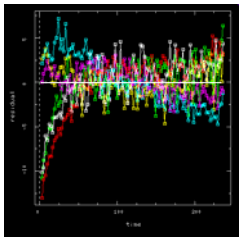

"something" happened with the first three time-points



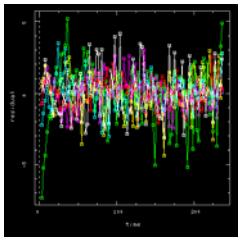
## Relaxed inhibitor concentrations: Example 1

WE **ALWAYS** HAVE "TITRATION ERROR" !

Residual plots: **fixed** [I]



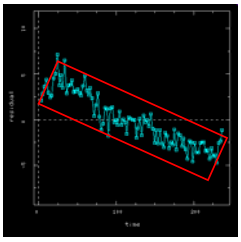
Residual plots: **relaxed** [I]



## Relaxed inhibitor concentrations: Example 1 (detail)

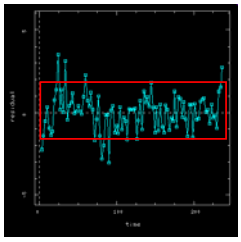
WE **ALWAYS** HAVE "TITRATION ERROR" !

Residual plots: **fixed** [I]



$n_D$ = 100, $n_P$ = 44
$n_R$ = 18
$p$ < 0.0000001

Residual plots: **relaxed** [I]



$n_D$ = 100, $n_P$ = 55
$n_R$ = 44
$p$ = 0.08



## Relaxed inhibitor concentrations: not all of them

ONE (USUALLY ANY ONE) OF THE INHIBITOR CONCENTRATIONS MUST BE KEPT FIXED

DynaFit script:

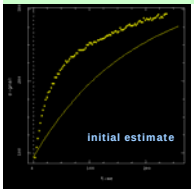
```
...
[data]
   directory  ./users/COM/.../100514/C1/data
   extension  txt

   file       0nM   | offset auto ?
   file       2p5nM | offset auto ? | conc I = 0.0025 ?
   file       5nM   | offset auto ? | conc I = 0.0050 ?
   file       10nM  | offset auto ? | conc I = 0.0100 ?   <- FIXED
   file       20nM  | offset auto ? | conc I = 0.0200 ?
   file       40nM  | offset auto ? | conc I = 0.0400 ?
...
```



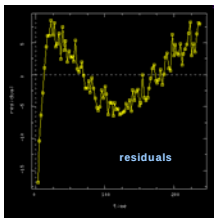
## Initial estimates: the "false minimum" problem

NONLINEAR REGRESSION REQUIRES US TO **GUESS** THE SOLUTION BEFOREHAND

```
E + S ---> E + P :  kdp
E + I <===> EI   :  kai   kdi
EI ---> X        :  kx
```




iterative refimenement




```
[constants]
   kdp =   10 ?
   kai =   10 ?
   kdi =    1 ?
   kx  = 0.01 ?
[concentrations]
   S   = 0.85 ?
```

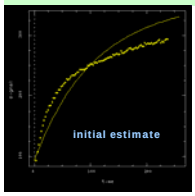
```
kdp =    72
kai =   4.5
kdi =   1.8
kx  = 0.048

[S] =  0.22
```



## Large effect of slight changes in initial estimates

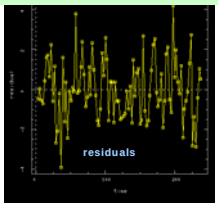
IN **UNFAVORABLE** CASES EVEN **ONE ORDER OF MAGNITUDE** DIFFERENCE IS IMPORTANT

```
E + S ---> E + P :  kdp
E + I <===> EI   :  kai   kdi
EI ---> X        :  kx
```




iterative refimenement

```
E + S ---> E + P :  kdp
E + I <===> EI   :  kai   kdi
```




```
[constants]
   kdp =   10 ?
   kai =  0.1 ?
   kdi = 0.01 ?
   kx  =  0.1 ?
[concentrations]
   S   = 0.85 ?
```

```
kdp =    96
kai =  0.36
kdi =0.0058
kx  =   ~ 0

[S] =  0.27
```



## Solution to initial estimate problem: systematic scan

DYNAFIT-4 ALLOWS **HUNDREDS, OR EVEN THOUSANDS,** OF DIFFERENT INITIAL ESTIMATES

```
[mechanism]
   E + S ---> E + P    :    kdp
   E + I <===> EI      :    kai   kdi
   EI ---> X           :    kx

[constants]
   kdp = { 10, 1, 0.1, 0.01 } ?
   kai = { 10, 1, 0.1, 0.01 } ?
   kdi = { 10, 1, 0.1, 0.01 } ?
   kx  = { 10, 1, 0.1, 0.01 } ?
```

MEANS:

"Try all possible combinations of initial estimates."

- 4 rate constants
- 4 estimates for each rate constant

- $4 \times 4 \times 4 \times 4 = 4^4 =$ **256** initial estimates



## Model discrimination: Use your judgment

DYNAFIT IMPLEMENTS **TWO** MODEL-DISCRIMINATION **CRITERIA**

1. **Fischer's *F*-ratio** for nested models

**F-Test Criterion**

Probabilities (%)

| | | #1 | #2 |
|---|---|---|---|
| one step | #1 | | |
| two steps | #2 | 100.0 | |

relative sum of squares

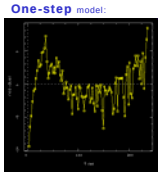
2. **Akaike Information Criterion** for all models

**Akaike Information Criterion**

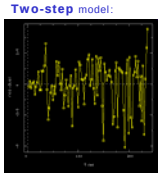
| | model | $n_D$ | $n_P$ | $SS_{rel}$ | $AIC_c$ | $\Delta AIC_c$ | weight |
|---|---|---|---|---|---|---|---|
| [1] | one step | 100 | 4 | 2.931 | 595.5 | 102.9 | 0.000 |
| [2] | two steps | 100 | 6 | 1.000 | 592.5 | 0.0 | 1.000 |

probability (0 .. 1)

**One-step** model:



**Two-step** model:



## Summary and Conclusions

NUMERICAL MODELS ENABLE US TO DO **MORE USEFUL EXPERIMENTS** IN THE LABORATORY

**ADVANTAGES** of "Numerical Enzyme Kinetics" (the new approach):

- No constraints on experimental conditions
  EXAMPLE: **large excess** of [I] over [E] no longer required
- No constraints on the theoretical model
  EXAMPLE: **dissociation** rate can be comparable with **deactivation** rate
- Theoretical model is automatically derived by the computer
  No more algebraic rate equations
- Learn more from the same data
  EXAMPLE: Determine $k_{ON}$ and $k_{OFF}$, not just equilibrium constant $\mathbf{K_i} = k_{OFF}/k_{ON}$

**DISADVANTAGES:**

- **Change** in standard operating procedures
  Is it better stick with **invalid but established** methods ? (Continuity problem)
- **Training / Education** required
  Where to find time for **continuing education** ? (Short-term vs. long-term view)



## Questions?

MORE INFORMATION AND CONTACT:

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