A New 'Microscopic' Look at Steady-state Enzyme Kinetics

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Outline

Part I: **Theory**
steady state enzyme kinetics: a new approach

Part II: **Experiment**
inosine-5'-monophosphate dehydrogenase

Enzyme kinetic modeling and its importance

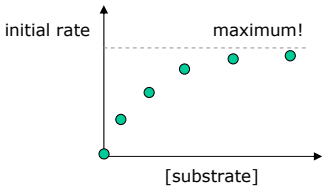
WHAT CAN ENZYME KINETICS DO FOR US?

macroscopic
laboratory
measurement

→ mathematical model →

microscopic
molecular
mechanisms

EXAMPLE: Michaelis-Menten (1913)



→ rectangular hyperbola

$v = V_m S/(S+K_m)$

MECHANISM:

substrate and enzyme form a **reactive complex**, which decomposes into products and **regenerates** the enzyme catalyst

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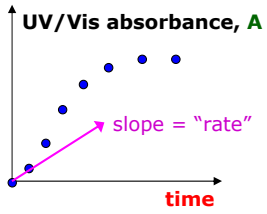
The main purpose of enzyme kinetics is to elucidate microscopic **molecular mechanisms** of enzyme action and inhibition.

It is a remarkable feat that we can go from observing events on a macroscopic scale (i.e. plate-reader well) to making inference about events that evolve on a **molecular scale**.

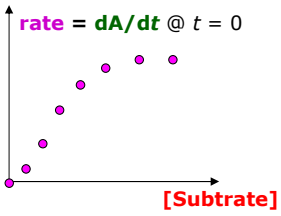
Another use of enzyme kinetics, no less important, is to measure **quantitatively** the strength enzyme binding with various ligands (substrates, **inhibitors**), as well as the reaction rates.

Two types of enzyme kinetic experiments

1. "reaction progress" method:



2. "initial rate" method:



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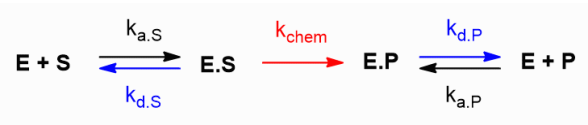
1. "reaction progress" method:

- mix enzyme and reactants
- monitor some experimental signal over time
- build mathematical models of the reaction **time course**
- see which of the models fits best

2. "initial rate" method:

- mix enzyme and reactants
- monitor some experimental signal over time
- compute the **slope** (reaction rate) at **time = 0**
- repeat at various concentrations of reactants
- build mathematical models of the **initial rates** changing with initial concentrations
- see which of the models fits best

The steady-state approximation in enzyme kinetics



Two different mathematical formalisms for **initial rate** enzyme kinetics:

1. **"rapid equilibrium"** approximation $k_{chem} << k_{d.S}$, $k_{chem} << k_{d.P}$

2. **"steady state"** approximation $k_{chem} \approx k_{d.S}$, $k_{chem} \approx k_{d.P}$ *or* $k_{chem} > k_{d.S}$, $k_{chem} > k_{d.P}$

Analysis of initial rates in enzyme kinetics usually proceeds
while invoking one of two theoretical formalisms, or approximations:

- **"rapid equilibrium"** approximation

- *slow* chemistry
- *fast* ligand dissociation

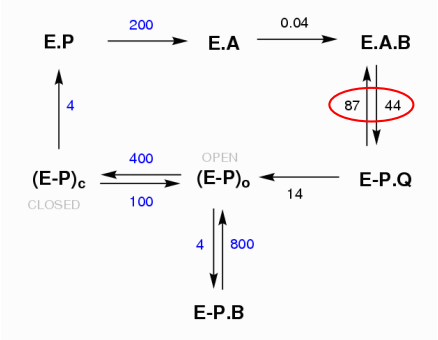
2. **"steady state"** approximation

- *fast or slow* chemistry
- it is a more general approach

Importance of steady-state treatment: Therapeutic inhibitors

MANY ENZYMES THAT ARE TARGETS FOR DRUG DESIGN DISPLAY "FAST CHEMISTRY"

Example: Inosine-5'-monophosphate dehydrogenase from *Cryptosporidium parvum*



chemical step:
fast hydride transfer

T. Riera *et al.* (2008) Biochemistry **47**, 8689–8696

Arguably the "holy grail" of enzyme kinetics, in the context of therapeutic inhibition, is to understand the **microscopic rate constants** for enzyme-inhibitor interactions.

However, such **detailed understanding** is not possible for "fast enzymes", unless we actually invoke the steady-state approximation.

The reason is that specifically the binding of inhibitors to **enzyme-substrate complexes** or **enzyme-product complexes** cannot be properly quantified without having a firm grasp of *all* microscopic rate constants in the given mechanism.

(This is not true for **"competitive"** interactions, where the inhibitor binds to the **free enzyme**. For those interactions, it is enough to know the actual "on" and "off" rate constants.)

Here is an example of an enzyme that has "fast" chemistry and so we must study the *inhibition* kinetics under the steady-state approximation.

Steady-state initial rate equations: The conventional approach

The King-Altman method conventionally proceeds in **two separate steps:**

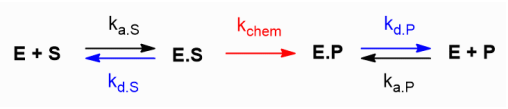
Step One:	*Derive* a rate equation in terms of **microscopic rate constants**
Step Two:	*Rearrange* the original equation in terms of **secondary "kinetic constants"**

EXPERIMENT:

- **Measure** "kinetic constants" (K_m, V_{max}, ...) experimentally.
- **Compute** micro-constant (k_{on}, k_{off}, ...) from "kinetic constants", when possible.

Steady-state initial rate equations: Example

1. postulate a particular kinetic mechanism:



2. derivation ("Step One"):

$$v = [E]\frac{n_1\,[S]}{d_1 + d_2\,[P] + d_3\,[S]}$$

$$\begin{aligned} n_1 &= k_{\mathrm{a.S}}k_{\mathrm{chem}}k_{\mathrm{d.P}} \\ d_1 &= k_{\mathrm{d.P}}\,(k_{\mathrm{d.S}} + k_{\mathrm{chem}}) \\ d_2 &= k_{\mathrm{a.P}}\,(k_{\mathrm{d.S}} + k_{\mathrm{chem}}) \\ d_3 &= k_{\mathrm{a.S}}\,(k_{\mathrm{d.P}} + k_{\mathrm{chem}}) \end{aligned}$$

micro-constants

3. rearrangement ("Step Two"):

$$v = [E]\,k_{\mathrm{cat}}\,\frac{[S]/K_{\mathrm{m(S)}}}{1 + [P]/K_{\mathrm{i(P)}} + [S]/K_{\mathrm{m(S)}}}$$

$$\begin{aligned} k_{\mathrm{cat}} &= \frac{k_{\mathrm{chem}}k_{\mathrm{d.P}}}{k_{\mathrm{d.P}} + k_{\mathrm{chem}}} \\ K_{\mathrm{m(S)}} &= \frac{k_{\mathrm{d.P}}\,(k_{\mathrm{d.S}} + k_{\mathrm{chem}})}{k_{\mathrm{a.S}}\,(k_{\mathrm{d.P}} + k_{\mathrm{chem}})} \\ K_{\mathrm{i(P)}} &= \frac{k_{\mathrm{d.P}}}{k_{\mathrm{a.P}}} \end{aligned}$$

"kinetic" constants

Details: Segel, I. (1975) *Enzyme Kinetics*, Chapter 9, pp. 509-529.

•Whether or not the steady-state approximation is necessary depends *only* on the relative magnitude of the **chemical** rate constants (red) vs. the **dissociation** rate constants (blue).

•Remember: The microscopic rates of the **association** steps (black) depend on concentrations and therefore have **no upper bound**!

•After the first step of the derivation, the rate equation consists of **microscopic** rate constants. Those are *usually* not accessible to direct measurement in initial-rate experiments.

•In the second step, the micro-constants are grouped in various ways into "kinetic" constants such as K_m nd k_{cat}.

•Very soon we will see that this second step of the derivation is not always possible. In contrast, the first step is always possible.

Several problems with the conventional approach

1. **Fundamental** problem:

   Step 2 (deriving "K_m" etc.) is in principle **impossible for branched** mechanisms.

2. **Technical** problem:

   Even when **Step 2** is possible in principle, it is **tedious and error prone**.

3. **Resource** problem:

   Measuring "kinetic constants" (K_m, K_i, ...) consumes a lot of **time and materials**.

1. **Fundamental** problem:

   Step 2 (rearrangement) is in principle **impossible for branched** mechanisms.
   The means: There can be **no K_m, V_{max}, etc.** derived for branched mechanisms.
   We can get a rate equation in terms of micro-constants, but then we are stuck.

2. **Technical** problem:

   Even when **Step 2** is possible in principle, it is tedious and error prone.
   These algebraic derivations typically are for "hard-core" kineticists only.
   It would be nice to **automate all derivations** and relegate this task to a machine.

3. **Economics** or resource management problem:

   Measuring "kinetic constants" (K_m, k_{cat}, ...) consumes a lot of time and materials.
   Plus, it is not very clear how to convert "kinetic constants" to micro-constants.
   It would be nice to do a **global fit**, to extract as many **micro-constants** as possible.

A solution to the fundamental problem

TURN THE CONVENTIONAL APPROACH ON ITS HEAD:

CONVENTIONAL APPROACH:

- **Measure** "kinetic constants" (K_m, V_{max}, ...) experimentally, when they do exist.
- **Compute** micro-constant (k_{on}, k_{off}, ...) from "kinetic constants", when possible.

THE NEW APPROACH:

- **Measure** micro-constant (k_{on}, k_{off}, ...) experimentally.
- **Compute** "kinetic constants" (K_m, V_{max}, ...), when they do exist.

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Advantages of the new approach:

1. Rate equations formulated in terms of micro-constants **always exist**.
   Rate equations in terms of "kinetic constants" do not always exist (branched mechanisms).

2. In some cases the best-fit values of micro-constants are actually their **"true" values**.

   Caveat: in other cases the best-fit micro-constants are only "apparent" values.

   But Even if we cannot estimate the "true" values (model redundancy, see below), we can
   often at least estimate either the **lower** or the **upper bound** for a given micro-constant.

Disadvantages of the new approach:

1. In most cases the model formulated in terms of micro-constants is **overparametrized (redundant)**.

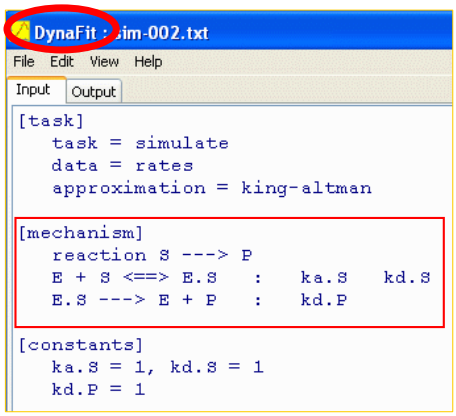
   However, model redundancy can be dealt with in numerous ways:

   - make educated guesses based on literature reports;
   - supply estimates from independent rapid-kinetic measurements;
   - construct a **minimal ("reduced")** kinetic model, with fewer steps

A solution to the technical / logistical problem

USE A SUITABLE **COMPUTER PROGRAM** TO **AUTOMATE** ALL ALGEBRAIC DERIVATIONS

INPUT:



OUTPUT:

$v = [E]_0 \boldsymbol{N}/\boldsymbol{D} = d[P]/dt = + k_{d.P}\,[E.S]$

Numerator

$\boldsymbol{N} = n_1\,[S]$

where

$n_1 = \dfrac{k_{a.S}\,k_{d.P}}{k_{d.S} + k_{d.P}}$

Denominator

$\boldsymbol{D} = d_1 + d_2\,[S]$

where

$d_1 = 1$

$d_2 = \dfrac{k_{a.S}}{k_{d.S} + k_{d.P}}$

$k_{cat(f)} = n_1/d_2 = k_{d.P}$

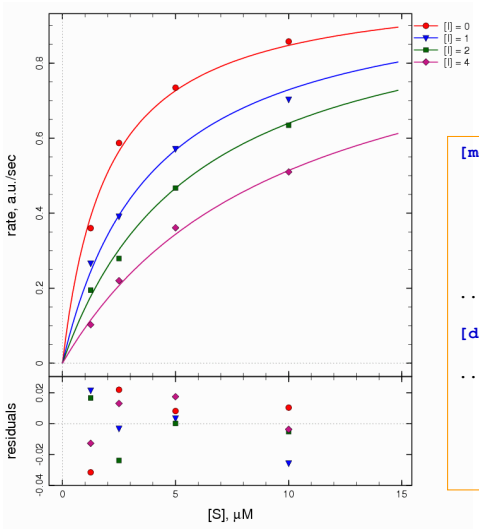
$K_{m(S)} = d_1/d_2 = \dfrac{k_{d.S} + k_{d.P}}{k_{a.S}}$

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

- The DynaFit software is available free of charge (to all *academic* researchers and students) from the BioKin website.
- It has been cited in close to **900** journal articles up to this point, most of them in the journal *Biochemistry*, followed by *JBC*.
- The **"King-Altman"** method in DynaFit has been beefed up in the last month or so, specifically to facilitate the IMPDH collaboration with Liz Hedstrom's group at Brandeis.

A solution to the resource problem

USE **GLOBAL FIT** OF MULTI-DIMENSIONAL DATA TO REDUCE THE TOTAL NUMBER OF DATA POINTS



16-20 data points are sufficient

DYNAFIT INPUT:

```
[mechanism]
   reaction  S ---> P
   modifiers I

   E + S <==> E.S    :   ka.S   kd.S
   E.S ---> E + P    :   kd.P
   E + I <==> E.I    :   ka.I   kd.I
...

[data]
   variable S
...
   file d01 | conc I = 0 | label [I] = 0
   file d02 | conc I = 1 | label [I] = 1
   file d03 | conc I = 2 | label [I] = 2
   file d04 | conc I = 4 | label [I] = 4
```

global fit

- Most of the time, we all do too many experiments (wastefully), because of the less-than-optimal methods for data analysis.
- The key is always use the **global fit** method.
- With global fit, we only need something like **3-5 times** as many data points as there are adjustable model parameters (rate constants).
- For example, if we are trying to determine 4 microscopic rate constants or 4 "kinetic constants", then with a well-designed experiment we only need something like 16 or 20 data points.
- This is *not true* in the "traditional" approach to kinetic analysis: analyzing subsets of data and then doing "re-plots" or "re-analyses" of intermediate results.

Part II: Experiment

1. **Background**

   Inosine-5'-monophosphate dehydrogenase (IMPDH) and its importance

2. **"Microscopic" kinetic model from stopped-flow data**

   A complex "microscopic" model of IMPHD inhibition kinetics

3. **Validating the transient kinetic model by initial rates**

   Is our complex model sufficiently supported by initial-rate data?

***Data**: Dr. Yang Wei (Hedstrom Group, Brandeis University)*

IMPDH: Inosine-5'-monophosphate dehydrogenase

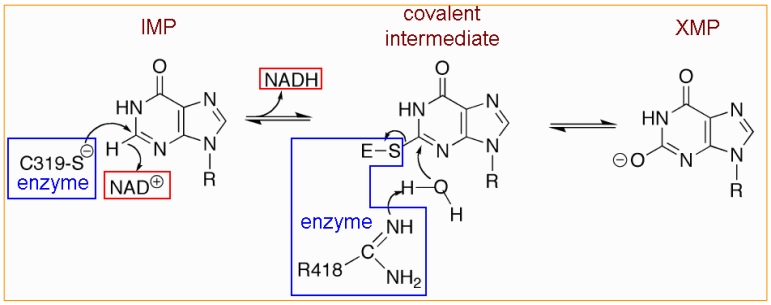
A POTENTIAL TARGET FOR THERAPEUTIC INHIBITOR DESIGN

Overall reaction:

inosine-5'-monophosphate + NAD$^+$ → xanthosine-5'-monophosphate + NADH

IMP **"A"** **"B"** XMP **"P"** **"Q"**

Chemical mechanism:



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•This enzyme is important for drug design because the human form is sufficiently different from the form found in pathogenic microorganisms.

•The human form is a "billion dollar" drug design target in its own right. There are drugs currently in use targeting human IMPDH.

•A salient feature is that there is a **covalent intermediate** on the reaction pathway.

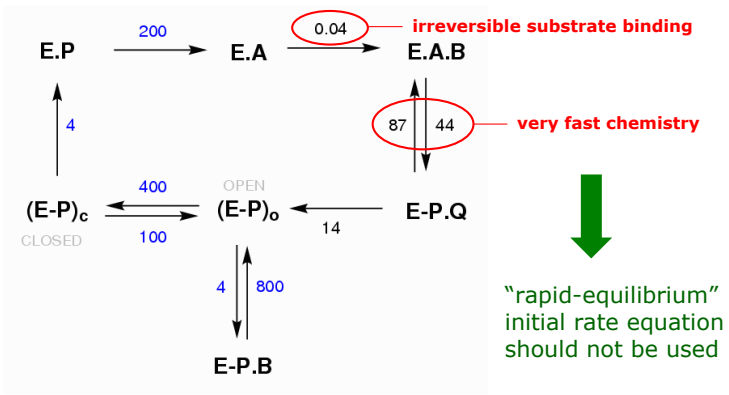
•Importantly, many inhibitors seem to be binding almost exclusively to this covalent intermediate. It turns out that there is no "rapid equilibrium" mechanism (and therefore a "rapid equilibrium" rate equation) that can properly account for this fact. Instead we really need **steady-state**.

IMPDH kinetics: Fast hydrogen transfer catalytic step

HIGH REACTION RATE MAKES IS NECESSARY TO INVOKE THE **STEADY-STATE** APPROXIMATION

A = IMP
B = NAD^+
P = XMP
Q = NADH

UNITS:
μM, sec



"rapid-equilibrium" initial rate equation should not be used

T. Riera *et al.* (2008) Biochemistry **47**, 8689–8696
IMPDH from *Cryptosporidium parvum*

•Once again, here is the "fast chemistry" feature highlighted.

•Also note that the substrate "A" (i.e. NAD^+) is binding effectively in an irreversible fashion!

•That means that the dissociation rate constant is effectively zero.

•It can't get any further from the classic "rapid equilibrium" approximation.

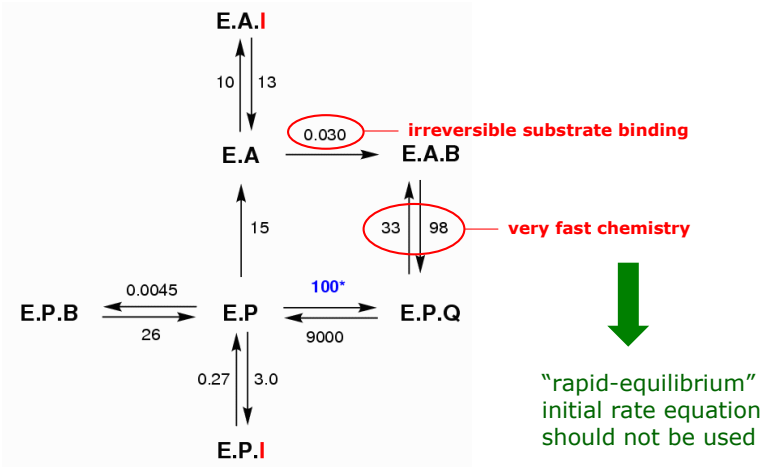
•In other words, the **steady-state** approximation is fundamentally needed to analyze and understand the kinetics of these enzymes.

Transient kinetic model for *Bacillus anthracis* IMPDH

THIS SCHEME FOLLOWS FROM **STOPPED-FLOW (TRANSIENT)** KINETIC EXPERIMENTS

A = IMP
B = NAD^+
P = XMP
Q = NADH

UNITS:
μM, sec



"rapid-equilibrium" initial rate equation should not be used

Y. Wei, *et al.* (2015) unpublished
IMPDH from *Bacillus anthracis*

New (unpublished) stopped flow experiments produced this model.
We'll take it as a "given" for the purposes of this talk.

DETAILS:

Conditions:

- Very large excess of IMP ("A") over $K_{m(A)}$: *no free enzyme*.
- Essentially a Uni-Uni kinetic mechanism ($NAD^+ \rightarrow$ NADH, *i.e.* "B" $\rightarrow$ "Q").
- The last step (E.P $\rightarrow$ E.S) is an irreversible product/substrate "exchange".
- It is irreversible because it involves *hydrolysis* of a covalent intermediate.

Steady-state approximation is truly needed:

- The binding of NAD^+ ("B") to E.A is *irreversible* (Riera *et al.* [2008]).
- Hydride transfers is faster than *any* ligand dissociation, except NADH release.
- Binding of NAD^+ ("B") to E.P (substrate inhibition) is extremely slow.

Inhibition mode:

- Inhibitor ("I") is essentially a product (NADH = "Q") analog.
- It binds preferentially to the enzyme-XMP ("E.P") complex, K_d ~90 nM.
- Somewhat weaker binding to the enzyme-IMP ("E.S") complex, Kd ~1.4 μM.

Rapid equilibrium NADH release step (E.P.Q ↔ E.P):

- "On" constant set arbitrarily to 100 $\mu M^{-1}.s^{-1}$.

Goal: Validate transient kinetic model by initial rate data

Two major goals:

1. **Validate existing transient kinetic model**

   Are stopped-flow results sufficiently supported by initial rate measurements?

2. **Construct the "minimal" initial rate model**

   How far we can go in model complexity based on initial rate data alone?

Probing the IMPDH inhibition mechanism from two independent directions.

1. Validate existing transient kinetic model

The transient kinetic model for *B. anthracis* IMPDH is highly complex:
Is it sufficiently supported by initial rate measurements?

2. Construct the "minimal" initial rate model

If we didn't have the transient kinetic results,
which "minimal" kinetic scheme would we end up with,
based solely on initial rate measurements?

Definition of a "minimal" model:

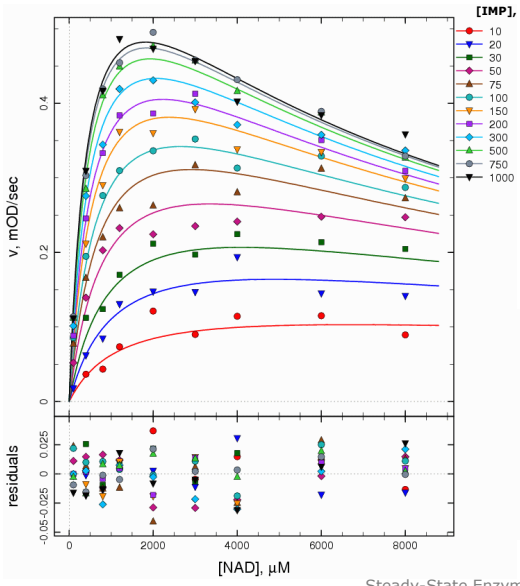
Contains only the reaction steps (i.e. rate constants) that are actually defined by the available experimental data, no "extraneous" or "assumed" steps

Three types of available initial rate data

1. Vary **[NAD^+]** and **[IMP]**
   substrate "B" and substrate "A"
2. Vary **[NAD^+]** and **[NADH]** at saturating [IMP]
   substrate "B" and product "Q" at constant substrate "A"
3. Vary **[NAD^+]** and **[Inhibitor]** at saturating [IMP]
   substrate "B" and inhibitor "I" at constant substrate "A"

Simultaneous variation of [NAD+] and [IMP]

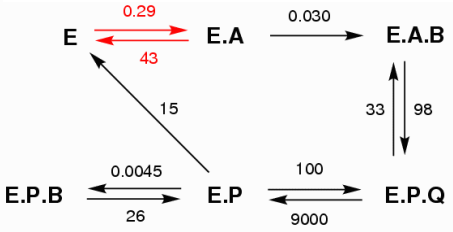
ADDED A NEW STEP – **BINDING OF IMP (substrate "A")** TO THE ENZYME



UNITS: µM, sec

A = IMP
B = NAD^+
P = XMP
Q = NADH

the only fitted rate constants



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•All rate constants except **ka.A / kd.A** were fixed to values from stopped-flow transient kinetics.

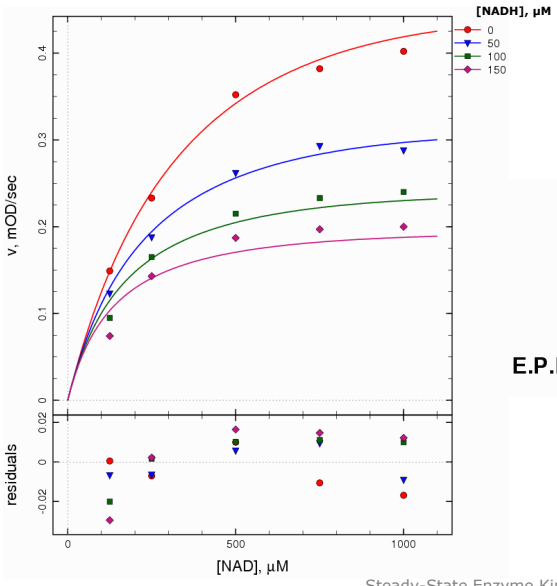
•It was necessary to add the E ↔ E.A step, because otherwise we could not build a model for "A" (i.e., IMP) being varied in the experiment.

CONCLUSIONS:

The kinetic model predicted from the stopped-flow measurements agrees very well with the initial rate data.

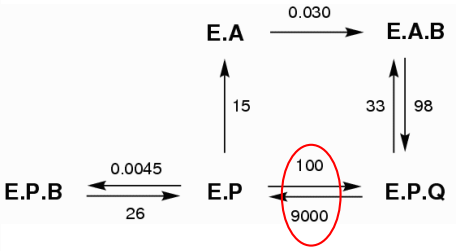
Simultaneous variation of [NAD+] and [NADH]

THIS CONFIRMS THAT **NADH IS REBINDING** TO THE **E.P** COMPLEX ("PRODUCT INHIBITION")



UNITS: µM, sec

A = IMP
B = NAD^+
P = XMP
Q = NADH



$K_{d(NADH)}$ = 90 µM

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•All rate constants were fixed to values from stopped-flow transient kinetics.

CONCLUSIONS:

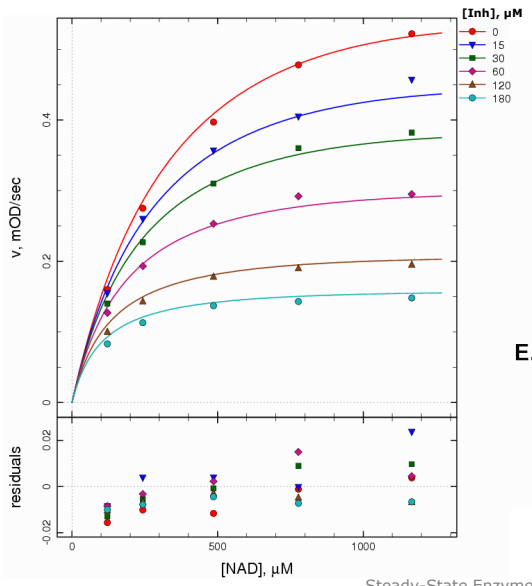
The kinetic model predicted from the stopped-flow measurements agrees very well with the initial rate data.

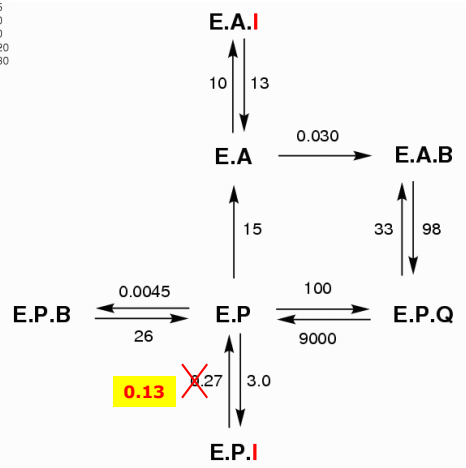
NOTE:

On the **qualitative** level, the stopped-flow model predicts that NADH ("Q") *does rebind* to the enzyme-XMP complex ("E.P"). This prediction is clearly verified in the initial rate experiment.

In general, **product inhibition** studies often are **essential** to a proper and complete understanding of many kinetic mechanisms.

Simultaneous variation of [NAD+] and inhibitor [A110]





•All rate constants were fixed to values from stopped-flow transient kinetics *except* the dissociation rate constant for "uncompetitive" inhibition, E.P.I → E.P + I.

•That rate constant had to be treated as an adjustable model parameter, in order to achieve a sufficient goodness of fit.

•However, note that the difference between the postulated and fitted values (0.27 s^{-1} vs. 0.13 s^{-1}) is less than 50%.

•Such relatively small differences are not surprising, because the stopped-flow experiment is conducted under experimental conditions (*micromolar* concentrations of enzyme) that are very different from the experimental conditions of the initial rate measurements (*nanomolar* concentrations).

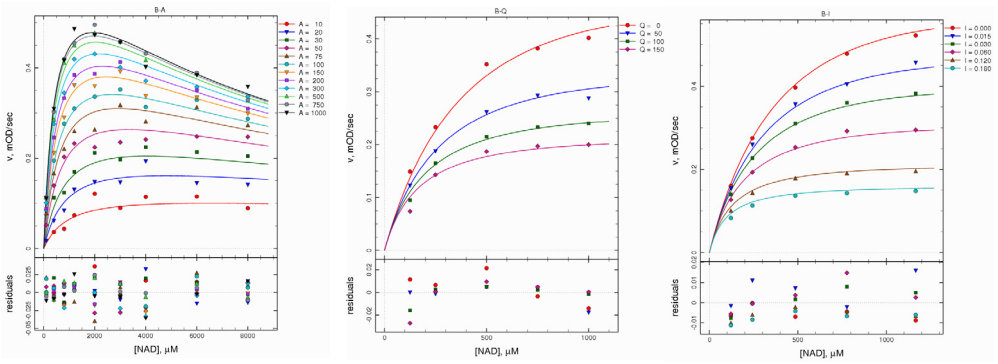
CONCLUSIONS:

The kinetic model predicted from the stopped-flow measurements agrees *reasonably* well with the initial rate data.

Toward the "minimal" kinetic model from initial rate data

WHAT IF WE DID NOT HAVE THE STOPPED-FLOW (TRANSIENT) KINETIC RESULTS?

vary **B + A** | vary **B + Q** | vary **B + I**



combine all three data sets, analyze as a single unit (**"global fit"**)

A **global fit** of enzyme kinetic data consists of combining all available data points into a **single data set**, regardless of which particular component (substrate, product, inhibitor) was varied in the experiment.

This method of kinetic data analysis has proved superior (ref. [1]) to analyzing various subsets of the global data set, and subsequently "stitching together" various re-replots or re-fits of intermediate results.

In this case the individual subsets of data "report" on various distinct segments of the mechanism. For example the "B + Q" (i.e., NAD^+ + NADH) data points "report" exclusively on the rebinding of NADH to the enzyme-XMP complex.

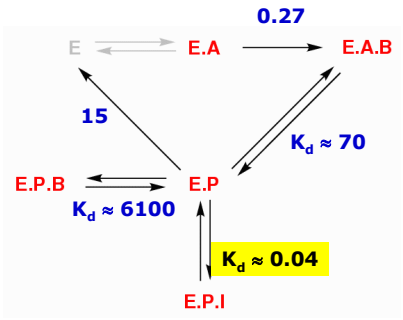
REFERENCES:

•Beechem, J. M.
"Global analysis of biochemical and biophysical data"
Meth. Enzymol. **210** (1992) 37-54.

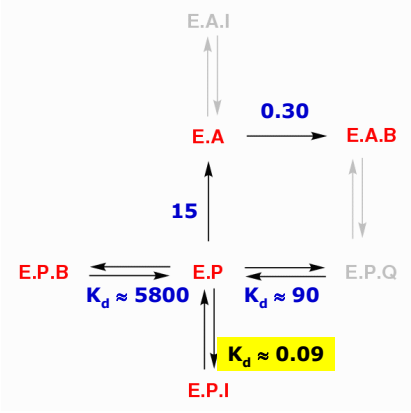
The "minimal" kinetic model from initial rate data

INITIAL RATE AND **STOPPED-FLOW** MODELS ARE IN REASONABLY GOOD AGREEMENT

UNITS:
μM, sec



from **initial rates**



from **stopped-flow**

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Steady-State Enzyme Kinetics 23

•The enzyme forms shown in **red** are those that can be "seen" in both types of experiments: stopped-flow and initial rates.

•We cannot "see" the free enzyme ('E') in the stopped flow experiment, because it is done at saturating concentration of IMP ('A').

•We cannot "see" the weakly bound complex E.A.I ($K_d \sim 1$ μM) in the initial rate experiment, because the highest inhibitor concentration was very much lower than that ($[I]_{max} \sim 150$ nM).

To see this interaction, we'd have to increase $[I]_{max} >> 1$ μM, but that is not practically achievable because by that time the enzyme is completely inhibited due to the *other* ("uncompetitive") step.

•We cannot "see" the complex E.P.Q in the initial rate experiment, because it is an isomeric form of another "central complex", E.A.B. Distinct "central complexes" can never be observed at **steady state**.

•In the **steady-state** initial rate experiment we **cannot see "on" constants** for any ***inhibition*** step (including substrate and product inhibition), so we can only compare **equilibrium** constants for those steps.

•With those caveats, the comparison turns out not too bad.

The "minimal" kinetic model: derived kinetic constants

DYNAFIT DOES COMPUTE "K_m" AND "K_i" FROM BEST-FIT VALUES OF MICRO-CONSTANTS

input

```
[mechanism]
   reaction A + B ---> P + Q
   modifiers I

   E + A <==> E.A          :   ka.A       kd.A
   E.A + B ---> E.A.B      :   ka.EA.B
   E.A.B <==> E.P + Q      :   kd.EP.Q    ka.EP.Q
   E.P ---> E + P          :   kd.P
   E.P + B <==> E.P.B      :   ka.EP.B    kd.EP.B
   E.P + I <==> E.P.I      :   ka.EP.I    kd.EP.I
```

derived kinetic constants

secondary output

primary output

microscopic rate constants

No.	Par#Set	Initial	Final
#1	ka.A	0.29	**0.256004**
#2	kd.A	42.9	**29.9727**
#3	ka.EA.B	0.0303	**0.0266841**
#4	kd.EP.Q	90	**67.1446**
#5	kd.P	15	**14.7149**
#6	kd.EP.B	5000	**6053.58**
#7	kd.EP.I	0.05	**0.0368578**

Kinetic Constants
King-Altman steady-state model

$k_{cat(f)}$	12.0698
$K_{m(A)}$	47.1467
$K_{m(B)}$	452.32
$K_{i(I,A,B)}$	0.0449353
$K_{i(A)}$	117.079
$K_{i(B)}$	1123.24
$K_{i(B,A)}$	7380.24
$K_{i(Q,A,B)}$	81.8595

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•**"Best from both worlds"**: DynaFit always reports the best-fit values of microscopic rate constants. *If* "kinetic constants" can exist in principle (i.e., **non-branched** mechanism) we get those reported, too.

The "minimal" kinetic model: derivation of kinetic constants

DYNAFIT DOES "KNOW" HOW TO PERFORM **KING-ALTMAN** ALGEBRAIC DERIVATIONS

As displayed in the program's output:

Rate Equation

$v = [E]_0 N/D$

Numerator

$N = n_1 [A][B]$

Denominator

$D = d_1 + d_2 [B] + d_3 [A] + d_4 [A][B] + d_5 [A][B][I] + d_6 [A][B][Q] + d_7 [A][B]^2$

where

$d_1 = 1$

$n_1 = \frac{k_{a,A}\, k_{a,EA,B}}{k_{d,A}}$

$d_2 = \frac{k_{a,EA,B}}{k_{d,A}}$

$d_3 = \frac{k_{a,A}}{k_{d,A}}$

$d_4 = \frac{k_{a,A}\, k_{a,EA,B}\, (k_{d,P} + k_{d,EP,Q})}{k_{d,A}\, k_{d,EP,Q}\, k_{d,P}}$

$d_5 = \frac{k_{a,A}\, k_{a,EA,B}\, k_{a,EP,I}}{k_{d,A}\, k_{d,P}\, k_{d,EP,I}}$

$d_6 = \frac{k_{a,A}\, k_{a,EA,B}\, k_{a,EP,Q}}{k_{d,A}\, k_{d,EP,Q}\, k_{d,P}}$

$d_7 = \frac{k_{a,A}\, k_{a,EA,B}\, k_{a,EP,B}}{k_{d,A}\, k_{d,P}\, k_{d,EP,B}}$

automatically derived kinetic constants:

$k_{cat(f)} = n_1/d_4 = \frac{k_{d,EP,Q}\, k_{d,P}}{k_{d,P} + k_{d,EP,Q}}$

$K_{m(A)} = d_2/d_4 = \frac{k_{d,EP,Q}\, k_{d,P}}{k_{a,A}\, (k_{d,P} + k_{d,EP,Q})}$

$K_{m(B)} = d_3/d_4 = \frac{k_{d,EP,Q}\, k_{d,P}}{k_{a,EA,B}\, (k_{d,P} + k_{d,EP,Q})}$

$K_{i(I,A,B)} = d_4/d_5 = \frac{k_{d,EP,I}\, (k_{d,P} + k_{d,EP,Q})}{k_{d,EP,Q}\, k_{a,EP,I}}$

$K_{i(A)} = d_1/d_3 = \frac{k_{d,A}}{k_{a,A}}$

$K_{i(B)} = d_1/d_2 = \frac{k_{d,A}}{k_{a,EA,B}}$

$K_{i(B,A)} = d_4/d_7 = \frac{k_{d,EP,B}\, (k_{d,P} + k_{d,EP,Q})}{k_{d,EP,Q}\, k_{a,EP,B}}$

$K_{i(Q,A,B)} = d_4/d_6 = \frac{k_{d,P} + k_{d,EP,Q}}{k_{a,EP,Q}}$

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•The automatically derived King-Altman model is available for inspection in the output files generated by DynaFit.

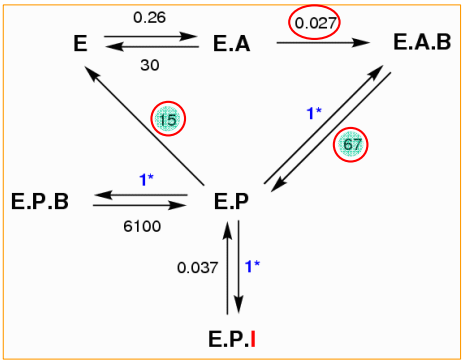
•This slide shows a **screen shot** of the relevant "model page".

Checking automatic derivations for *B. anthracis* IMPDH

"TRUST, BUT VERIFY"

turnover number, "k_{cat}":

$$k_{cat(f)} = n_1/d_4 = \frac{k_{d.EP.Q}\, k_{d.P}}{k_{d.P} + k_{d.EP.Q}} = 15\frac{67}{15+67} = 12\ s^{-1}$$ ✓



"K_m" for NAD^+:

$$K_{m(B)} = d_3/d_4 = \frac{k_{d.EP.Q}\, k_{d.P}}{k_{a.EA.B}\,(k_{d.P} + k_{d.EP.Q})} = \frac{67}{0.027} \times \frac{15}{15+67} = 450\ \mu M$$ ✓

similarly for other "kinetic constants"

•**Turnover number, k_{cat}**: It is an amalgamation of at least two microscopic steps:

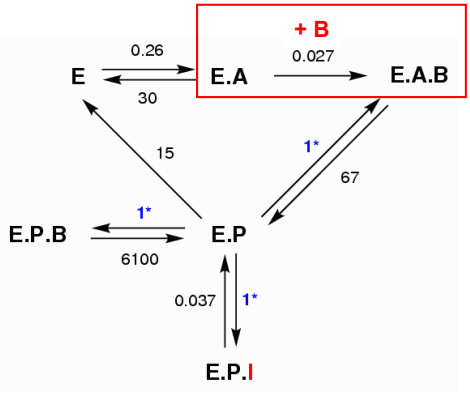
1. the NADH release step ($k_{d.EP.Q}$) and
2. XMP release step ($k_{d.P}$)

•However **"XMP release"** itself is an amalgamation of at least two microscopic steps, not counting any conformational changes:

1. hydrolysis of the covalent intermediate and
2. XMP release proper.

•Also note that k_{cat} = 12 s^{-1} is *slower* than the "rate limiting" step ($k_{d.P}$ = 15 s^{-1}). This is because "kinetic constants" are almost always – except for the simplest possible mechanisms – complex amalgamations of multiple rate constants.

Reminder: A "K_m" is *most definitely* not a "K_d"



$k_{on} = 2.7 \times 10^4\ M^{-1}s^{-1}$

$k_{off} \approx 0$

$K_d^{(NAD)} = k_{off}/k_{on} \approx \mathbf{0}$

$K_m^{(NAD)} = \mathbf{450}\ \mu M$

A K_d is a **d**issociation equilibrium constant.
However, NAD^+ does not appear to dissociate.

A K_m *sometimes* is the half-maximum rate substrate concentration (although **not** in this case).

•The Michaelis constants (K_m) is equal to the enzyme-substrate complex dissociation constant (K_d) only if the chemical step is **very slow** compared to all ligand dissociations.

•In other words the $K_m = K_d$ equivalence only holds under the "rapid equilibrium" approximation.

•However, in this case there is effectively no dissociation, so there can be no "dissociation constant".

•The best we can do (based on stopped-flow data) is to estimate the **upper limit** of the enzyme-substrate dissociation rate constant.

•It is "very low" in this case, but it is hard to say just how low, for various technical reasons. Other than that, the stopped-flow data are perfectly consistent with the dissociation constant being **zero**.

Advantage of "K_m"s: Reasonably portable across models

	full model	**minimal** model	
k_{cat}, s^{-1}	13	12	turnover number
$\mathbf{K}_{m(B)}$, μM	430	440	Michaelis constant of NAD^+
$\mathbf{K}_{i(B)}$, mM	6.6	7.4	substrate inhibition constant of NAD^+
$\mathbf{K}_{i(Q)}$, μM	77	81	product inhibition constant of NADH
$\mathbf{K}_{i(I,EP)}$, nM	50	45	"uncompetitive" K_i for A110

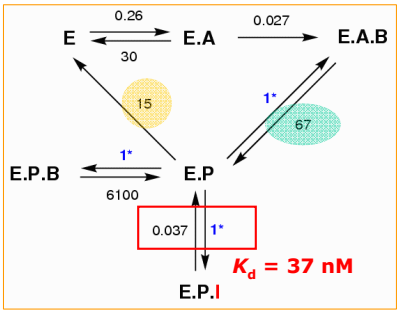
•Although the "kinetic constants" (Km, kcat, Ki, ...) are not always possible to obtain, when this is possible, they are quite useful.

•One advantage is that even if we get the microscopic model wrong, of have multiple equally plausible kinetic mechanisms, the "kinetic constants" stay more-or-less the same.

•In that sense the kinetic constants have high "portability" across models.

Reminder: A "K_i" is *not necessarily* a "K_d", either ...

minimal model (initial rates):



King-Altman rate equation automatically derived by **DynaFit**:

$$K_{i(I,EP)} = \frac{k_{d.EP.I}}{k_{a.EP.I}} \times \frac{k_{d.EP.Q} + k_{d.P}}{k_{d.EP.Q}}$$

K_d

$$= 37\ \text{nM} \frac{67+15}{67} = 45\ \text{nM}$$

•We all learned at some point that the Michaelis constant is *not* strictly speaking a dissociation equilibrium constant.

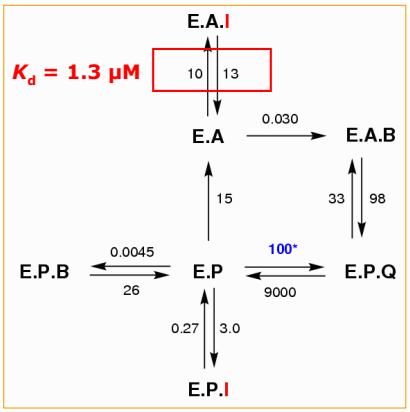
•However, it may not be equally widely recognized that, under the **steady-state** approximation, the (external ligand) inhibition constants are not *necessarily* equilibrium dissociation constants, either.

•Some K_i's *are* equal to the corresponding equilibrium constants, but some are not. It just depends on the given mechanism.

•Here we see an example of a particular inhibition constant that is *not* a simple ratio of the dissociation and association rate constants.

... although in *most* mechanisms *some* "K_i"s are "K_d"s

full model (transient kinetics)



King-Altman rate equation automatically derived by **DynaFit**:

$$K_{i(1)} = d_1/d_2 = \frac{k_{d.EA.I}}{k_{a.EA.I}} \quad K_d$$

$$K_{i(1,B)} = d_3/d_4 = \frac{k_{d.EP.I}\,(\,k_{rev}\,k_{d.P} + k_{d.EP.Q}\,k_{d.P} + k_{for}\,k_{d.P} + k_{for}\,k_{d.EP.Q}\,)}{k_{for}\,k_{d.EP.Q}\,k_{a.EP.I}}$$

CONCLUSIONS:

- The "competitive" K_i is a ***simple*** K_d
- The "uncompetitive" K_i is a ***composite***

•However, in the *same* kinetic mechanism as on the previous slide we can find another inhibition constant that is, in fact, equivalent to a simple Kd for the given ligand.

•One of the rules of thumb (applicable especially to single-substrate enzymes) is as follows:

- If the inhibitor binds to the **free enzyme** form, then the K_i for this step is always identical to the corresponding K_d.

- But if the inhibitor binds to an **enzyme-substrate or enzyme-product complex**, then the K_i for *that* step is *not* equal to the corresponding K_d.

•For **multi-substrate** enzymes the rules are not as clear, as we can see here: binding to E.A vs. binding to E.P produces different results in terms of relationship between K_i and K_d.

Part III: Summary and Conclusions

Importance of steady-state approximation

- "Fast" enzymes *require* the use of **steady-state** formalism.
- The usual **rapid-equilibrium** approximation cannot be used.
- The same applies to mechanisms involving **slow release** of products.
- The **meaning of some** (but not all) **inhibition constants** depends on this.

A "microscopic" approach to steady-state kinetics

- Many enzyme mechanisms (e.g. Random Bi Bi) **cannot have "K_m"** derived for them.
- However a rate equation formulated in terms of **micro-constants** always exists.
- Thus, we can always **fit** initial rate data to the **micro-constant** rate equation.
- If a **"K_m"**, "V_{max}" etc. do actually exist, they can be **recomputed** after the fact.
- This is a **reversal** of the usual approach to the analysis of initial rate data.
- This approach combined with **global fit** can produce savings in time and materials.

Computer automation of all algebraic derivations

- The **DynaFit** software package performs derivations by the **King-Altman method**.
- The newest version (4.06.027 or later) derives "kinetic constants" (K_m, etc.) if possible.
- DynaFit is available from www.biokin.com, free of charge to all academic researchers.

IMPDH kinetic mechanism

- IMPDH from *B. anthracis* follows a mechanism that includes **NADH rebinding**.
- This "product inhibition" can only be revealed if excess NADH is present in the assay.
- The **inhibitor "A110"** binds almost exclusively to the **covalent intermediate**.
- The observed inhibition pattern is **"uncompetitive" or "mixed-type"** depending on the exact conditions of the assay.
- Thus a proper **interpretation of the observed inhibition constant** depends on microscopic details of the catalytic mechanism.

Note:

- Crystal structures of inhibitor complexes are all **ternary**: **E·IMP**·Inhibitor
- Therefore X-ray data may not show the relevant interaction.

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  Brandeis University
  Departments of Biology and Chemistry