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

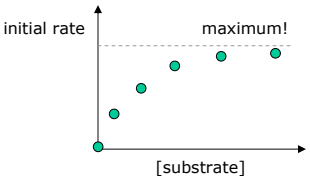
WHAT CAN ENZYME KINETICS DO FOR US?

macroscopic
laboratory
measurement

→ mathematical model →

microscopic
molecular
mechanisms

EXAMPLE: Michaelis-Menten (1913)



rectangular hyperbola

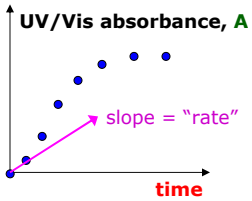
$v = V_m S/(S+K_m)$

MECHANISM:

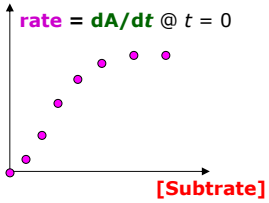
substrate and enzyme form a **reactive complex**, which decomposes into products and **regenerates** the enzyme catalyst

Two types of enzyme kinetic experiments

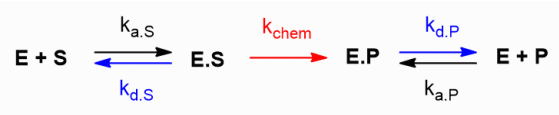
1. "reaction progress" method:



2. "initial rate" method:



The steady-state approximation in enzyme kinetics



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

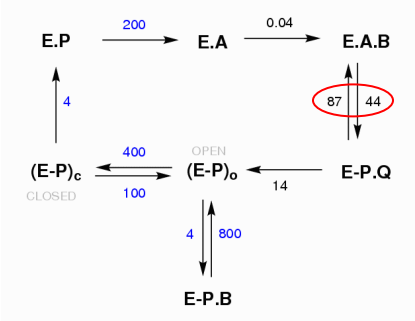
1. **"rapid equilibrium"** approximation $k_{chem} << k_{d.S}$, $k_{chem} << k_{d.P}$

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

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

Steady-state initial rate equations: The conventional approach

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

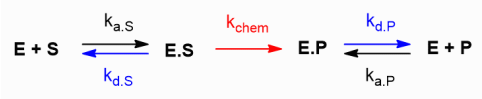
Step One:	*Derive* a rate equation in terms of **microscopic rate constants**
Step Two:	*Rearrange* the original equation in terms of **secondary "kinetic constants"**

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"):

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

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

micro-constants

3. rearrangement ("Step Two"):

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

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

"kinetic" constants

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

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**.

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.

A solution to the technical / logistical problem

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

INPUT:

DynaFit : im-002.txt

File Edit View Help

Input | Output

```
[task]
   task = simulate
   data = rates
   approximation = king-altman

[mechanism]
   reaction S ---> P
   E + S <==> E.S    :    ka.S    kd.S
   E.S ---> E + P    :    kd.P

[constants]
   ka.S = 1, kd.S = 1
   kd.P = 1
```

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

$d_1 = 1$

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

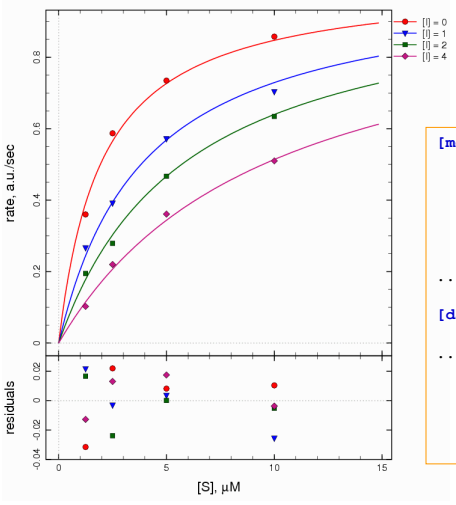
$k_{cat(f)} = n_1/d_2 = k_{d.P}$

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

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

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

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

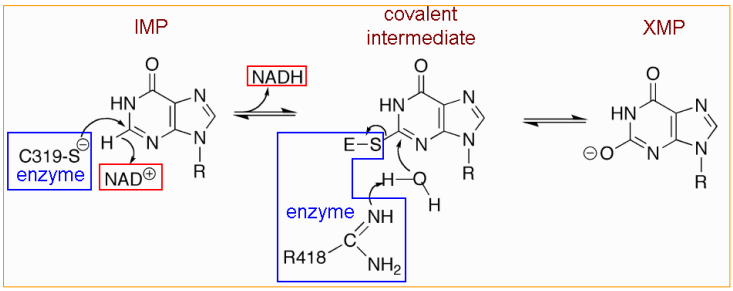
A POTENTIAL TARGET FOR THERAPEUTIC INHIBITOR DESIGN

Overall reaction:

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

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

Chemical mechanism:

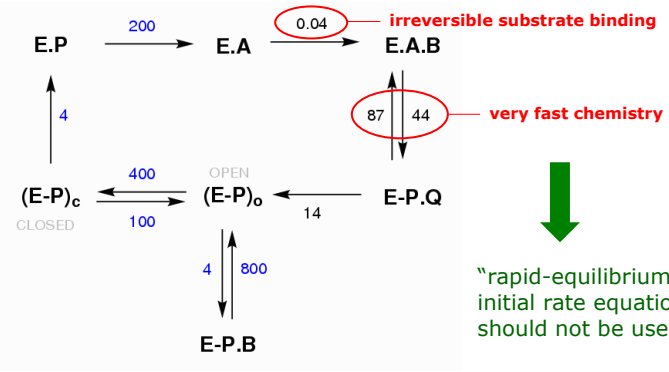


IMPDH kinetics: Fast hydrogen transfer catalytic step

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

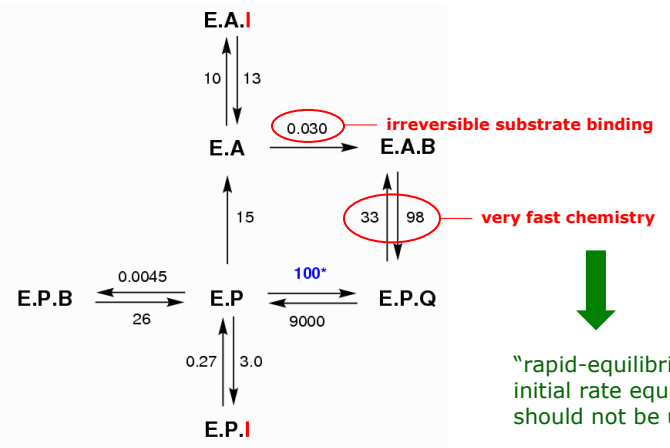
T. Riera *et al.* (2008) Biochemistry **47**, 8689–8696
IMPDH from *Cryptosporidium parvum*

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*

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?

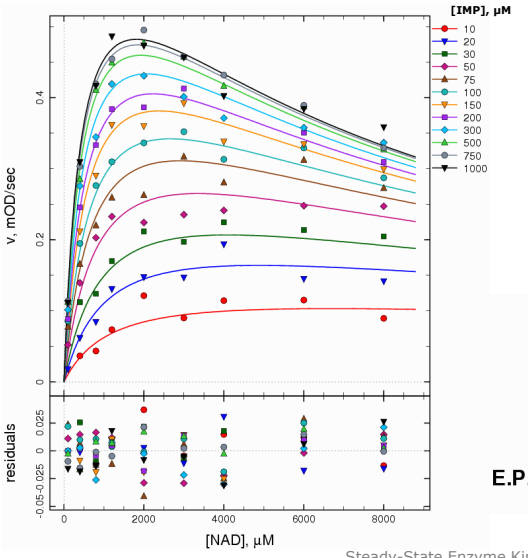
Probing the IMPDH inhibition mechanism from two independent directions.

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]

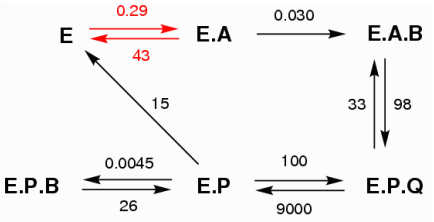
ADDED A NEW STEP – **BINDING OF IMP (substrate "A")** TO THE ENZYME



UNITS: µM, sec

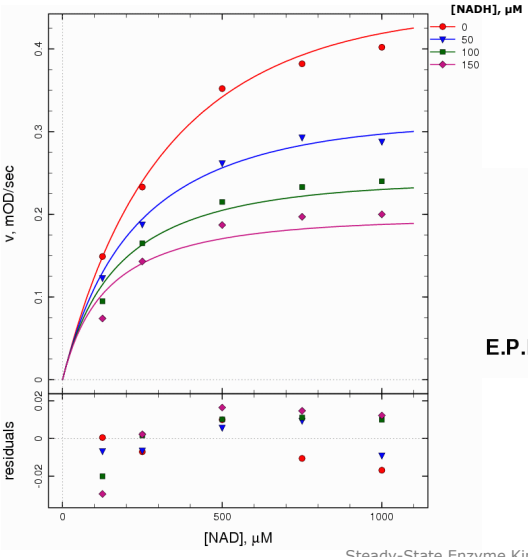
A = IMP
B = NAD^+
P = XMP
Q = NADH

the only fitted rate constants



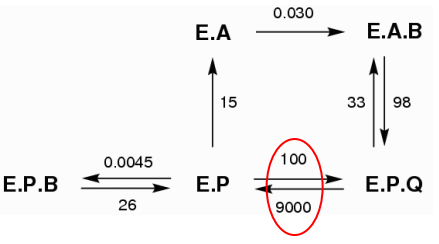
Simultaneous variation of [NAD+] and [NADH]

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



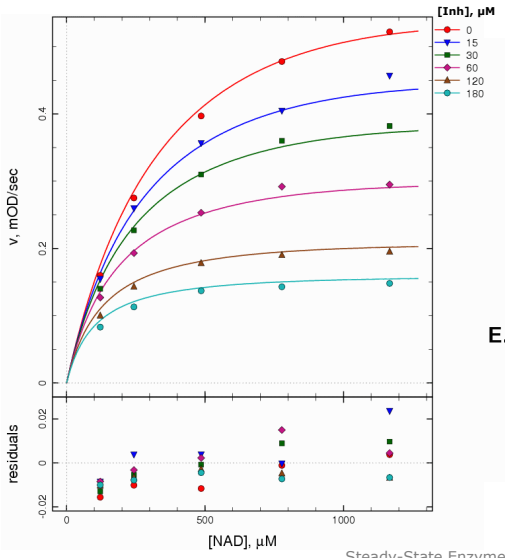
UNITS: µM, sec

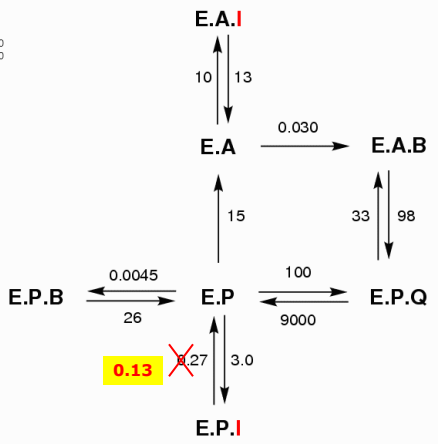
A = IMP
B = NAD^+
P = XMP
Q = NADH



$K_{d(NADH)}$ = 90 µM

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

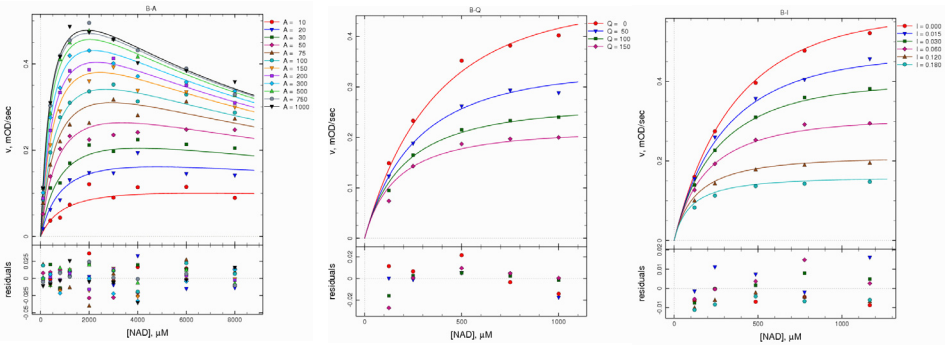




Toward the "minimal" kinetic model from initial rate data

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

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

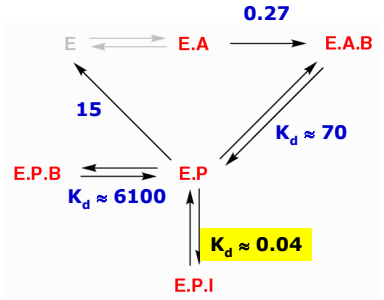


combine all three data sets, analyze as a single unit (**"global fit"**)

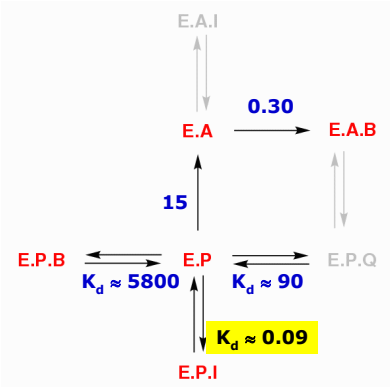
The “minimal” kinetic model from initial rate data

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

UNITS:
µM, sec



from **initial rates**



from **stopped-flow**

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
```

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**

derived
kinetic
constants
secondary output

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

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

$d_1 = 1 \qquad n_1 = \frac{k_{a,A} k_{a,EA,B}}{k_{d,A}}$

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

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

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

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

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

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

automatically derived kinetic constants:

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

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

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

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

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

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

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

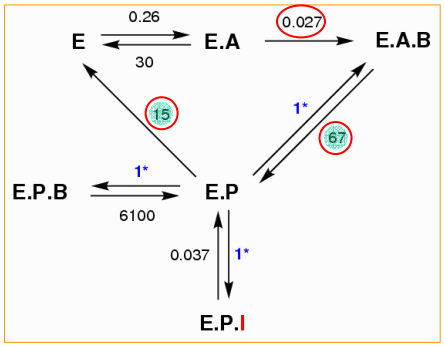
$K_{I(Q,A,B)} = d_4/d_6 = \frac{k_{d,P} + k_{d,EP,Q}}{k_{a,EP,Q}}$

Checking automatic derivations for *B. anthracis* IMPDH

"TRUST, BUT VERIFY"

turnover number, "k_{cat}":

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

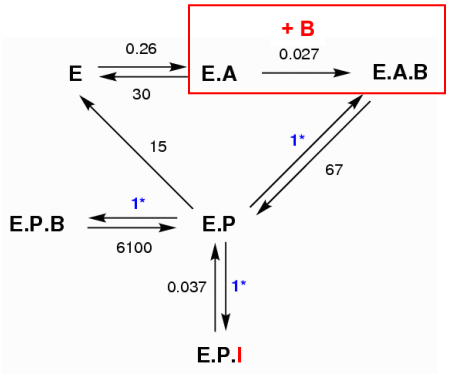


"K_m" for NAD$^+$:

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

similarly for other "kinetic constants"

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



k_{on} = 2.7 × 10^4 $M^{-1}s^{-1}$

$k_{off} \approx 0$

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

$K_m^{(NAD)}$ = **450** μM

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

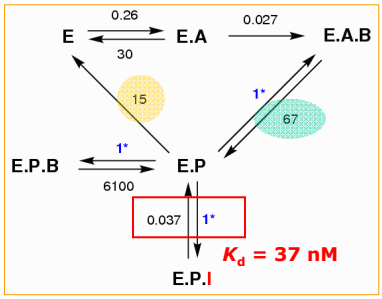
A K_m *sometimes* is the half-maximum rate substrate concentration (although **not** in this case).

Advantage of "K_m"s: Reasonably portable across models

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

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

minimal model (initial rates):



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

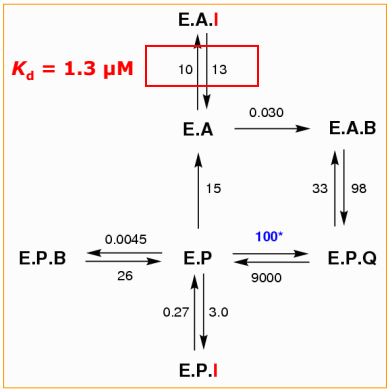
$$K_{i(I,EP)} = \frac{k_{d.EP.I}}{k_{a.EP.I}} \times \frac{k_{d.EP.Q} + k_{d.P}}{k_{d.EP.Q}}$$

K_d

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

... 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_d

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

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

CONCLUSIONS:

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

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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