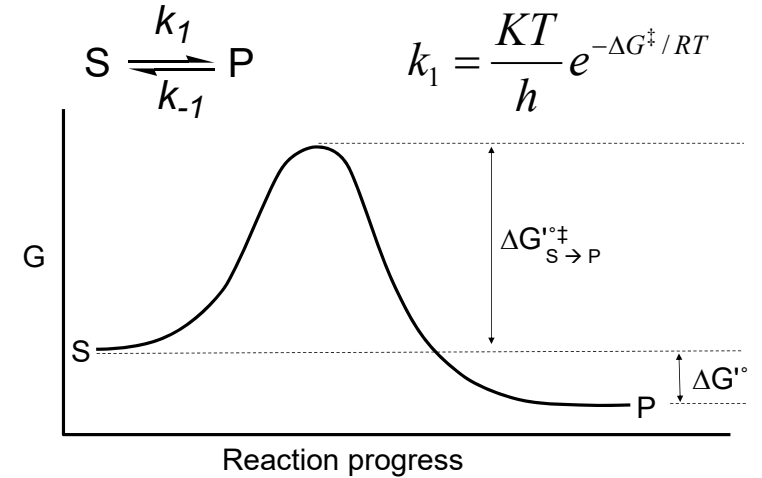


Enzyme Kinetics: The single substrate case

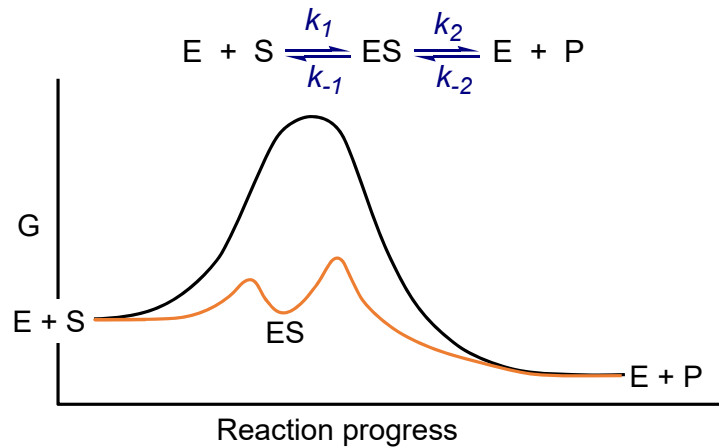
1
S. Ensign, enzyme kinetics

Reaction coordinate for $S \rightarrow P$



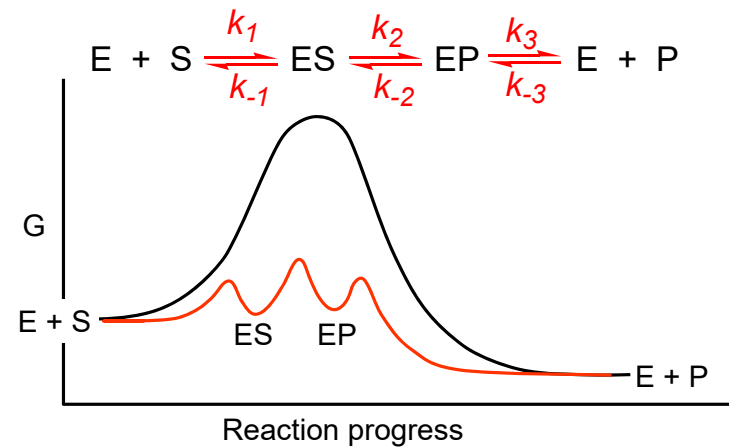
2
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Reaction coordinate for $S \rightarrow P$ following the kinetic mechanism:



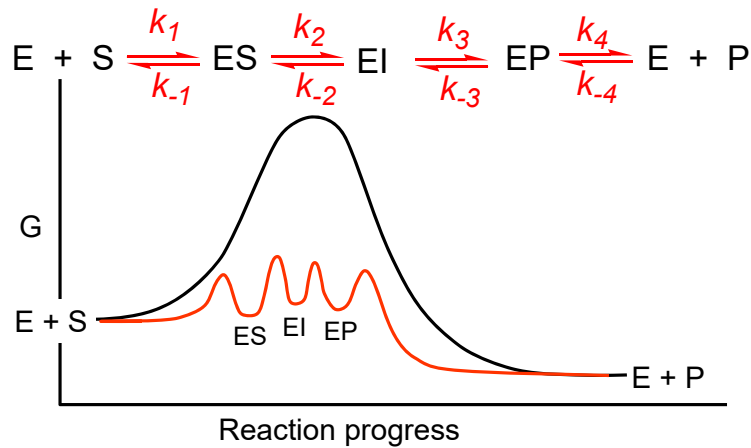
3
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Reaction coordinate for $S \rightarrow P$ following the kinetic mechanism:



4
S. Ensign, enzyme kinetics

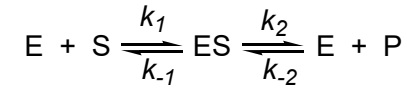
Reaction coordinate for $S \rightarrow P$ following the kinetic mechanism:



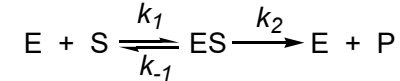
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Single substrate model: Steady state assumption



- E & P do not react back to form ES to any appreciable degree (design enzyme assay to assure this)



- Equilibrium of first step is rapidly established

$$k_{-1} \gg k_2 \quad K_a = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$

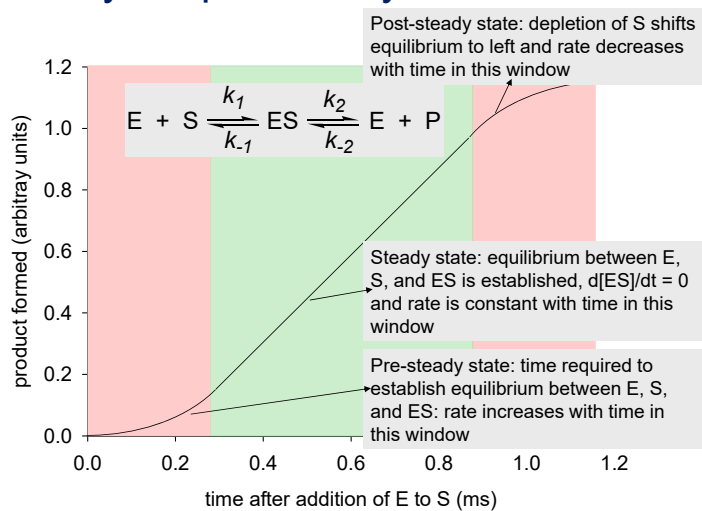
- [ES] remains constant during period in which enzyme activity is being measured

$$\frac{d[ES]}{dt} = 0$$

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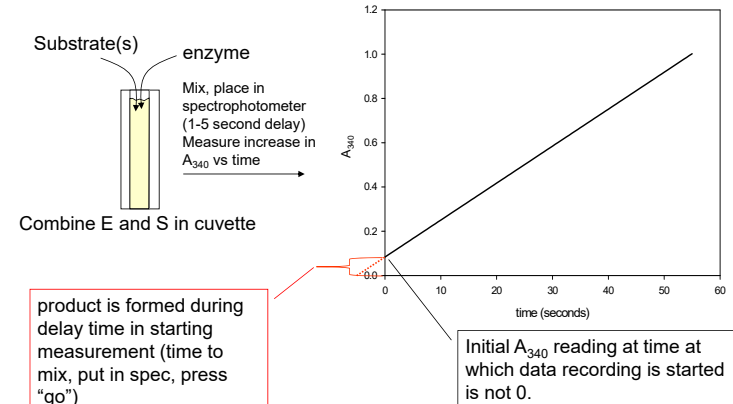
Steady vs. pre-steady state



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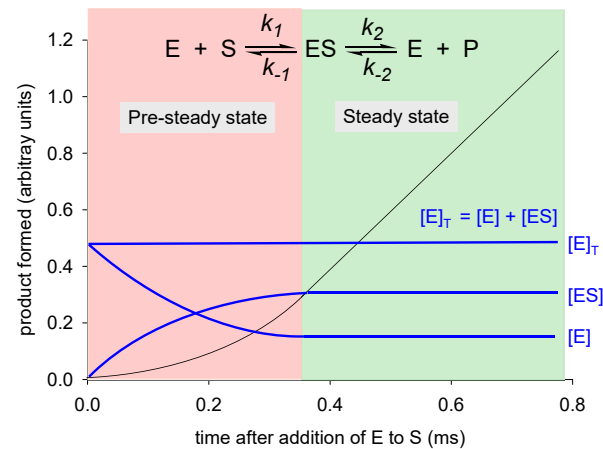
- The pre-steady state is usually only observed in the ms time frame (not seen under conditions most enzyme assays are conducted under)
- Example: lactate dehydrogenase: lactate + NAD^+ $\rightarrow$ pyruvate + NADH



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[E], [ES], and [E]_T during establishment of the steady state

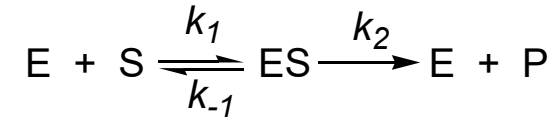


The relative concentrations of E, S and ES in the steady state are determined by how much E_T and S are present in the assay

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Derivation of a rate law for the steady-state



Equation 1 $\frac{d[ES]}{dt} = 0 = k_1[E][S] - k_{-1}[ES] - k_2[ES]$

Equation 2 $[E]_T = [E] + [ES]$

Equation 3 $\frac{d[P]}{dt} = v_0 = k_2[ES]$

Algebraic manipulation of these three expressions allows the rate law to be determined

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Derivation of a rate law for the steady-state

Equation 1

$$(1) \frac{d[ES]}{dt} = 0 = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

Solve for $k_2[ES]$

$$(2) k_1[E][S] = k_{-1}[ES] + k_2[ES]$$

Combine like terms

$$(3) k_1[E][S] = (k_{-1} + k_2)[ES]$$

Equation 2

$$(4) [E]_T = [E] + [ES]$$

Solve for [E]

$$(5) [E] = [E]_T - [ES]$$

Substitute (5) into (3)

$$(6) k_1([E]_T - [ES])[S] = (k_{-1} + k_2)[ES]$$

Expand left side

$$(7) k_1[E]_T[S] - k_1[ES][S] = (k_{-1} + k_2)[ES]$$

Solve for $k_1[E]_T[S]$

$$(8) k_1[E]_T[S] = k_1[ES][S] + (k_{-1} + k_2)[ES]$$

Combine like terms

$$(9) k_1[E]_T[S] = (k_1[S] + k_{-1} + k_2)[ES]$$

Solve for [ES]

$$(10) [ES] = \frac{k_1[E]_T[S]}{(k_1[S] + k_{-1} + k_2)}$$

Multiply N and D by $1/k_1$

$$E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow{k_2} E + P$$

$$(11) [ES] = \frac{k_1[E]_T[S]}{(k_1[S] + k_{-1} + k_2)} \cdot \frac{1}{k_1}$$

Define constant K_m

$$(12) [ES] = \frac{[E]_T[S]}{[S] + \left(\frac{k_{-1} + k_2}{k_1}\right)}$$

Substitute 13 into 12

$$(13) K_m = \frac{k_{-1} + k_2}{k_1}$$

Equation 3

$$(14) [ES] = \frac{[E]_T[S]}{[S] + K_m}$$

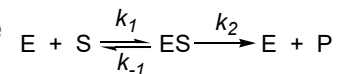
Solve for [ES]

$$(15) \frac{d[P]}{dt} = v_0 = k_2[ES]$$

$$(16) [ES] = \frac{v_0}{k_2}$$

Continued next page 11
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Derivation of a rate law for the steady-state



Substitute (16) into (14)

$$(14) [ES] = \frac{[E]_T[S]}{[S] + K_m} \quad (16) [ES] = \frac{v_0}{k_2}$$

$$(17) \frac{v_0}{k_2} = \frac{[E]_T[S]}{[S] + K_m}$$

Solve for v_0

$$(18) v_0 = \frac{k_2[E]_T[S]}{[S] + K_m}$$

The maximal velocity for the enzyme (V_{max}) will occur when the concentration of S is infinitely high, such that essentially all of [E]_T is in the form of [ES]

$$v_0 = k_2[ES]$$

$$(19) V_{max} = k_2[E]_T$$

Substitute (19) into (18)

$$(20) v_0 = \frac{V_{max}[S]}{[S] + K_m}$$

Michaelis-Menten equation

$$v_o = \frac{V_{max}[S]}{[S] + K_m}$$

Equation for fractional saturation of oxygen binding to myoglobin

$$\theta = \frac{[O_2]}{[O_2] + K_d}$$

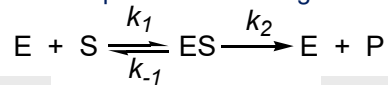
Equation giving a rectangular hyperbola

$$y = \frac{x}{x + C}$$

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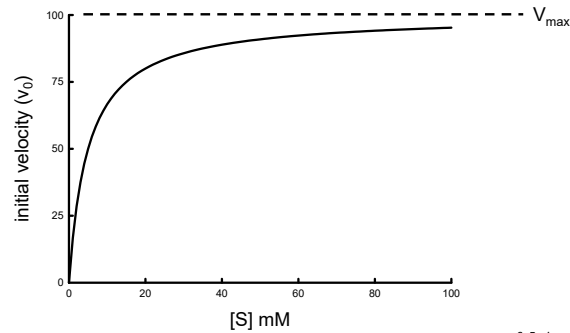
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Michaelis-Menten Equation for the single substrate case



$$v = \frac{V_{\max} [S]}{K_m + [S]}$$

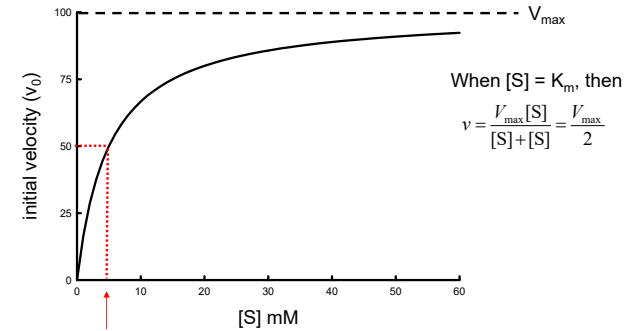
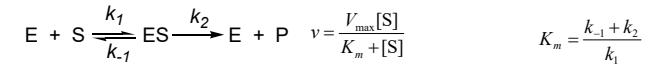
$$K_m = \frac{k_{-1} + k_2}{k_1}$$



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Michaelis-Menten Equation for the single substrate case

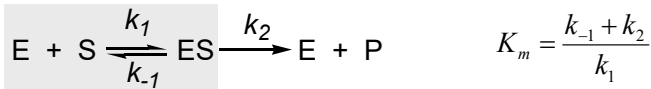


K_m = concentration of substrate that results in a rate equal to $\frac{1}{2} V_{\max}$

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K_D vs. K_m (single substrate case)



Consider the equilibrium for the formation of ES from E and S (disregard 2nd step)

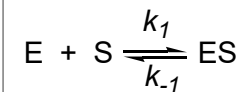
Association equilibrium constant: $K_a = \frac{k_1}{k_{-1}} = \frac{[ES]}{[E][S]}$

Dissociation equilibrium constant: $K_d = \frac{1}{K_a} = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$

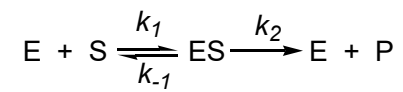
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K_D vs. K_m (single substrate case)



$$K_d = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$



$$K_m = \frac{k_{-1} + k_2}{k_1}$$

When $k_{-1} \gg k_2$, then $K_m \approx \frac{k_{-1}}{k_1} = K_d$

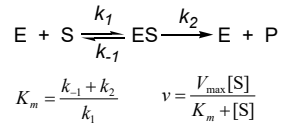
K_m approximates the affinity of an enzyme for its substrate only when $k_{-1} \gg k_2$

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The numerical values of K_m and V_{max} vary greatly for different enzymes

- K_m : high nM/low $\mu M \rightarrow$ high mM
- Physiological significance?
 - Available [S]
 - Need to regulate substrate conversion rate?
- V_{max} units: $\mu\text{mol}/\text{min}$; tells nothing about $[E]_T$ in assay (can report as $\mu\text{mol}/\text{min}/\text{mg}$)
-
- $\frac{v_0}{V_{max}} = \frac{k_2 [ES]}{k_1 [E]_T} = \frac{V_{max}}{K_m + [S]}$

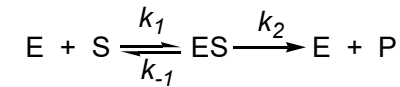


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k_{cat} : turnover number

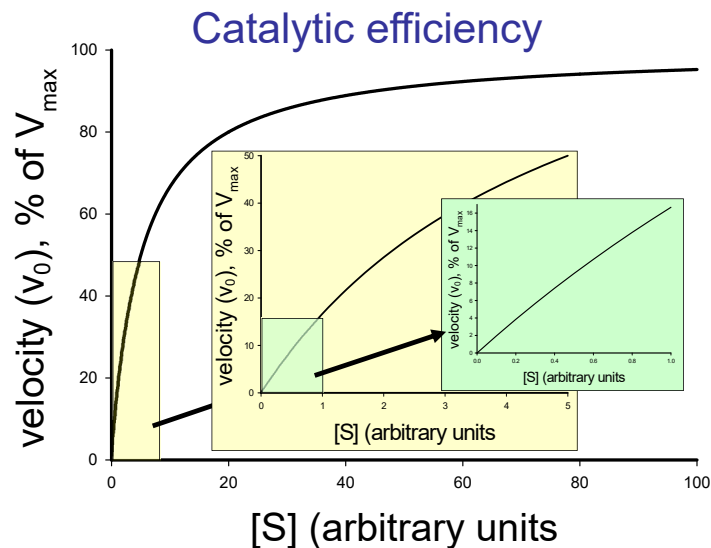
- The number of reaction processes (turnovers) that each active site catalyzes per unit time (per s, per min)
- A first order rate constant (s^{-1} or min^{-1})
- Calculate k_{cat} for a homodimeric (α_2) enzyme of Mr = 126,000 exhibiting $V_{max} = 10,000 \text{ U/mg}$
- For the simple kinetic mechanism,



$k_{cat} = k_2$ when step 2 is the RDS

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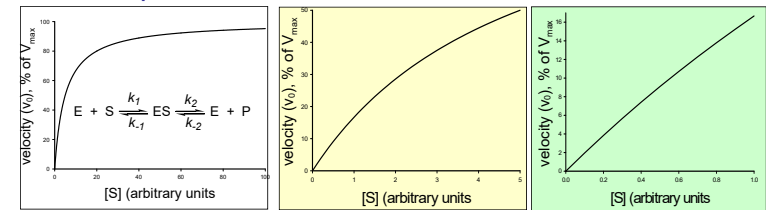
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An enzyme is most “efficient” at converting substrate to product at low concentrations of substrate



$$v = \frac{V_{max}[S]}{K_m + [S]} ; \text{ when } [S] \ll K_m, v \approx \frac{V_{max}}{K_m} [S] ; V_{max} = [E]_T \cdot k_{cat} ; v \approx \frac{k_{cat}}{K_m} [E]_T [S]$$

$$[E]_T = [E] + [ES] ; \text{ when } [S] \ll K_m, [E] \gg [ES] \text{ and } [E]_T \approx [E]$$

thus $v \approx \frac{k_{cat}}{K_m} [E][S]$ A 2nd order rate equation of form $rate = k[A][B]$

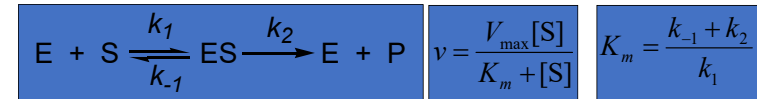
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Catalytic efficiency

- The ratio $\frac{k_{cat}}{K_m}$
- A second order rate constant (units of $M^{-1} \cdot s^{-1}$)
- A high k_{cat} and low K_m contribute to high catalytic efficiency
- The magnitude of k_{cat}/K_m differs dramatically for different enzymes

Is there an upper limit to the catalytic efficiency of an enzyme?



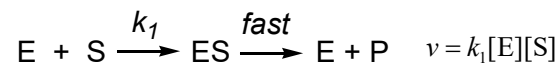
For the single intermediate model shown above, $k_{cat} = k_2$

$$\text{Thus, } \frac{k_{cat}}{K_m} = \frac{k_2}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2}$$

$\frac{k_{cat}}{K_m}$ is greatest when $k_2 \gg k_{-1}$, reducing above expression to

$$\frac{k_{cat}}{K_m} \approx k_1 \quad \text{and thus the rate of reaction is dictated by the magnitude of } k_1$$

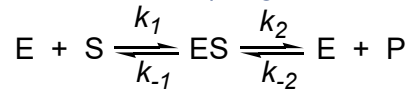
Is there an upper limit to the catalytic efficiency of an enzyme?



- The diffusion controlled upper limit to a bimolecular second order reaction is 10^8 to $10^9 M^{-1} s^{-1}$
- Enzymes with k_{cat}/K_m values in this range are “perfect enzymes”: they “instantaneously” catalyze a reaction every time E and S collide!
- It is impossible to exceed this diffusion controlled limit for a catalyzed (thus bimolecular) reaction

How do you improve a perfect enzyme?

Reversible reactions (single S and P case)



$$v = \frac{\frac{V_{\max}^f [S]}{K_m^S} - \frac{V_{\max}^r [P]}{K_m^P}}{1 + \frac{[S]}{K_m^S} + \frac{[P]}{K_m^P}}$$

$$K_m^S = \frac{k_{-1} + k_2}{k_1}$$

$$K_m^P = \frac{k_{-1} + k_2}{k_{-2}}$$

$$V_{\max}^f = [E]_T \cdot k_2$$

$$V_{\max}^r = [E]_T \cdot k_{-2}$$

At equilibrium, rate of $S \rightarrow P$ = rate of $P \rightarrow S$ and $v = 0$.
Substitute 0 into the equation for v and solve for $[P]/[S]$
(i.e., K_{eq})

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The Haldane Relationship

$$K_{eq} = \frac{[P]}{[S]} = \frac{V_{\max}^f K_m^P}{V_{\max}^r K_m^S}$$

- The kinetic parameters of a reversible enzymatic reaction are related by the equilibrium constant for the overall reaction

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Models with two or more intermediates

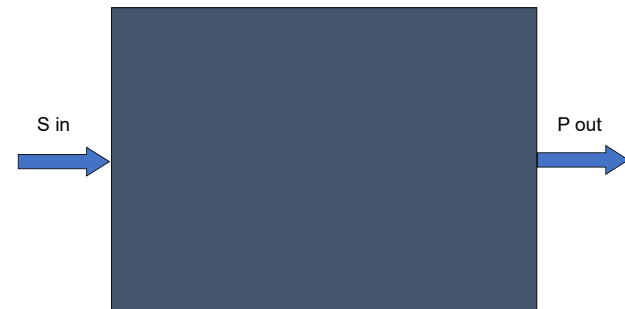


- The overall Michaelis-Menten equations are the same as for the one intermediate model
- Kinetic parameters ($V_{\max}^f, K_m^S, V_{\max}^r, K_m^P$) are defined in terms of 6 or 8 kinetic constants for the examples shown
- The RDS for product determination defines k_{cat}
- Steady state kinetics cannot distinguish the number or nature of intermediates in an enzymatic reaction.

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Black box model for steady state enzyme kinetics

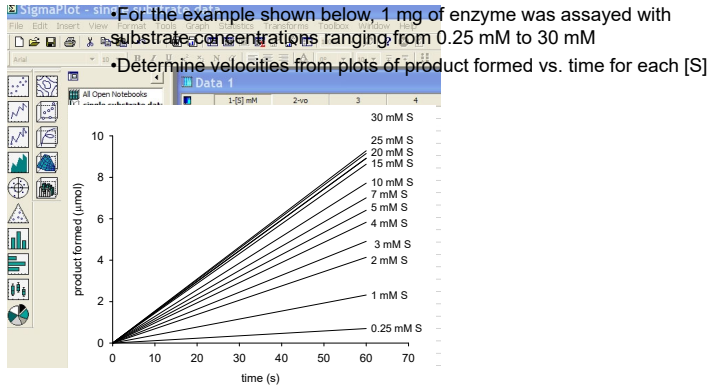


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Analysis of kinetic data: single substrate case

- Conduct enzyme assays with constant [E] and varying [S]
- Choose a wide range of substrate concentrations both below, around, and above estimated K_m

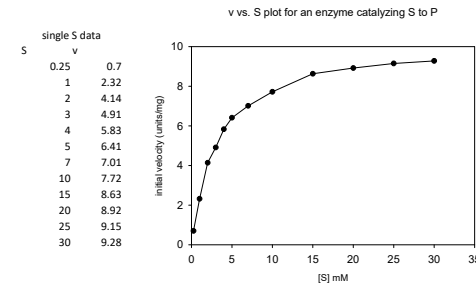


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Analysis of kinetic data: single substrate case

- Create a plot of initial velocity vs. [S]



Estimate V_{max} and K_m from looking at this graph?

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Methods used to determine K_m and V_{max}

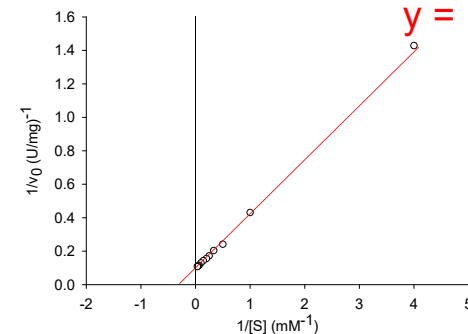
- Estimate from v vs. S plot (qualitative at best)
- Algebraic rearrangement of Michaelis-Menten equation; replot data and estimate parameters from replots (useful for visual purposes)
- Nonlinear regression of v vs. S data- the only acceptable way for determining kinetic constants for publication

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Algebraic rearrangement of the Michaelis-Menten Equation

$$v = \frac{V_{max}[S]}{K_m + [S]} \Rightarrow \frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$



- Lineweaver-Burke (double reciprocal) plot
- $y\text{-int} = 1/V_{max}$
- $x\text{-int} = -1/K_m$
- $\text{slope} = K_m/V_{max}$
- DON'T fit the double reciprocal data using linear regression or you will introduce errors (trumpet effect)

1/S	1/v
4	1.428571
1	0.431034
0.5	0.241546
0.333333	0.203666
0.25	0.171527
0.2	0.156006
0.142857	0.142653
0.1	0.129534
0.066667	0.115875
0.05	0.112108
0.04	0.10929
0.033333	0.107759

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Fitting Kinetic data to the Michaelis-Menten Equation (rectangular hyperbola) using a free on line tool: <http://statpages.info/nonlin.html>

- Statpages credit: John Pezullo
- Page is no longer maintained or updated, so I have created a modified mirror page in case the above site gets taken down:
 - http://ensignchemistry.com/enzymology/curve_fitting.html
- See file: “kinetic curve fitting codes”

Fitting Kinetic data to the Michaelis-Menten Equation (rectangular hyperbola) using nonlinear regression

Instructions:

- Enter the number of data points: 12
- Enter the number of independent variables: 1 (1 for single substrate M-M equation; 2 for substrate, 2 for C, UC, and mixed inhibition)
- Enter the number of parameters: 2 (deduce from form of M-M equation used; will be 2 for single substrate M-M)
- Enter the formula for the function to be fitted:
$$Y = \frac{a \cdot x^b}{(b \cdot x^c + 1)}$$

$$v = \frac{V_{\max} [S]}{K_m + [S]}$$

Use "*" for multiplication and "/" for division when entering formulas. Define parameters (a, b, c) and variables (x1, x2, x3) as described above. Be sure to use parentheses appropriately to get the correct hierarchy.

5. Type (or paste) the [x,y] data:

0.25	0.7
1	2.32
2	4.14
3	4.91
4	5.83
5	6.41
7	7.03
10	7.72

Data can be typed in directly, or pasted from a text file or spreadsheet. The first column(s) contain the independent variable(s) (x1, x2, ...) while the final column contains the dependent variable y (velocity for enzyme data).

6. Enter initial guesses for the parameters from evaluation of the data (e.g. Lineweaver-Burke plots and appropriate slope and intercept plots). If the data fails to converge, provide better initial guesses. :

a (or p1) =	10	±		p =	
b (or p2) =	3	±		p =	
c (or p3) =	0	±		p =	
d (or p4) =	0	±		p =	
e (or p5) =	0	±		p =	
f (or p6) =	0	±		p =	
g (or p7) =	0	±		p =	
h (or p8) =	0	±		p =	

Place the value "0" (zero) in all parameter boxes for which a value is not defined in the equation to be fit. Leave the fields to the right of the parameters blank; these will be populated with the standard errors upon iteration.

7. Click the [Iterate] button once for each iteration cycle. Watch how the initial guesses change in response to the iteration. Continue to click the "iterate" button until the values converge to unchanging values. If the parameters do not converge on reasonable numbers, provide better initial guesses and try again.

Fitting Kinetic data to the Michaelis-Menten Equation (rectangular hyperbola) using nonlinear regression

- Enter initial guesses for the parameters from evaluation of the data (e.g. L. V_{max} ± std error

a (or p1) =	10.31581092113	±	0.084894623156	p =	5e-7
b (or p2) =	3.175900437348	±	0.093749889629	p =	5e-7
c (or p3) =	0	±	1	p =	1.000000
d (or p4) =	0	±	1	p =	1.000000
e (or p5) =	0	±	1	p =	1.000000
f (or p6) =	0	±	1	p =	1.000000
g (or p7) =	0	±	1	p =	1.000000
h (or p8) =	0	±	1	p =	1.000000

Place the value "0" (zero) in all parameter boxes for which a value is not defined in the eq.

- Click the [Iterate] button once for each iteration cycle. Watch how the init parameters do not converge on reasonable numbers, provide better init. If you encounter problems getting the parameters to converge, you can sp making the convergence slower but more stable. Change this value back to 1.0 once the i

RMS Err = 0.105620199095 Weighted Standard Error of Estimate: the average scal points to within their intrinsic errors.

Y = a*x1/(b+x1)
x1 Y Yc SEest YcLo YcHi
0.25 0.7 0.7527809921646346 -0.0528 0.0158 0.7177 0.7879
0.2500 0.7000 0.7528 -0.0528 0.0158 0.7177 0.7879
1.0000 2.3200 2.4703 -0.1503 0.0399 2.3813 2.5593
2.0000 4.1400 3.9861 -0.1539 0.0481 3.8789 4.0933
3.0000 4.9100 5.0110 -0.1010 0.0471 4.9059 5.1161
4.0000 5.8300 5.7503 -0.0797 0.0438 5.6526 5.8479
5.0000 6.4100 6.3087 -0.1013 0.0404 6.2188 6.3986
7.0000 7.0300 7.0962 -0.0862 0.0358 7.0165 7.1760
10.0000 7.7200 7.8293 -0.1093 0.0352 7.7510 7.9076

Optional) click here for ☒ Tab-delimited output (more convenient for pasting the results into spreadsheet)

Y = a*x1/(b+x1)
x1 Y Yc SEest YcLo YcHi
0.25 0.7 0.7527809921646346 -0.0528 0.0158 0.7177 0.7879
0.2500 0.7000 0.7528 -0.0528 0.0158 0.7177 0.7879
1.0000 2.3200 2.4703 -0.1503 0.0399 2.3813 2.5593
2.0000 4.1400 3.9861 -0.1539 0.0481 3.8789 4.0933
3.0000 4.9100 5.0110 -0.1010 0.0471 4.9059 5.1161
4.0000 5.8300 5.7503 -0.0797 0.0438 5.6526 5.8479
5.0000 6.4100 6.3087 -0.1013 0.0404 6.2188 6.3986
7.0000 7.0300 7.0962 -0.0862 0.0358 7.0165 7.1760
10.0000 7.7200 7.8293 -0.1093 0.0352 7.7510 7.9076

Tab-delimited data → textedit (pc) → open in excel:

	A	B	C	D	E	F	G
1	x1	Y	yc	Y-yc	SEest	YcLo	YcHi
2	0.25	0.7	0.752781	-0.05278	0.015752	0.717683	0.787879
3	1	2.32	2.47032	-0.15032	0.039938	2.381332	2.559308
4	2	4.14	3.986093	0.153907	0.048095	3.878931	4.093255
5	3	4.91	5.010999	-0.101	0.04715	4.905943	5.116055
6	4	5.83	5.750253	0.079747	0.043807	5.652646	5.84786
7	5	6.41	6.30867	0.10133	0.040356	6.21875	6.398589
8	7	7.03	7.096244	-0.06824	0.035796	7.016486	7.176003
9	10	7.72	7.829302	-0.1093	0.03515	7.750983	7.907622
10	15	8.63	8.513315	0.116685	0.040961	8.422047	8.604582
11	20	8.92	8.902188	0.017812	0.047329	8.796731	9.007644
12	25	9.15	9.153045	-0.00304	0.052485	9.036101	9.269988
13	30	9.28	9.328287	-0.04829	0.056523	9.202347	9.454227
14							
15	Corr. Coef		0.99935	r ² =	0.9987		
16	RMS Error		0.10562	d.f. =	10	SSq =	0.111556
17	AIC =		-50.1376	AIC(correc	-47.1376		
18							
19	Parameter Estimates...						
20	p1=	10.31581		0.084895	p=	3.33E-16	
21	p2=	3.1759		0.09375	p=	1.19E-11	
22							
23	Covariance Matrix Terms and Error-Correlations...						
24	B(1,1)=	0.007207	r=	1			
25	B(1,2)=B(2,1)=	0.006673	r=	0.838484			
26	B(2,2)=	0.008789	r=	1			

Fitting Kinetic data to the Michaelis-Menten Equation (rectangular hyperbola) using nonlinear regression

Try putting in some bad initial guesses and see what happens...

Instructions:

- Enter the number of data points: 12
- Enter the number of independent variables: 1 (1 for single substrate M-M equation; 2 for bisubstrate, 2 for C, UC, and mixed inhibition)
- Enter the number of parameters: 2 (deduce from form of M-M equation used; will be 2 for single substrate M-M)
- Enter the formula for the function to be fitted:

$$Y = \frac{a \cdot x1}{b + x1}$$
- Type (or paste) the (x,y) data:

x	y
0.25	0.7
1	2.32
2	4.14
3	4.91
4	5.83
5	6.41
7	7.01
10	7.72

Data can be typed in directly, or pasted from a text file or spreadsheet. The first column(s) contain the independent variable(s) (x1, x2, ...) while the final column contains the dependent variable y (velocity for enzyme data).

- Enter initial guesses for the parameters from evaluation of the data (e.g. Lineweaver-Burke plots and appropriate slope and intercept replots). If the data fails to converge, provide better initial guesses:

Parameter	Initial Guess	Standard Error
a (or p1)	10	
b (or p2)	3	
c (or p3)	0	
d (or p4)	0	
e (or p5)	0	
f (or p6)	0	
g (or p7)	0	
h (or p8)	0	

Place the value "0" (zero) in all parameter boxes for which a value is not defined in the equation to be fit. Leave the fields to the right of the parameters blank; these will be populated with the standard errors upon iteration.

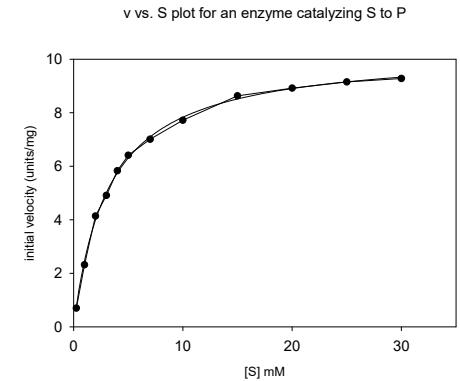
- Click the **Iterate** button once for each iteration cycle. Watch how the initial guesses change in response to the iteration. Continue to click the "iterate" button until the values converge to unchanging values. If the parameters do not converge on reasonable numbers, provide better initial guesses and try again.

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Making a publication quality graph showing the nonlinear regression fit of the experimental data

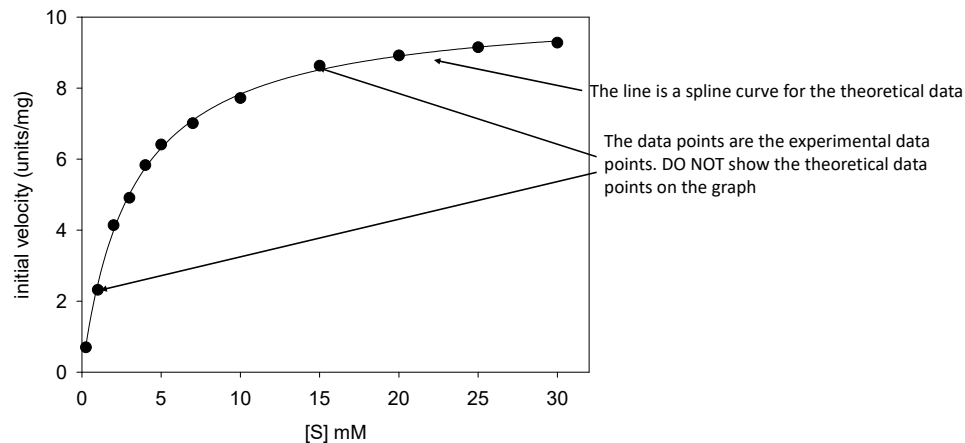
- Make a v vs s plot with both the experimental and theoretical velocity data plotted on the x axis
- Remove the line connecting data points for the experimental data
- Add a smoothed line (spline) going through the theoretical data points
- Hide the theoretical data points
- You will now have a graph showing the experimental data points, and with a line showing the shape of the hyperbola based on the nonlinear regression (see next slide)



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Making a publication quality graph showing the nonlinear regression fit of the experimental data



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Adding a Lineweaver-Burke Plot to your v vs. S plot

- Transform your s, v_{expt} and $v_{\text{theoretical}}$ data to $1/s$, $1/v_{\text{expt}}$, and $1/v_{\text{theor}}$

single S data						
S	v	v(theor)	1/S	1/v	1/v(theor)	
0.25	0.7	0.752781		4	1.428571	1.328408
1	2.32	2.47032		1	0.431034	0.404806
2	4.14	3.986093		0.5	0.241546	0.250872
3	4.91	5.010999	0.333333	0.203666	0.199561	
4	5.83	5.750253		0.25	0.171527	0.173905
5	6.41	6.30867		0.2	0.156006	0.158512
7	7.01	7.096244	0.142857	0.142653	0.14092	
10	7.72	7.829302		0.1	0.129534	0.127725
15	8.63	8.513315	0.066667	0.115875	0.117463	
20	8.92	8.902188		0.05	0.112108	0.112332
25	9.15	9.153045		0.04	0.10929	0.109253
30	9.28	9.328287	0.033333	0.107759	0.107201	

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Adding a Lineweaver-Burke Plot to your v vs. S plot II

•Plot both $1/v_{\text{expt}}$ AND $1/v_{\text{theor}}$ vs $1/s$ and make a scatter plot that looks like this:

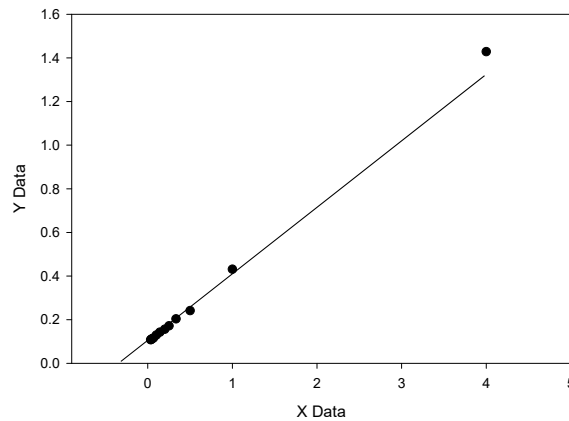
•The black circles are the experimental data

•The red circles are the theoretical data

•Add a linear regression line to the theoretical data, NOT the experimental data

•Extend the line to intersect the x axis

•Hide the theoretical data points, but keep the regression line



S. Ensign, enzyme kinetics 41

Adding a Lineweaver-Burke Plot to your v vs. S plot II

•Plot both $1/v_{\text{expt}}$ AND $1/v_{\text{theor}}$ vs $1/s$ and make a scatter plot that looks like this:

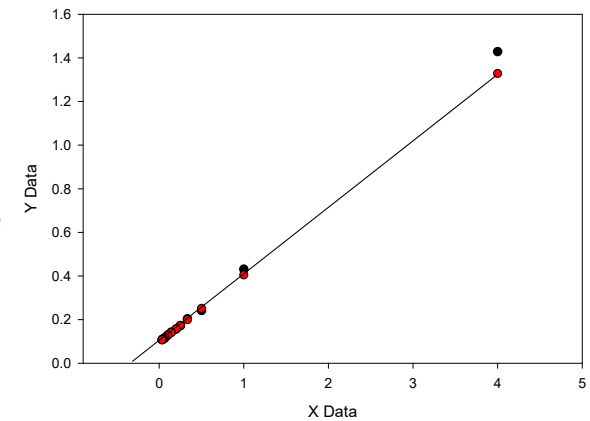
•The black circles are the experimental data

•The red circles are the theoretical data

•Add a linear regression line to the theoretical data, NOT the experimental data

•Extend the line to intersect the x axis

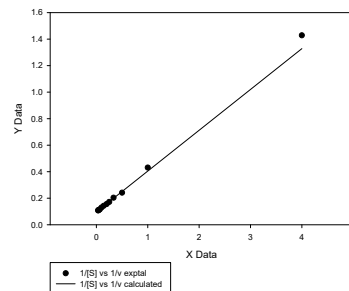
•Hide the theoretical data points, but keep the regression line



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Adding a Lineweaver-Burke Plot to your v vs. S plot III

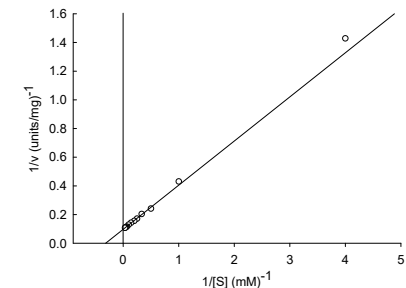
•Note: the line you have generated for your plot is NOT the same line you would have obtained by performing a linear regression of the experimental data points. The line shown at right is a correct rendering, because it is fitting the data points using the rectangular hyperbola, NOT linear regression of the double reciprocal data. If you had fit the experimental double reciprocal data using linear regression, it would have treated all points equally and generated an incorrectly fit line.



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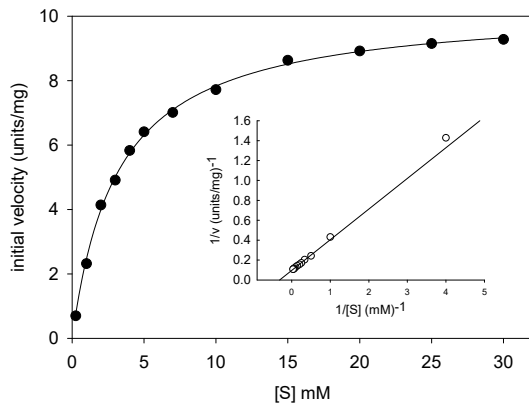
Adding a Lineweaver-Burke Plot to your v vs. S plot IV

•When formatted, you will have a publication quality, properly fit line for your Lineweaver-Burke plot that you can paste into your hyperbola plot as an inset.



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Publication-ready Kinetic Plot



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Expanding beyond $S \rightarrow P$

- | | |
|---------------------------------|---------------------------|
| • One substrate, one product | $S \rightarrow P$ |
| • One substrate, two products | $S \rightarrow P_1 + P_2$ |
| • Two substrates, one product | $A + B \rightarrow C$ |
| • Two substrates, two products | $A + B \rightarrow C + D$ |
| • Three substrates, one product | $A + B + C \rightarrow D$ |

Most biochemical reactions involve two substrates

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Two substrate reactions

- In what order do substrates bind?
- In what order are products released?
- How are different kinetic mechanisms distinguished?
- How are kinetic constants determined for two substrate reactions?

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Two substrate reactions

- Sequential: both substrates must combine with enzyme before a reaction occurs and product(s) are released
 - “Ordered substrate binding”
 - “Random substrate binding”
- Ping-pong (double displacement): One or more products are released between binding of first and second substrates

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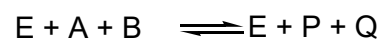
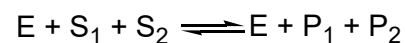
48

Kinetic mechanisms of two substrate reactions

- Sequential
 - Ordered or random substrate binding?
 - Ordered or random product release?
- Ping-pong
 - Always ordered, by nature of the reaction

Designate substrates as S_1 and S_2 , or A and B

Designate product(s) as P_1 and P_2 , or P and Q



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Writing kinetic mechanisms for two substrate reactions

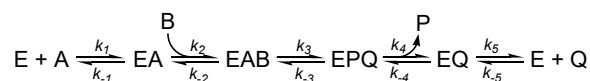
1. Use equilibrium expressions (as for single substrate reactions)
2. Use horizontal lines to denote the enzyme surface, vertical arrows to denote incoming substrates and outgoing products

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Example kinetic mechanisms for sequential reactions involving 2 substrates and 2 products

- Ordered with respect to substrate addition and product release:

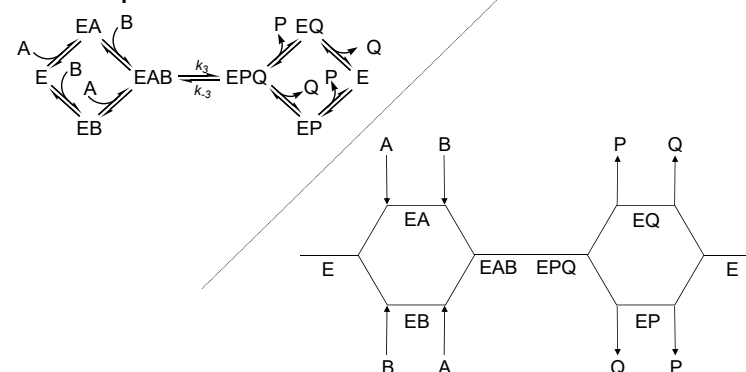


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Example kinetic mechanisms for sequential reactions involving 2 substrates and 2 products

- random with respect to substrate addition and product release:



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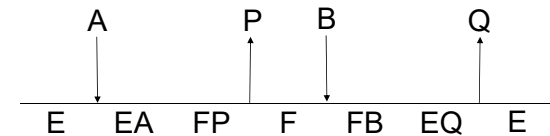
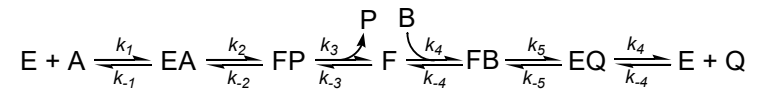
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Other possible kinetic mechanisms for sequential reactions involving 2 substrates and 2 products

- Ordered with respect to substrate binding, random with respect to product release
- Random with respect to substrate binding, ordered with respect to product release

Kinetic mechanism for a ping-pong (double displacement) reaction involving 2 substrates and 2 products

- “F” designates a modified form of the enzyme (e.g., enzyme with a covalent bond to a fragment of the first substrate)



Two substrate reactions

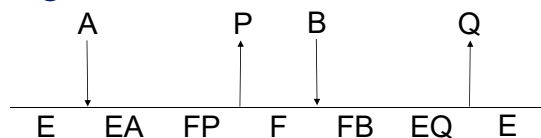
- In what order do substrates bind?
- In what order are products released?
- How are different kinetic mechanisms distinguished?
- How are kinetic constants determined for two substrate reactions?

- Collect kinetic data
- Analyze kinetic data using Michaelis-Menten equations derived for ping-pong, ordered sequential, and random sequential mechanisms
- Analyze kinetic data and curve fits statistically and graphically
- Deduce kinetic mechanism from best fits

Collection and analysis of kinetic data for two substrate reactions

- Conduct enzyme assays with constant [E] and varying [A] with a fixed, subsaturating [B] present in each assay
- Select a second fixed [B] (e.g., twice that used for the first set of assays), and repeat the kinetic measurements for each varied [A]
- Repeat this procedure for as many different fixed [B] desired
- Construct a graph containing each of the v vs. [A] plots obtained for different [B]
- Replot each v vs. [A] plot in double reciprocal form
- The pattern of double reciprocal lines (parallel or intersecting) reveals whether the kinetic mechanism is ping-pong or ordered

Michaelis-Menten Equation for a Bimolecular Ping-Pong reaction



$$v = \frac{V_{\max} [A] \cdot [B]}{K_{m,a} \cdot [B] + K_{m,b} \cdot [A] + [A] \cdot [B]}$$

$K_{m,a} = K_m$ for substrate A

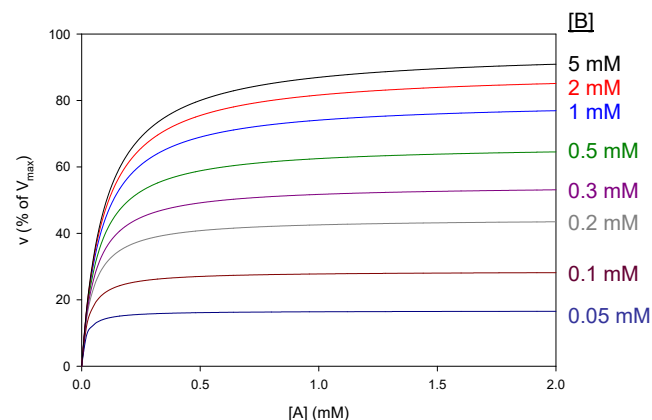
$K_{m,b} = K_m$ for substrate B

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V vs. S plots for a 2 substrate ping-pong kinetic mechanism

For the hypothetical enzyme example shown below, $K_{m,a} = 0.1$ mM, $K_{m,b} = 0.25$ mM, and $V_{\max} = 100$ U/mg.



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Algebraic rearrangement of the Michaelis-Menten Equation for a Bimolecular Ping-Pong reaction in the form of $y = mx + b$

$$\frac{1}{v} = \underbrace{\frac{K_{m,a}}{V_{\max}}}_{y = m} \cdot \underbrace{\frac{1}{[A]}}_{x} + \underbrace{\frac{1}{V_{\max}} \cdot \left(1 + \frac{K_{m,b}}{[B]}\right)}_{b}$$

$K_{m,a} = K_m$ for substrate A

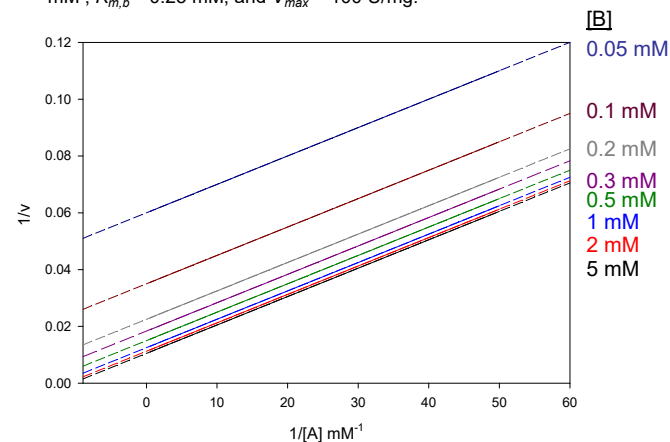
$K_{m,b} = K_m$ for substrate B

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Reciprocal plots for a 2 substrate ping-pong kinetic mechanism

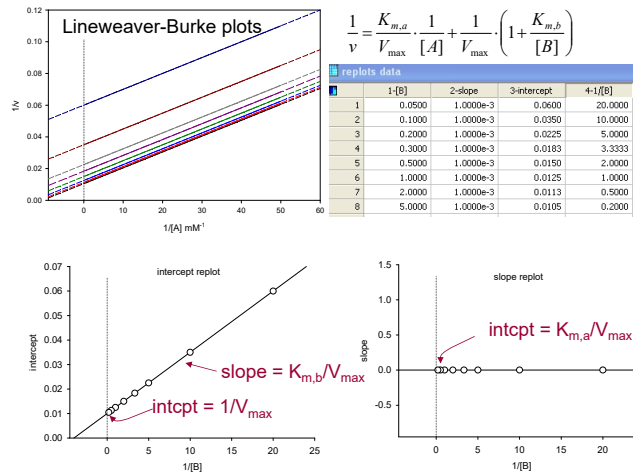
For the hypothetical enzyme example shown below, $K_{m,a} = 0.1$ mM, $K_{m,b} = 0.25$ mM, and $V_{\max} = 100$ U/mg.



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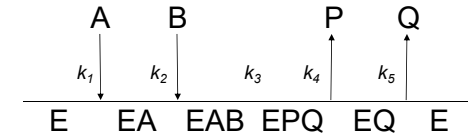
Graphical estimation of kinetic parameters for a 2 substrate ping pong reaction from Lineweaver-Burke plots



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Michaelis-Menten Equation for an ordered sequential bimolecular reaction



$$v = \frac{V_{max} [A] \cdot [B]}{K_{ia} \cdot K_{m,b} + K_{m,a} \cdot [B] + K_{m,b} \cdot [A] + [A] \cdot [B]}$$

$K_{m,a} = K_m$ for substrate A

$K_{m,b} = K_m$ for substrate B

K_{ia} = the dissociation constant for the first substrate for an ordered sequential mechanism.

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Algebraic rearrangement of the Michaelis-Menten Equation for an ordered sequential bimolecular reaction in the form of $y = mx + b$

Equation where A is varied at fixed concentrations of B:

$$\frac{1}{v} = \underbrace{\frac{K_{m,a}}{V_{max}} \cdot \left(1 + \frac{K_{ia} \cdot K_{m,b}}{K_{m,a} \cdot [B]} \right)}_m \cdot \underbrace{\frac{1}{[A]}}_x + \underbrace{\frac{1}{V_{max}} \cdot \left(1 + \frac{K_{m,b}}{[B]} \right)}_b$$

$y = m x + b$

$K_{m,a} = K_m$ for substrate A

$K_{m,b} = K_m$ for substrate B

K_{ia} = the dissociation constant for the first substrate for an ordered sequential mechanism (assumes A is substrate that binds first).

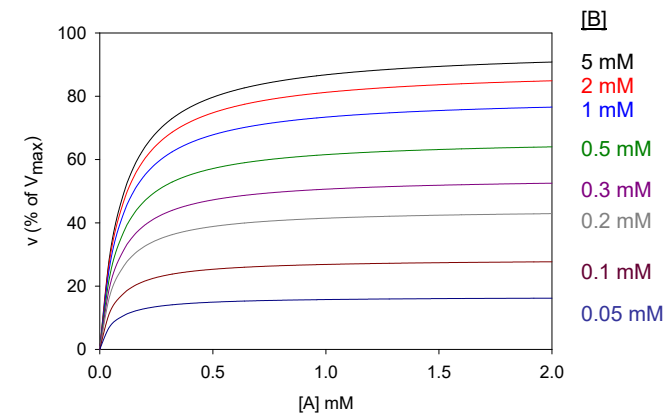
If the order of binding is random, then $K_{ia} \cdot K_{m,b} = K_{ib} \cdot K_{m,a}$

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V vs. S plots for a 2 substrate ordered sequential kinetic mechanism

For the hypothetical enzyme example shown below, $K_{m,a} = 0.1$ mM, $K_{m,b} = 0.25$ mM, $K_{ia} = 0.05$ mM and $V_{max} = 100$ U/mg.

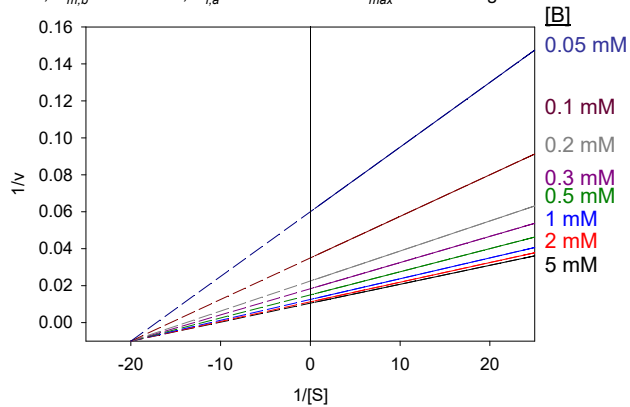


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Reciprocal plots for a 2 substrate ordered sequential kinetic mechanism

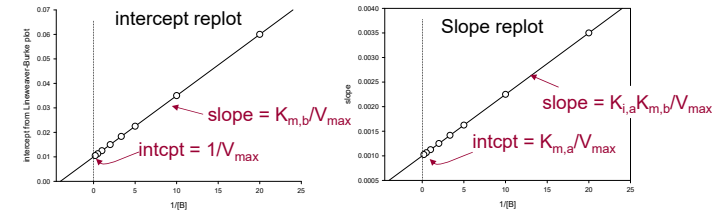
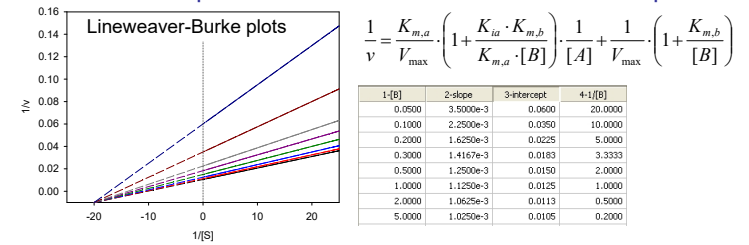
For the hypothetical enzyme example shown below, $K_{m,a} = 0.1$ mM, $K_{m,b} = 0.25$ mM, $K_{i,a} = 0.05$ mM and $V_{max} = 100$ U/mg.



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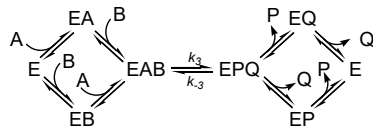
Graphical estimation of kinetic parameters for a 2 substrate ordered sequential reaction from Lineweaver-Burke plots



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Michaelis-Menten Equation for a random sequential bimolecular reaction



$$v = \frac{V_{max}[A] \cdot [B]}{\alpha K_a \cdot K_b + \alpha K_a \cdot [B] + \alpha K_b \cdot [A] + [A] \cdot [B]}$$

K_a = dissociation constant for substrate A

K_b = dissociation constant for substrate B

α = binding interaction factor

if $\alpha = 1$, substrate binding is independent

if $\alpha > 1$, binding of one substrate decreases affinity of other

if $\alpha < 1$, binding of one substrate increases affinity of other

S. Ensign, enzyme kinetics

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Algebraic rearrangement of the Michaelis-Menten Equation for a random sequential bimolecular reaction in the form of $y = mx + b$

Equation where A is varied at fixed concentrations of B:

$$\frac{1}{v} = \underbrace{\frac{\alpha K_a}{V_{max}} \cdot \left(1 + \frac{K_b}{[B]}\right)}_{y = m} \cdot \underbrace{\frac{1}{[A]}}_{x} + \underbrace{\frac{1}{V_{max}} \cdot \left(1 + \frac{\alpha K_b}{[B]}\right)}_{b}$$

K_a = dissociation constant for substrate A

K_b = dissociation constant for substrate B

α = binding interaction factor

if $\alpha = 1$, substrate binding is independent

if $\alpha > 1$, binding of one substrate decreases affinity of other

if $\alpha < 1$, binding of one substrate increases affinity of other

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How do you discriminate an ordered from a random sequential mechanism?

- Examine product inhibition profiles (we can discuss this more after discussing the various types of reversible enzyme inhibitors-C, NC, UC)
- Lactate dehydrogenase provides a good example to illustrate this

Analysis of two substrate kinetic data

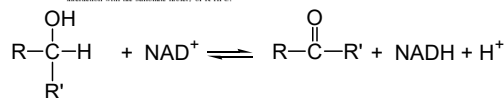
- Conduct enzyme assays with constant [E] and varying [A] with a fixed, subsaturating [B] present in each assay
- Select a second fixed [B] (e.g., twice that used for the first set of assays), and repeat the kinetic measurements for each varied [A]
- Repeat this procedure for as many different fixed [B] desired
- Construct a graph containing each of the v vs. [A] plots obtained for different [B]
- Replot each v vs. [A] plot in double reciprocal form
- The pattern of double reciprocal lines (parallel or intersecting) reveals whether the kinetic mechanism is ping-pong or ordered
- Slope and intercept replots are useful for visual purposes and estimating kinetic parameters
- Fit your data to the appropriate M-M equation to get your kinetic parameters

An actual example to illustrate how to analyze two substrate data

Biochemistry 2002, 41, 2727–2740
2727
Characterization of the 2-[(R)-2-Hydroxypropylthio]ethanesulfonate Dehydrogenase from *Xanthobacter* Strain Py2: Product Inhibition, pH Dependence of Kinetic Parameters, Site-Directed Mutagenesis, Rapid Equilibrium Inhibition, and Chemical Modification^a

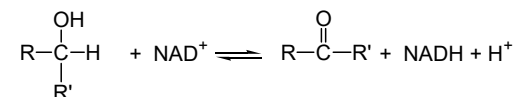
Daniel D. Clark and Scott A. Ensign^a
^aDepartment of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322-0000
Received September 17, 2001; Revised Manuscript Received December 13, 2001

ABSTRACT: Although the short-chain dehydrogenase/reductase (SDR) superfamily contains a very large number of members defined in annotated databases and by biochemical and structural studies, very few SDR enzymes have been identified that have a homologue partner catalyzing the same reaction but with an opposite stereospecificity. In the present study we have cloned and expressed one of these enzymes, the 2-[(R)-2-hydroxypropylthio]ethanesulfonate (R-HPC) dehydrogenase, that is part of the coenzyme N-dependent pathway of allene and cycloole metabolism in *Xanthobacter* strain Py2. Investigation of the kinetic mechanism using product inhibition suggested that a compulsory-ordered ternary complex mechanism was followed. The pH dependence of K_m/K_a indicated the presence of a single ionizable residue of catalytic importance ($pK_a = 6.9$) that was proposed to be Y155 of the catalytic triad. Amino acid substitutions of the putative catalytic triad residues produced inactive enzymes (S142C, Y155F, Y155E, and K155A) or enzyme with a greatly decreased activity (S142A). Inhibitors were investigated as probes of the molecular features of R-HPC that contribute to substrate binding. 2-[(S)-2-Hydroxypropylthio]ethanesulfonate (S-HPC) and 2-(2-methyl-2-hydroxypropylthio)ethanesulfonate were found to be competitive inhibitors of R-HPC with K_i values close to the K_m for R-HPC. The arginine-specific modifiers 2,3-diaminobutane and phenylglyoxal were found to be inactivators, and inactivation could be protected against by the addition of R-HPC. 2,3-Diaminobutane was found to reduce enzyme activity with R-HPC as a substrate much more chemically than with substrates that lacked a sulfonate moiety [e.g., 2-propanol, (R)-2-pentanol, and (R)-2-heptanol]. Amino acid analyses of enzyme modified by 2,3-diaminobutane in the presence and absence of S-HPC suggested protection of a single arginine residue. On the basis of these results, we propose that one or more active site arginines play a key role in substrate binding via an ionic interaction with the sulfonate moiety of R-HPC.



An actual example to illustrate how to analyze two substrate data

- NAD^+ = substrate A
- R-HPC = substrate B (the alcohol)
- Graphical analysis of the data indicates sequential (not ping pong)
- Product inhibition profiles suggest ordered sequential, with NAD^+ binding before R-HPC



Graph the data

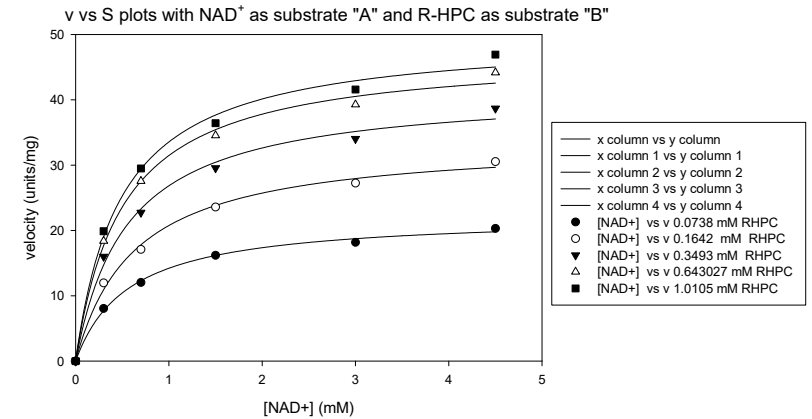
- Make scatter plots of each v vs. S curve on a single page
- Fit each individual curve to a rectangular hyperbola
- Add the lines for the curve fits
- Make double reciprocal plots ($1/v$ vs. $1/S$) on a single page
- Add the linear regressions for the theoretical data to complete the Lineweaver-Burke plots
- Determine the slopes and intercepts of each $1/v$ vs. $1/S$ plot, and replot the slopes and intercepts vs. $1/[B]$ for visual estimation of kinetic parameters

1-[NAD ⁺]	2-v 0.0738 mM RHPC	3-v 0.1642 mM RHPC	4-v 0.3493 mM RHPC	5-v 0.643027 mM RHPC	6-v 1.0105 mM RHPC
0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.3000	8.0520	11.9680	15.9980	18.3740	19.8820
0.7000	12.0390	17.0890	22.7320	27.5490	29.4900
1.5000	16.1990	23.5827	29.5530	34.5370	36.4190
3.0000	18.1830	27.2560	34.0350	39.2700	41.5810
4.5000	20.3120	30.5370	38.6740	44.1600	46.9240

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v vs. S curves for R-HPCDH data

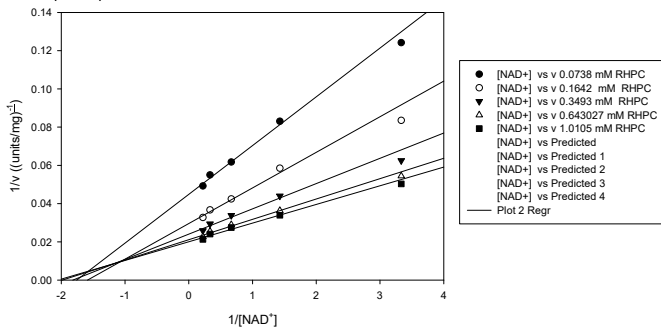


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Reciprocal plots of R-HPCDH data

reciprocal plots with NAD⁺ as substrate "A" and R-HPC as substrate "B"



Note how the lines intersect pretty well at a common point, indicative of a sequential reaction. Is it a concern that the top line does not intersect with the others as well?

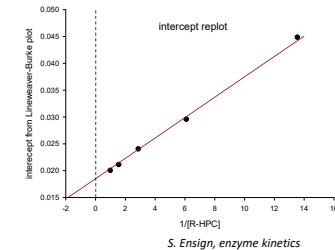
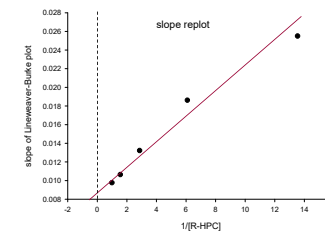
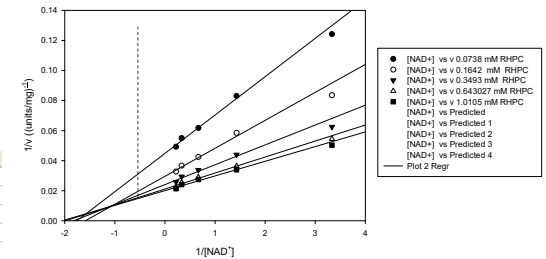
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Slope and intercept replots of R-HPCDH data

- Use the slopes and intercepts determined from linear regression of the predicted data points

1-[R-HPC]	2-slope	3-intercept	4-1/[R-HPC]
0.0738	0.0255	0.0449	13.5413
0.1642	0.0186	0.0296	6.0897
0.3493	0.0132	0.0240	2.8628
0.6430	0.0106	0.0211	1.5551
1.0105	9.7675e-3	0.0200	0.9896



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Analysis of two substrate kinetic data

- Conduct enzyme assays with constant [E] and varying [A] with a fixed, subsaturating [B] present in each assay
- Select a second fixed [B] (e.g., twice that used for the first set of assays), and repeat the kinetic measurements for each varied [A]
- Repeat this procedure for as many different fixed [B] desired
- Construct a graph containing each of the v vs. [A] plots obtained for different [B]
- Replot each v vs. [A] plot in double reciprocal form
- The pattern of double reciprocal lines (parallel or intersecting) reveals whether the kinetic mechanism is ping-pong or ordered
- Slope and intercept replots are useful for visual purposes and estimating kinetic parameters
- Fit your data to the appropriate form of the M-M equation to get your kinetic parameters

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Analysis of two substrate kinetic data using nonlinear regression

- Arrange all data into 3 columns:

1-[NAD+]	2-v 0.0738 mM RHP	3-v 0.1642 mM RHP	4-v 0.3493 mM RHP	5-v 0.643027 mM RHP	6-v 1.0105 mM RHP
0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
0.3000	8.0520	11.9680	15.9980	18.3740	19.8820
0.7000	12.0390	17.0890	22.7320	27.5490	29.4900
1.5000	16.1990	23.5827	29.5530	34.5370	36.4190
3.0000	18.1830	27.2560	34.0350	39.2700	41.5810
4.5000	20.3120	30.5370	38.6740	44.1600	46.9240



1-[NAD+]	2-[R-HPC]	3-v
0.3000	0.0738	8.0520
0.3000	0.1642	11.9680
0.3000	0.3493	15.9980
0.3000	0.6430	18.3740
0.3000	1.0105	19.8820
0.7000	0.0738	12.0390
0.7000	0.1642	17.0890
0.7000	0.3493	22.7320
0.7000	0.6430	27.5490
0.7000	1.0105	29.4900
1.5000	0.0738	16.1990
1.5000	0.1642	23.5827
1.5000	0.3493	29.5530
1.5000	0.6430	34.5370
1.5000	1.0105	36.4190
3.0000	0.0738	18.1830
3.0000	0.1642	27.2560
3.0000	0.3493	34.0350
3.0000	0.6430	39.2700
3.0000	1.0105	41.5810
4.5000	0.0738	20.3120
4.5000	0.1642	30.5370
4.5000	0.3493	38.6740
4.5000	0.6430	44.1600
4.5000	1.0105	46.9240

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Analysis of two substrate kinetic data using nonlinear regression

$$v = \frac{V_{\max} [A] \cdot [B]}{K_{ia} \cdot K_{m,b} + K_{m,a} \cdot [B] + K_{m,b} \cdot [A] + [A] \cdot [B]}$$

- Formula: $Y = a \cdot x_1 \cdot x_2 / (d \cdot c + c \cdot x_1 + b \cdot x_2 + x_1 \cdot x_2)$
- Number of data points = (varies for data) (25 here)
- number of independent variables = 2 (x1, x2)
 - x1 = [A] (NAD+)
 - x2 = [B] (R-HPC)
- number of parameters = 4 (a, b, c, d)
 - a = Vmax
 - b = Kma
 - c = Kmb
 - d = Kia
- Y = velocity

x1	x2	Y
0.3000	0.0738	8.0520
0.3000	0.1642	11.9680
0.3000	0.3493	15.9980
0.3000	0.6430	18.3740
0.3000	1.0105	19.8820
0.7000	0.0738	12.0390
0.7000	0.1642	17.0890
0.7000	0.3493	22.7320
0.7000	0.6430	27.5490
0.7000	1.0105	29.4900
1.5000	0.0738	16.1990
1.5000	0.1642	23.5827
1.5000	0.3493	29.5530
1.5000	0.6430	34.5370
1.5000	1.0105	36.4190
3.0000	0.0738	18.1830
3.0000	0.1642	27.2560
3.0000	0.3493	34.0350
3.0000	0.6430	39.2700
3.0000	1.0105	41.5810
4.5000	0.0738	20.3120
4.5000	0.1642	30.5370
4.5000	0.3493	38.6740
4.5000	0.6430	44.1600
4.5000	1.0105	46.9240

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Analysis of two substrate kinetic data using nonlinear regression

- You must put in good initial guesses using statpages.info fitting. Get these from the slope and intercept replots

- Enter the number of data points: 25
- Enter the number of independent variables: 2 (1 for single substrate M-M equation; 2 for bisubstrate, 2 for C, UC, and mixed inhibition)
- Enter the number of parameters: 4 (deduce from form of M-M equation used, will be 2 for single substrate M-M)
- Enter the formula for the function to be fitted:
 $Y = \frac{a \cdot x_1 \cdot x_2}{d \cdot c + c \cdot x_1 + b \cdot x_2 + x_1 \cdot x_2}$
 Use "*" for multiplication and "/" for division when entering formulas. Define parameters (a, b, c) and variables (x1, x2, x3) as described above. Be sure to use parentheses appropriately to get the correct hierarchy.
- Type (or paste) the [x,y] data:

0.3000	0.0738	8.0520
0.3000	0.1642	11.9680
0.3000	0.3493	15.9980
0.3000	0.6430	18.3740
0.3000	1.0105	19.8820
0.7000	0.0738	12.0390
0.7000	0.1642	17.0890
0.7000	0.3493	22.7320
0.7000	0.6430	27.5490
0.7000	1.0105	29.4900
1.5000	0.0738	16.1990
1.5000	0.1642	23.5827
1.5000	0.3493	29.5530
1.5000	0.6430	34.5370
1.5000	1.0105	36.4190
3.0000	0.0738	18.1830
3.0000	0.1642	27.2560
3.0000	0.3493	34.0350
3.0000	0.6430	39.2700
3.0000	1.0105	41.5810
4.5000	0.0738	20.3120
4.5000	0.1642	30.5370
4.5000	0.3493	38.6740
4.5000	0.6430	44.1600
4.5000	1.0105	46.9240
- Enter initial guesses for the parameters from evaluation of the data (e.g. Lineweaver-Burke plots and appropriate slope and intercept replots). If the data fails to converge, provide better initial guesses:

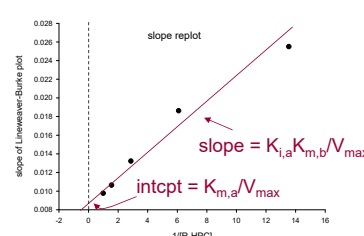
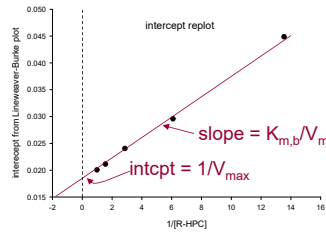
a (or p1) =			
b (or p2) =			
c (or p3) =			
d (or p4) =			
e (or p5) =	0		
f (or p6) =	0		
g (or p7) =	0		
h (or p8) =	0		
- Place the value "0" (zero) in all parameter boxes for which a value is not defined in the equation to be fit. Leave the fields to the right of the parameters blank; these will be populated with the standard errors upon iteration.
 Click the "Iterate" button once for each iteration cycle. Watch how the initial guesses change in response to the iteration. Continue to click the "Iterate" button until the values converge to such a degree that

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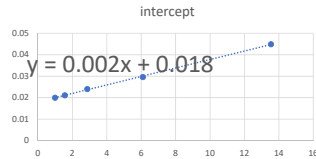
80

Slope and intercept replots of R-HPCDH data

1/RHPC	intercept	1/RHPC	slope
13.5413	0.0449	13.5413	0.0255
6.0897	0.0296	6.0897	0.0186
2.8628	0.024	2.8628	0.0132
1.5551	0.0211	1.5551	0.0106
0.9896	0.02	0.9896	9.77E-03

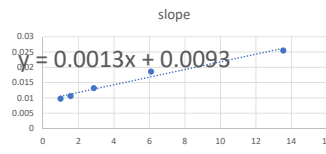


Linear regression in excel: right click data points, select "add trend line", "display equation on chart"



intcpt = $1/V_{\max}$; $V_{\max} = 1/\text{intcpt} = 1/0.018 = 55.6$

slope = $K_{m,b}/V_{\max}$; $K_{m,b} = V_{\max} \cdot \text{slope} = 55.6 \cdot 0.002 = 0.112$



intcpt = $K_{m,a}/V_{\max}$; $K_{m,a} = \text{intcpt} \cdot V_{\max} = 0.0093 \cdot 55.6 = 0.517$

slope = $K_{i,a}K_{m,b}/V_{\max}$; $K_{i,a} = \text{slope} \cdot V_{\max}/K_{m,b} = 0.0013 \cdot 55.6/0.112 = 0.645$

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Analysis of two substrate kinetic data using nonlinear regression

$$v = \frac{V_{\max} [A] \cdot [B]}{K_{i,a} \cdot K_{m,b} + K_{m,a} \cdot [B] + K_{m,b} \cdot [A] + [A] \cdot [B]}$$

- Formula: $Y = a \cdot x_1 \cdot x_2 / (d \cdot c + c \cdot x_1 + b \cdot x_2 + x_1 \cdot x_2)$
- Number of data points = (varies for data) (25 here)
- number of independent variables = 2 (x_1 , x_2)
 - $x_1 = [A]$ (NAD+)
 - $x_2 = [B]$ (R-HPC)
- number of parameters = 4 (a, b, c, d)
 - a = $V_{\max}$ ~55.6
 - b = $K_{m,a}$ ~0.517
 - c = $K_{m,b}$ ~0.112
 - d = $K_{i,a}$ ~0.645
- Y = velocity

6 Enter initial guesses for the parameters from evaluation of the data (e.g.

a (or p_1) =	55.6	±		p =	
b (or p_2) =	0.517	±		p =	
c (or p_3) =	0.112	±		p =	
d (or p_4) =	0.645	±		p =	
e (or p_5) =	0	±		p =	
f (or p_6) =	0	±		p =	
g (or p_7) =	0	±		p =	
h (or p_8) =	0	±		p =	

x1	x2	Y
0.3000	0.0738	8.0520
0.3000	0.1642	11.9680
0.3000	0.3493	15.9980
0.3000	0.6430	18.3740
0.3000	1.0105	19.8820
0.7000	0.0738	12.0390
0.7000	0.1642	17.0890
0.7000	0.3493	22.7320
0.7000	0.6430	27.5490
0.7000	1.0105	29.4900
1.5000	0.0738	16.1990
1.5000	0.1642	23.5827
1.5000	0.3493	29.5530
1.5000	0.6430	34.5370
1.5000	1.0105	36.4190
3.0000	0.0738	18.1830
3.0000	0.1642	27.2560
3.0000	0.3493	34.0350
3.0000	0.6430	39.2700
3.0000	1.0105	41.5810
4.5000	0.0738	20.3120
4.5000	0.1642	30.5370
4.5000	0.3493	38.6740
4.5000	0.6430	44.1600
4.5000	1.0105	46.924082

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Analysis of two substrate kinetic data using nonlinear regression

Be sure to iterate until the values no longer change. Here is what you end up with. Compare these numbers to your initial guesses from the intercept and slope replots. The numbers below, with their standard errors, are the numbers you report when you publish your data.

6. Enter initial guesses for the parameters from evaluation of the data (e.g. Line

a (or p_1) =	55.00977463651 ± 1.452688132807	p =	5e-7
b (or p_2) =	0.463554321482 ± 0.051612969840	p =	5e-7
c (or p_3) =	0.106694812940 ± 0.011758483401	p =	5e-7
d (or p_4) =	0.776863494422 ± 0.206706457062	p =	0.001156
e (or p_5) =	0 ± 1	p =	1.000000
f (or p_6) =	0 ± 1	p =	1.000000
g (or p_7) =	0 ± 1	p =	1.000000
h (or p_8) =	0 ± 1	p =	1.000000

Place the value "0" (zero) in all parameter boxes for which a value is not defined in the equation

- Estimates:
 - a = $V_{\max}$ ~55.6
 - b = $K_{m,a}$ ~0.517
 - c = $K_{m,b}$ ~0.112
 - d = $K_{i,a}$ ~0.645

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Michaelis-Menten equation for a bisubstrate ping-pong reaction

$$v = \frac{V_{\max} [A] \cdot [B]}{K_{m,a} \cdot [B] + K_{m,b} \cdot [A] + [A] \cdot [B]}$$

Sample data:

- Formula: $Y = a \cdot x_1 \cdot x_2 / (b \cdot x_2 + c \cdot x_1 + x_1 \cdot x_2)$
- Number of data points = (varies for data)
- number of independent variables = 2 (x_1 , x_2)
 - $x_1 = [A]$
 - $x_2 = [B]$
- number of parameters = 3 (a, b, c)
 - a = $V_{\max}$
 - b = $K_{m,a}$
 - c = $K_{m,b}$
- Y = velocity

0.2	0.025	8.6
0.25	0.025	9.2
0.333	0.025	9.9
0.5	0.025	10.6
1	0.025	11.7
2	0.025	12.2
2.5	0.025	12.3
3.33	0.025	12.42
4	0.025	12.44
5	0.025	12.5
0.2	0.05	12.2
0.25	0.05	13.5
0.333	0.05	15.1
0.5	0.05	17.1
1	0.05	18.75
2	0.05	21.3
2.5	0.05	21.75
3.33	0.05	22
4	0.05	22.2
5	0.05	22.5
0.2	0.1	15.6
0.25	0.1	17.8
0.333	0.1	20.6
0.5	0.1	25.55
1	0.1	30.3
2	0.1	34.2
2.5	0.1	35.1
3.33	0.1	36.1
4	0.1	36.6
5	0.1	37.2
0.2	0.25	18.8
0.25	0.25	22
0.333	0.25	26.4
0.5	0.25	32
1	0.25	44.5
2	0.25	53.75
2.5	0.25	56
3.33	0.25	58.6
4	0.25	59.9
5	0.25	61.3
0.2	0.5	20.1
0.25	0.5	23.8
0.333	0.5	29.1
0.5	0.5	37.5
1	0.5	52.8
2	0.5	66.3
2.5	0.5	69.8
3.33	0.5	71.9
4	0.5	73.1

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“Apparent” vs. “true” K_m and V_{max} values for bisubstrate reactions

- “True” K_m and V_{max} values are derived from a complete three variable analysis of kinetic data, as described above
- If K_m and V_{max} values are determined for only one substrate at a high, nominally “saturating” concentration of the second substrate, then the constants are “apparent” K_m and V_{max}

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Allosteric enzymes

- Multisubunit enzymes which exhibit cooperativity for substrate binding
- Sigmoidal curves for substrate binding instead of hyperbolic
- Michaelis-Menten kinetics does not apply

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Enzyme Inhibition

Inhibitors: Compounds that reduce the activity of an enzyme by combining with in a manner that influences the binding of substrate and/or its k_{cat} .

- Uses:
- Probes of catalytic mechanism
 - Elucidate metabolic pathways
 - Pharmaceuticals-chemotherapeutic action

Two broad classes of “reversible” inhibitors:

- Rapid equilibrium
 - Competitive
 - Noncompetitive
 - Uncompetitive
- Slow-(tight) binding

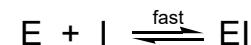
Irreversible inhibitors: inactivators (do not dissociate), include “mechanism-based inactivators (suicide substrates)

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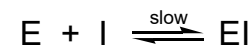
Distinguishing rapid-equilibrium and slow-binding inhibitors

- Rapid-equilibrium: equilibrium between E, I, and EI is rapidly established (i.e. within mixing time prior to initiating assay).



- Slow- (tight) binding:

- Scenario 1: equilibrium between E, I and EI is not established rapidly (i.e. within time frame of assay)



- Scenario 2: equilibrium between E, I, and EI is rapidly established, but EI complex slowly reacts further to form a second inhibited form EI*.

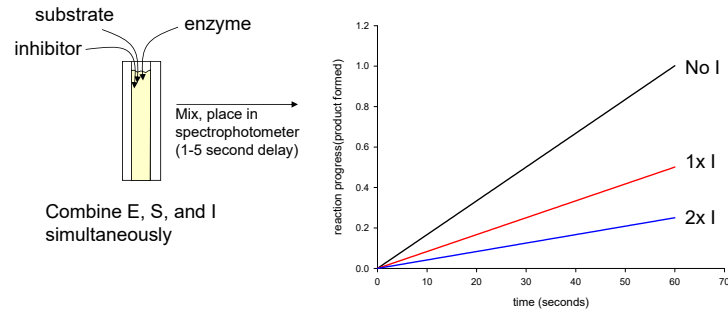


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Distinguishing rapid-equilibrium and slow-binding inhibitors

- Rapid-equilibrium: equilibrium between E, I, and EI is rapidly established (i.e. within mixing time prior to initiating assay).

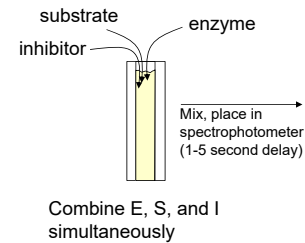


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Distinguishing rapid-equilibrium and slow-binding inhibitors

- Slow-binding: equilibrium between E, I, and EI (or EI*) is not rapidly established (i.e. within mixing time prior to initiating assay). The time required for establishment of the equilibrium is observed in the “steady state”

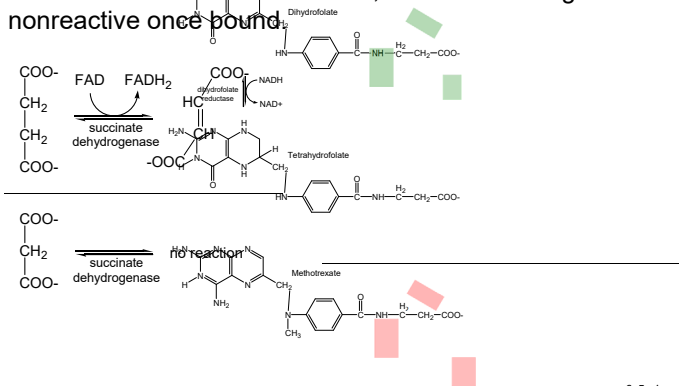


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Competitive Inhibition

Competitive inhibitor: a compound that competes with substrate for binding to enzyme active site. Usually resembles structure of substrate, but differs enough to be nonreactive once bound.

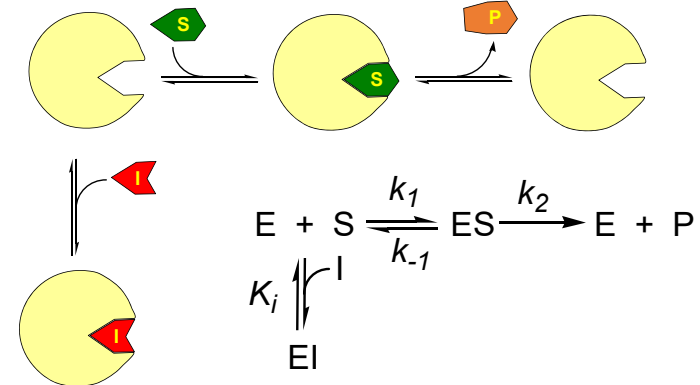


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Competitive Inhibition

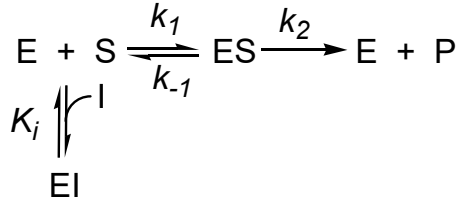
Competitive inhibitor: a compound that competes with substrate for binding to enzyme active site. Usually resembles structure of substrate, but differs enough to be nonreactive once bound.



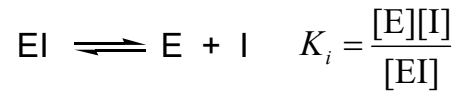
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Inhibition constant K_i



The inhibition constant K_i is the thermodynamic dissociation constant for the nonproductive EI complex



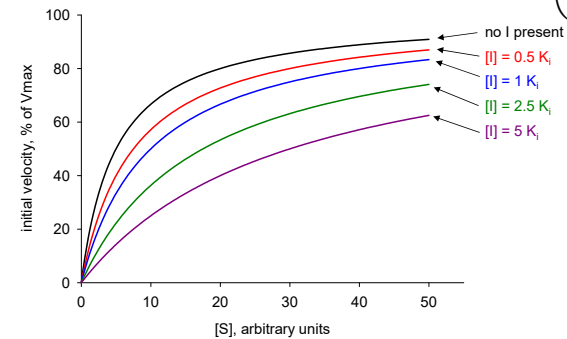
The smaller the value of K_i , the more potent the inhibitor

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Michaelis-Menten equation in the presence of a competitive inhibitor (single substrate case)

Without inhibitor: $v = \frac{V_{\max}[S]}{K_m + [S]}$ With inhibitor: $v = \frac{V_{\max}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}$



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Algebraic rearrangement of the Michaelis-Menten equation in the presence of a competitive inhibitor

Without inhibitor:

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

$y = \underbrace{\quad}_m x + b$

With inhibitor:

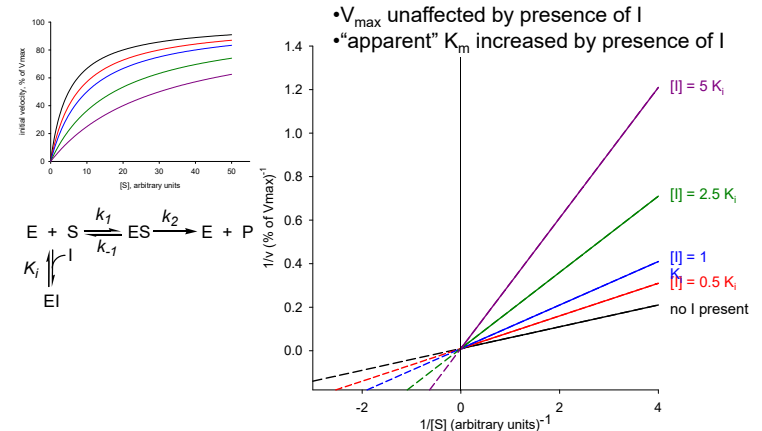
$$\frac{1}{v} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i}\right) \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

$y = \underbrace{\quad}_m x + b$

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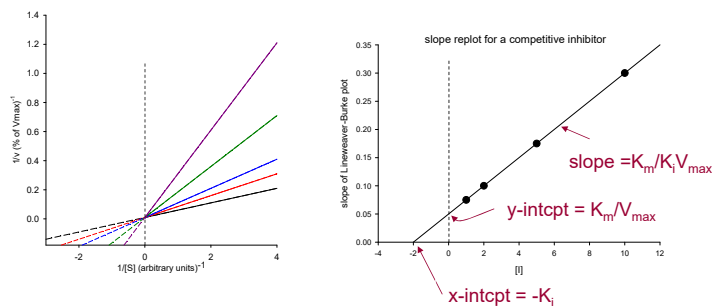
Lineweaver-Burke plots in the presence of a competitive inhibitor



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Slope replot for estimation of kinetic parameters with a competitive inhibitor

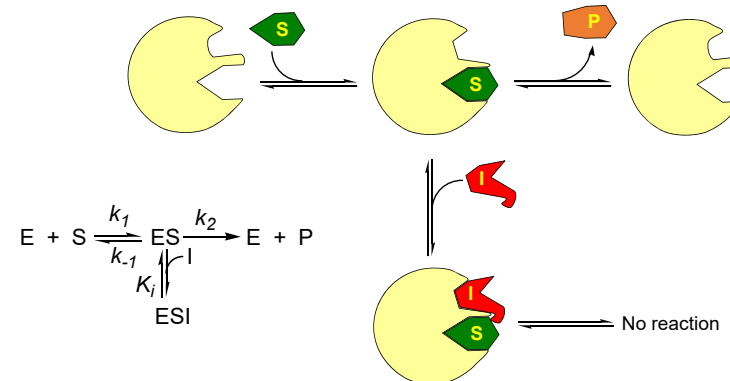


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Uncompetitive Inhibition

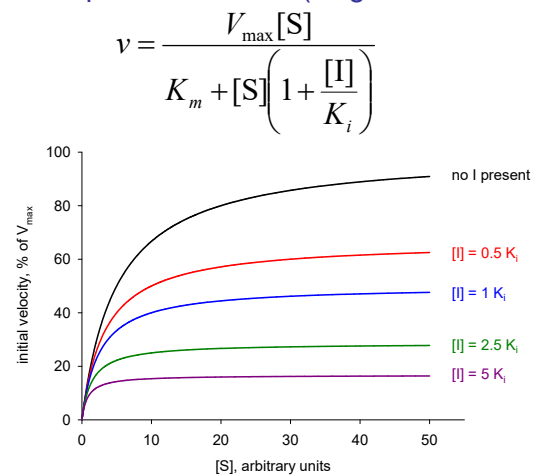
Uncompetitive inhibitor: a compound that inhibits activity by binding the ES complex but not the free enzyme E.



S. Ensign, enzyme kinetics

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Michaelis-Menten equation in the presence of an uncompetitive inhibitor (single substrate case)



S. Ensign, enzyme kinetics

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Algebraic rearrangement of the Michaelis-Menten equation in the presence of an uncompetitive inhibitor

Without inhibitor:

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

$y = \underbrace{\quad}_m \quad x + b$

With inhibitor:

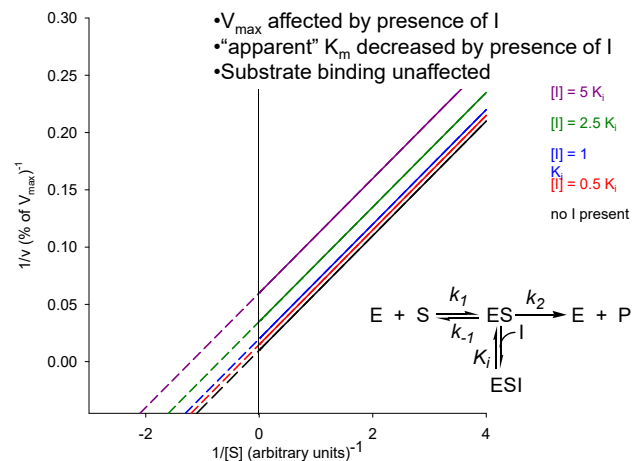
$$\frac{1}{v} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_i} \right)$$

$y = \underbrace{\quad}_m \quad x + b$

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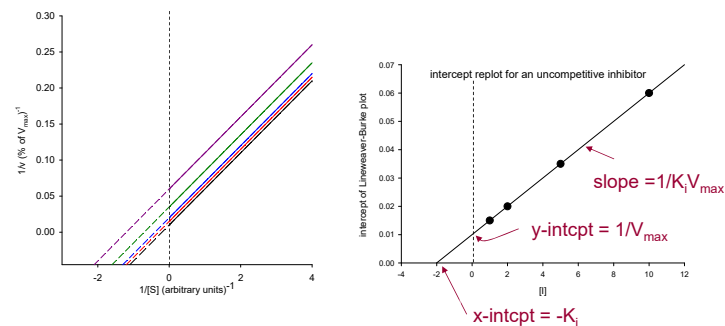
Lineweaver-Burke plots in the presence of an uncompetitive inhibitor



S. Ensign, enzyme kinetics

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Intercept replot for estimation of kinetic parameters with an uncompetitive inhibitor



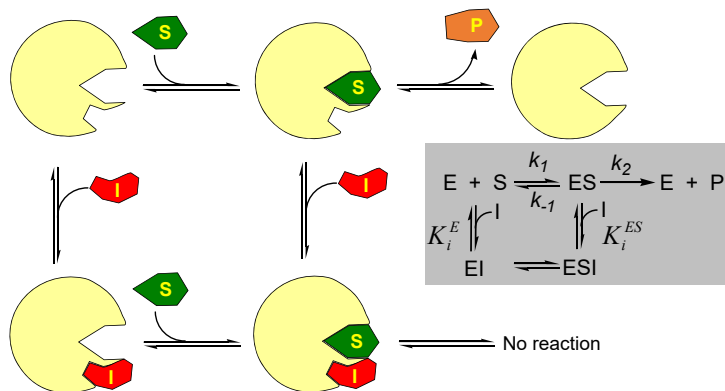
For this example, $V_{\max} = 100$, $K_m = 5$, and $K_i = 2$

S. Ensign, enzyme kinetics

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Mixed (noncompetitive) Inhibition

Mixed inhibitor: a compound that inhibits activity upon binding either the free enzyme or the ES complex

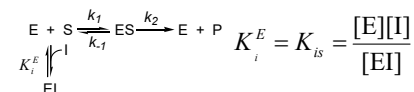


S. Ensign, enzyme kinetics

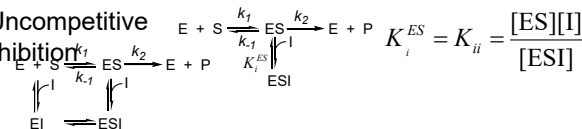
103

Inhibition constant nomenclature

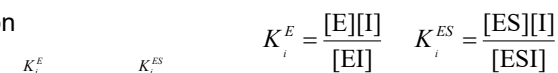
•Competitive inhibition



•Uncompetitive inhibition



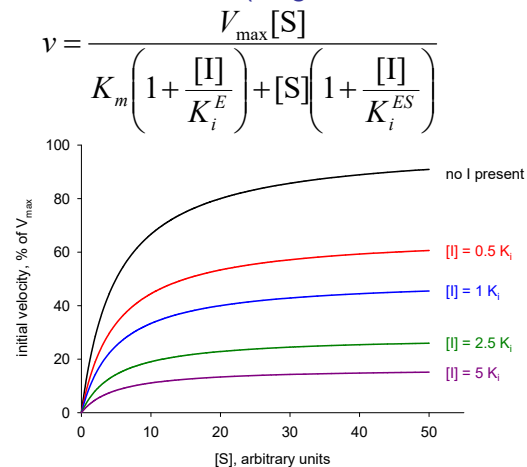
•Mixed inhibition



S. Ensign, enzyme kinetics

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Michaelis-Menten equation in the presence of a mixed inhibitor (single substrate case)



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S. Ensign, enzyme kinetics

Algebraic rearrangement of the Michaelis-Menten equation in the presence of a mixed inhibitor

Without inhibitor:

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

$$y = \underbrace{\frac{K_m}{V_{\max}}}_m x + \underbrace{\frac{1}{V_{\max}}}_b$$

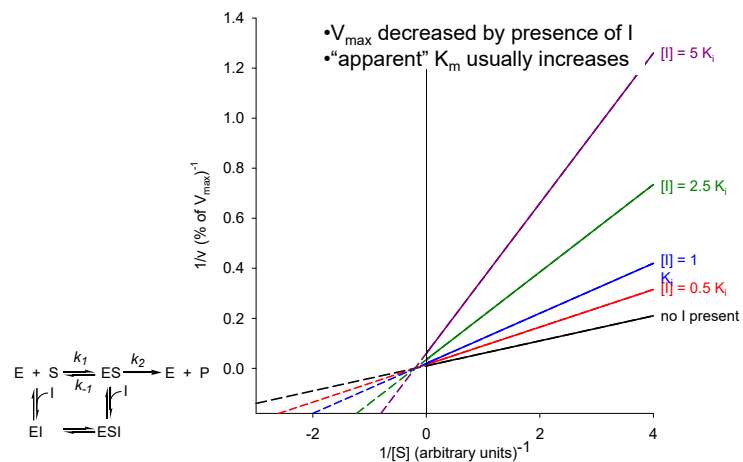
With inhibitor:

$$\frac{1}{v} = \frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i^E} \right) \cdot \frac{1}{[S]} + \frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_i^{ES}} \right)$$

$$y = \underbrace{\frac{K_m}{V_{\max}} \left(1 + \frac{[I]}{K_i^E} \right)}_m x + \underbrace{\frac{1}{V_{\max}} \left(1 + \frac{[I]}{K_i^{ES}} \right)}_b$$

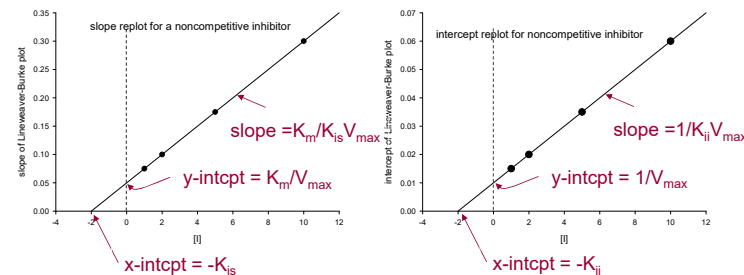
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Lineweaver-Burke plots in the presence of a mixed inhibitor



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Slope and intercept replots with a mixed inhibitor



For this example, $V_{\max} = 100$, $K_m = 5$, $K_{is} = 2$, and $K_{ii} = 2$

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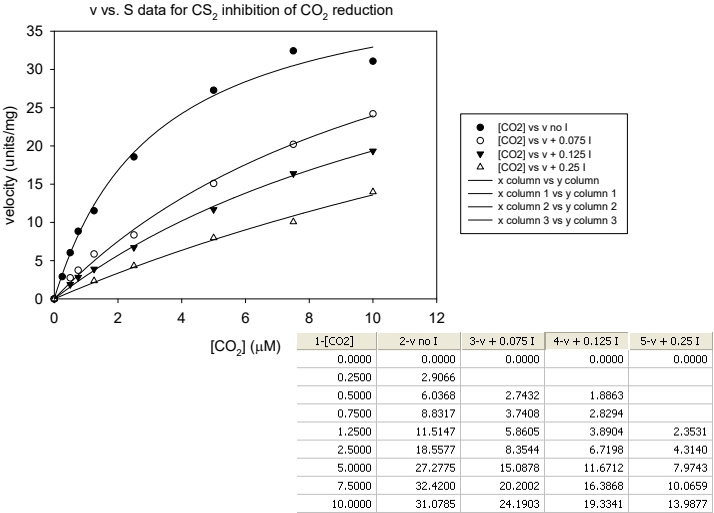
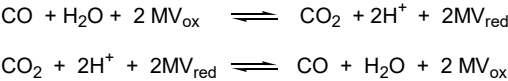
Analysis of kinetic data for a single substrate enzyme with a rapid-equilibrium inhibitor

- Conduct enzyme assays with constant [E] and varying [S] and with no I present
- Conduct enzyme assays with the same constant [E] and varying [S] as above, but with a fixed, subsaturating [I] present in each assay
- Select a second fixed [I] (e.g., twice that used for the first set of assays), and repeat the kinetic measurements for each varied [S]
- Repeat this procedure for as many different fixed [I] desired
- Construct a graph containing each of the v vs. [S] plots obtained for different [I]
- Replot each v vs. [S] plot in double reciprocal form
- The pattern of double reciprocal lines (intersecting on y-axis, parallel or intersecting in 3rd or 4th quadrant) reveals whether the inhibitor is C, UC, or NC
- Slope and intercept replots are useful for visual purposes and estimating kinetic parameters
- Fit your data to the appropriate M-M equation to get your kinetic parameters

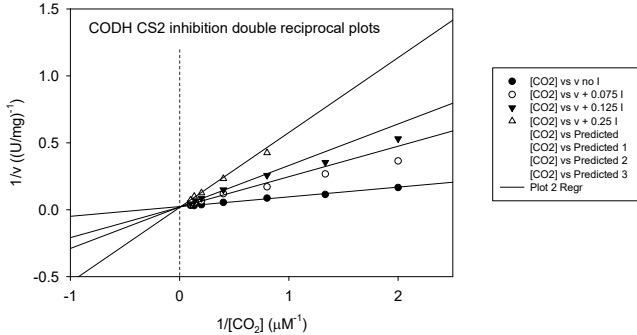
An actual example to illustrate how to analyze inhibitor data

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Biochemistry 1995, 34, 5372-5381
Reactivity of Carbon Monoxide Dehydrogenase from *Rhodospirillum rubrum* with Carbon Dioxide, Carbonyl Sulfide, and Carbon Disulfide
Scott A. Ensign^a
Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300
Received November 23, 1994; Revised Manuscript Received February 8, 1995^a

ABSTRACT: The reactivities of CO₂ and the related compounds COS and CS₂ with the nickel- and iron-sulfur-containing carbon monoxide dehydrogenase (CODH) from *Rhodospirillum rubrum* have been investigated. Both CO₂ and COS were substrates for CODH in a reductant-dependent reaction resulting in the formation of CO. CO₂ was reduced to CO and H₂O, while COS was reduced to CO and H₂S. CO was a potent inhibitor of CO₂ reduction at dissolved concentrations as low as 1 μM, but this inhibition could be prevented by quantitatively trapping CO as it was formed by including reduced hemoglobin in the assays. The addition of hemoglobin to the assays also allowed the formation of CO to be monitored in real time by following the decrease in absorbance at 433 nm resulting from carboxyhemoglobin formation. A variety of low-potential reductants, including dithionite, titanium(III) citrate, and dithionite-reduced viologens (methyl and benzyl), were suitable electron donors for the reduction of CO₂ and COS. Dithionite-reduced methyl viologen supported the highest rates of CO₂ and COS reduction, and the stimulation of CO₂ reduction (170-fold increased rate over dithionite alone) was much more dramatic than the stimulation of COS reduction (2.6-fold increased rate over dithionite alone). CO₂ was reduced to CO with a *K_m* for CO₂ of 190 μM and a *V_{max}* of 44 μmol of CO formed min⁻¹ (mg of protein)⁻¹, while COS was reduced with a *K_m* for COS of 2.2 μM and a *V_{max}* of 0.51 μmol of CO formed min⁻¹ (mg of protein)⁻¹. The CO₂/COS analog CS₂ interacted with CODH as a reversible, rapid-equilibrium inhibitor of CO₂ reduction, and the pattern of inhibition observed for CS₂ was competitive vs CO₂ (*K_i* = 43 μM).

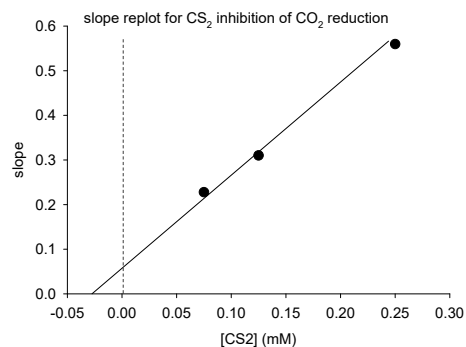


Reciprocal plots of CODH inhibition data



The straight lines are the linear regressions of the predicted data points obtained from the hyperbola regression fits of the original data. Note how data points nearer the origins (i.e. the larger numbers) are weighted greater than the points further from the origin (the smaller numbers)

Slope replot of CODH inhibition data



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Analysis of inhibition data using nonlinear regression

$$v = \frac{V_{\max} [S]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [S]}$$

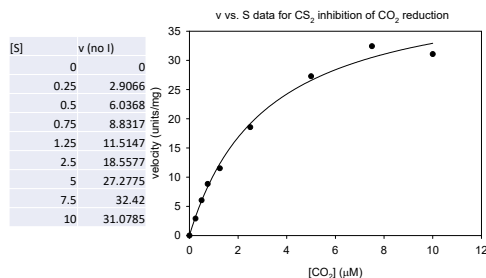
- Arrange data in 3 columns (S, I, and v)
- Formula: $Y = a \cdot x_1 / (b \cdot (1 + x_2/c) + x_1)$
- Number of data points = (varies for data)
- Number of independent variables = 2 (x_1 , x_2)
 - $x_1 = [S]$
 - $x_2 = [I]$
- Number of parameters = 3 (a, b, c)
 - $a = V_{\max}$
 - $b = K_m$
 - $c = K_i$
- Y = velocity

x1	x2	Y
0	0	0
0.25	0	2.9066
0.5	0	6.0368
0.75	0	8.8317
1.25	0	11.5147
2.5	0	18.5577
5	0	27.2775
7.5	0	32.42
10	0	31.0785
0.5	0.075	2.7432
0.75	0.075	3.7408
1.25	0.075	5.8605
2.5	0.075	8.3544
5	0.075	15.0878
7.5	0.075	20.2002
10	0.075	24.1903
0.5	0.125	1.8863
0.75	0.125	2.8294
1.25	0.125	3.8904
2.5	0.125	6.7198
5	0.125	11.6712
7.5	0.125	16.3868
10	0.125	19.3341
1.25	0.25	2.3531
2.5	0.25	4.314
5	0.25	7.9743
7.5	0.25	10.0659
10	0.25	13.9877

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Analysis of inhibition data using nonlinear regression

- You must put in good initial guesses using statpages.info fitting.
- Get the values of $V_{\max}$ (parameter a) and K_m (parameter b) from the v vs S data where no I was present



1. Enter the number of data points: 9
2. Enter the number of independent variables: 1 (1 for single substrate M.)
3. Enter the number of parameters: 2 (deduce from form of M-M equation u)
4. Enter the formula for the function to be fitted:
 $Y = [a \cdot x_1 / (b \cdot (1 + x_2/c) + x_1)]$
5. Type (or paste) the [x,y] data:

0	0
0.25	2.9066
0.5	6.0368
0.75	8.8317
1.25	11.5147
2.5	18.5577
5	27.2775
7.5	32.42

Data can be typed in directly, or pasted from a text file or spreadsheet. The first column is [S], the second is [I], and the third is v.

6. Enter initial guesses for the parameters from evaluation of the data (e.g. 1)
 - a (or p_1) = 35
 - b (or p_2) = 2
 - c (or p_3) = 0

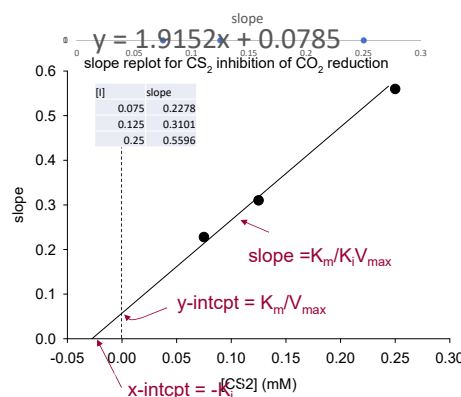
Iterate to convergence:

a (or p_1) =	43.28633202796	b (or p_2) =	2.164841440176	p =	5e-7
b (or p_2) =	3.148406172936	c (or p_3) =	0.399801101918	p =	0.000101
c (or p_3) =	0			p =	1.000000

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S. Ensign, enzyme kinetics

Analysis of inhibition data using nonlinear regression

- You must put in good initial guesses using statpages.info fitting.
- Get the values of $V_{\max}$ (parameter a) and K_m (parameter b) from the v vs S data where no I was present (43.3 and 3.15 for this example)
- Get the value of K_i from the slope replot:



slope = $K_m / K_i V_{\max}$
 $K_i = K_m / V_{\max} / \text{slope} = 3.15 / 43.3 / 1.9152 = 0.038$

1. Enter the number of data points: 28
2. Enter the number of independent variables: 2 (1 for single substrate M.)
3. Enter the number of parameters: 3 (deduce from form of M-M equation u)
4. Enter the formula for the function to be fitted:
 $Y = [a \cdot x_1 / (b \cdot (1 + x_2/c) + x_1)]$
5. Type (or paste) the [x,y] data:

0	0
0.25	2.9066
0.5	6.0368
0.75	8.8317
1.25	11.5147
2.5	18.5577
5	27.2775
7.5	32.42

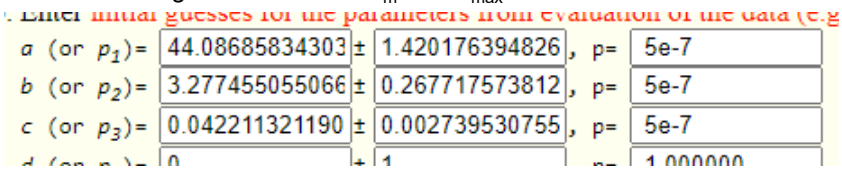
Data can be typed in directly, or pasted from a text file or spreadsheet. The first column is [S], the second is [I], and the third is v.

6. Enter initial guesses for the parameters from evaluation of the data (e.g. 1)
 - a (or p_1) = 43.3
 - b (or p_2) = 3.15
 - c (or p_3) = 0.038

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S. Ensign, enzyme kinetics

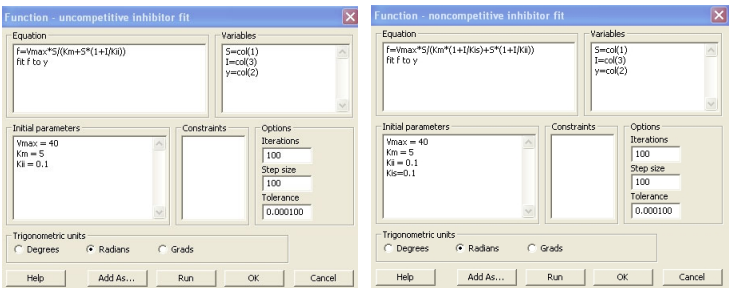
Analysis of two substrate kinetic data using nonlinear regression

Be sure to iterate until the values no longer change. Here is what you end up with. Compare these numbers to your initial guesses. The K_i value below is the one you report when you publish. Use the v vs S data without I to get the values of K_m and V_{max} .



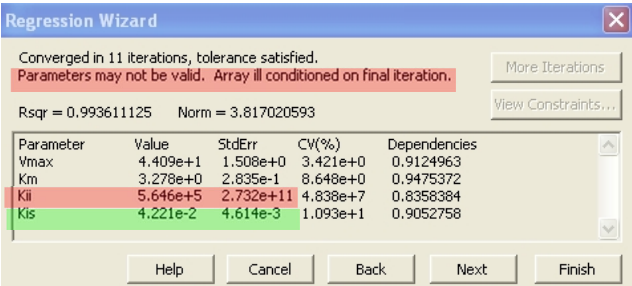
- Inputs:
 - $a = V_{max}$ 43.3 ±
 - $b = K_m$ 3.15 ±
 - $c = K_i$ ~0.038
- Numbers to report
 - $a = V_{max}$ 43.3 ± 2.16
 - $b = K_m$ 3.15 ± 0.40
 - $c = K_i$ 0.0422 ± 0.0027

Analysis of inhibition data using Sigmaplot 9.0:
equations for noncomp. and uncomp. inhibitors



Note: if you aren't sure what type of inhibition you have up front, use the noncompetitive inhibition fit, and examine the magnitudes of K_{is} and K_{ii} to give an indication of what type of inhibitor you are dealing with

Results of analyzing the “CS₂ inhibition of CODH” data
using the noncompetitive inhibitor fit



The magnitudes and standard errors of the inhibition constants tell you whether they contribute to the type of inhibition

Product inhibition patterns for ordered and random sequential bisubstrate reactions

Mechanism	Substrate		Inhibitor	Pattern?
	Variable	Fixed		
Ordered	A	B (NS or S)	Q	C
Ordered	B	A (NS)	Q	NC
Ordered	B	A (S)	Q	None
Ordered	A	B (NS)	P	NC
Ordered	A	B (S)	P	UC
Ordered	B	A (NS or S)	P	NC
Random	A	B (NS)	Q	C
Random	A	B (S)	Q	None
Random	A	B (NS)	P	C
Random	A	B (S)	P	None

Ordered applies to both substrate binding and product release in this example.
A= first substrate to bind for the ordered mechanism
B=second substrate to bind for the ordered mechanism
P= first product released for the ordered mechanism
Q=second product released for the ordered mechanism
NOTE: these assignments of A, B, P, and Q apply only to the ordered mechanism.
NS = nonsaturating concentration of substrate
S = saturating concentration of substrate of interest

