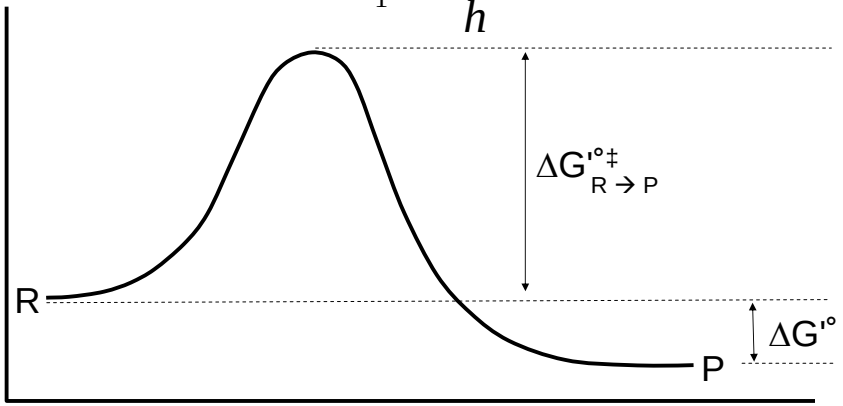
Enzymatic catalysis

- Reaction mechanism: The chemical pathway by which substrate(s) is/are converted to products. The mechanism includes all intermediates believed to be formed during the course of the reaction, and uses "electron pushing" to show how individual steps are believed to occur.
- Kinetics: The measurement of rates of chemical reactions and how changes in experimental conditions influence rates of reaction
- Kinetic mechanism: The description of the reaction mechanism in kinetic terms, by the assignment of rate constants to the sequence of steps believed to be involved in the chemical mechanism

Chemical kinetics: rate laws, reaction order, and rate constants

$$aA + bB \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} cC + dD \qquad K_{eq} = \frac{[C]^c[D]^d}{[A]^a[B]^b}$$

$$d[P]/dt = k_1[A]^n\,[B]^m \qquad k_1 = \frac{KT}{h} e^{-\Delta G^{\ddagger}/RT}$$



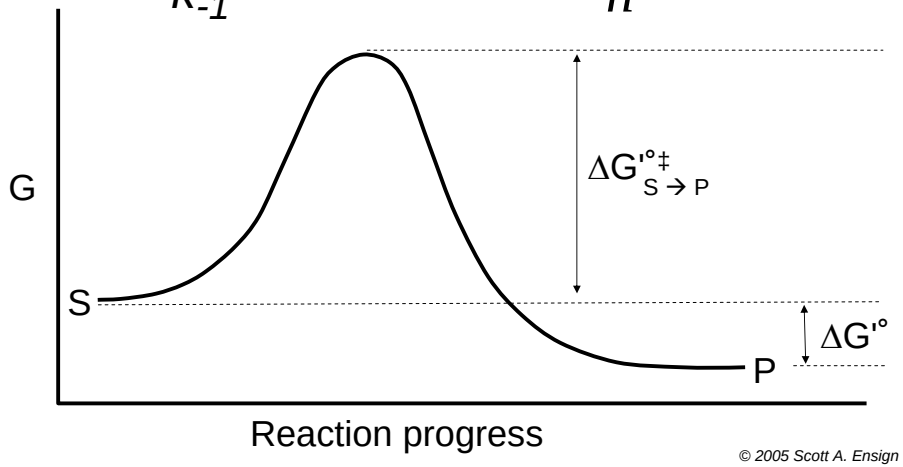
Chemical kinetics: elementary steps and kinetic mechanism

- The rate law for an overall reaction **cannot** be deduced from the stoichiometry of reactants and products in the balanced chemical equation- it must be **experimentally determined**.
- The rate law for an elementary step in a kinetic mechanism **can** be deduced from the stoichiometry of reactant(s) and product(s) in the elementary step

Enzyme Kinetics: The single substrate case

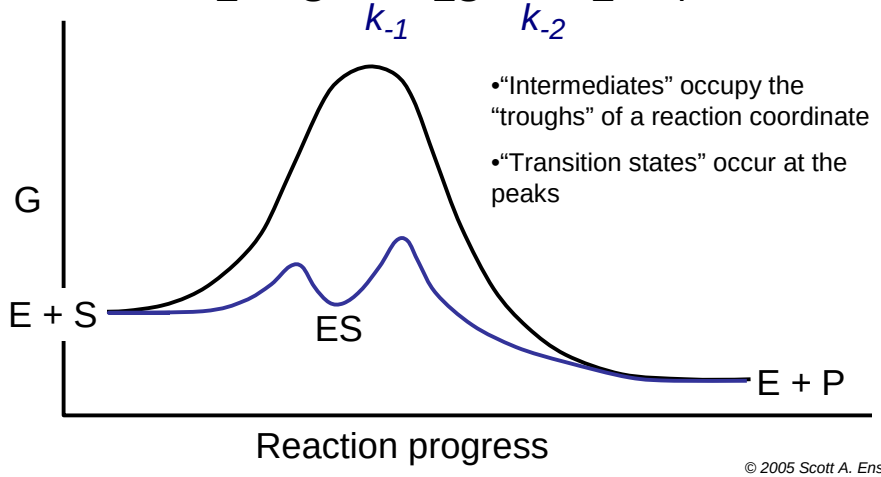
Reaction coordinate for S → P

$$S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P \qquad k_1 = \frac{KT}{h} e^{-\Delta G^{\ddagger}/RT}$$



Reaction coordinate for S → P following the kinetic mechanism:

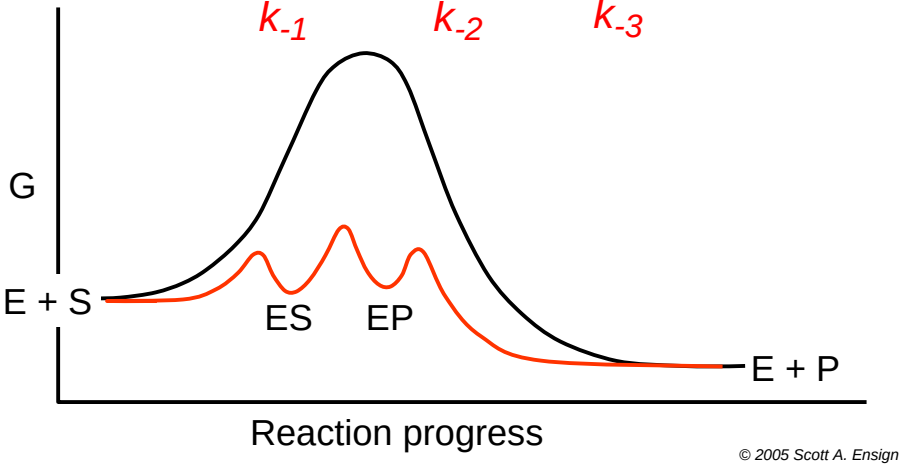
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$



- "Intermediates" occupy the "troughs" of a reaction coordinate
- "Transition states" occur at the peaks

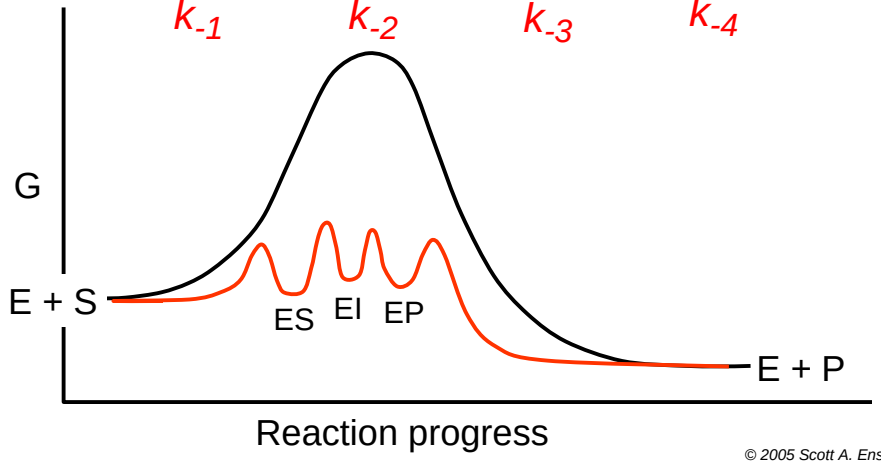
Reaction coordinate for S → P following the kinetic mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$



Reaction coordinate for S → P following the kinetic mechanism:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P$$



Single substrate model: Steady state assumption

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

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

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

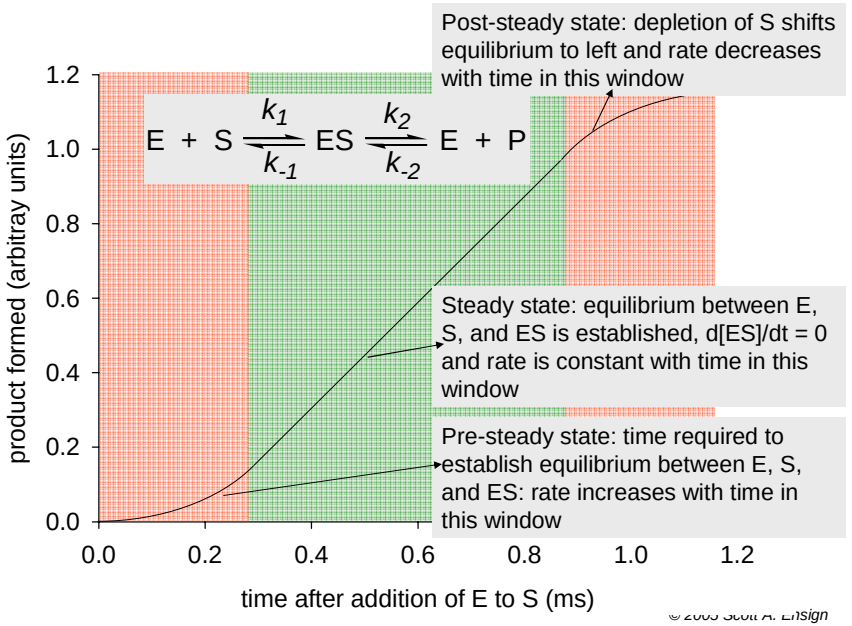
- Equilibrium of first step is rapidly established

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

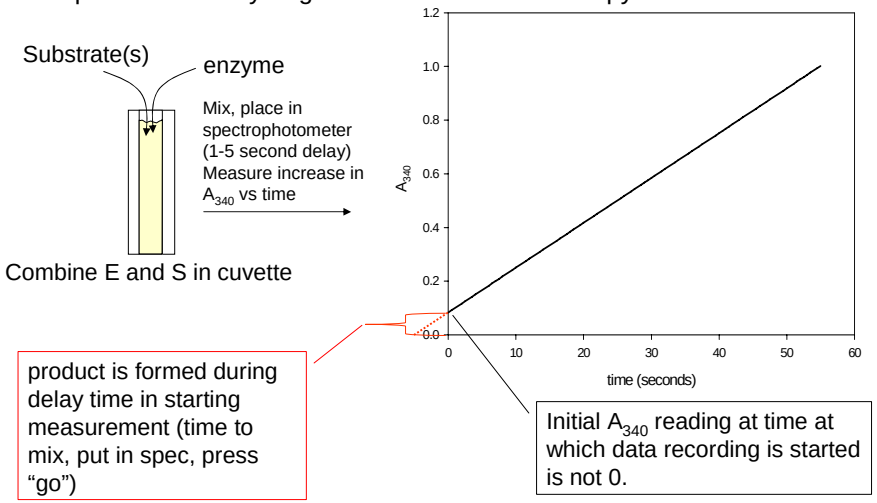
- [ES] remains constant during period in which enzyme activity is being measured

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

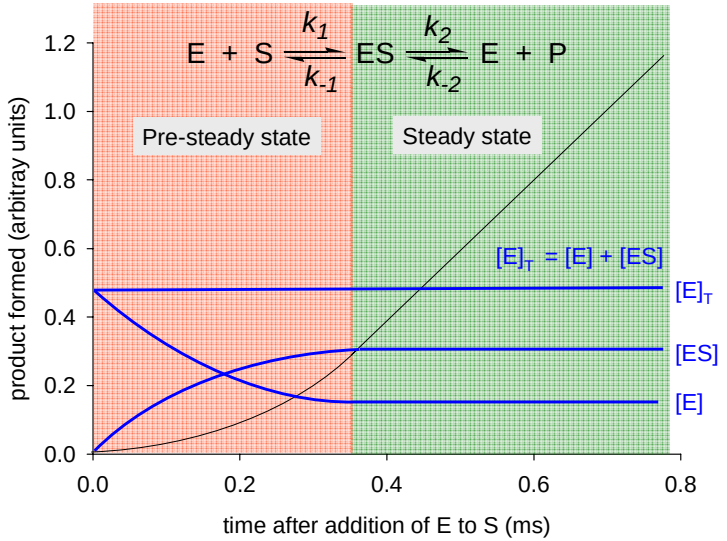
Steady vs. pre-steady state



- 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^+ → pyruvate + NADH



[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

Derivation of a rate law for the steady-state

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

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

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

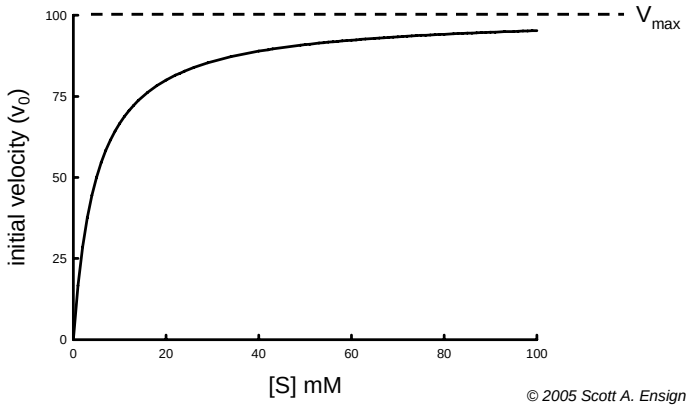
Algebraic manipulation of these three expressions allows the rate law to be determined

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

Michaelis-Menten Equation for the single substrate case

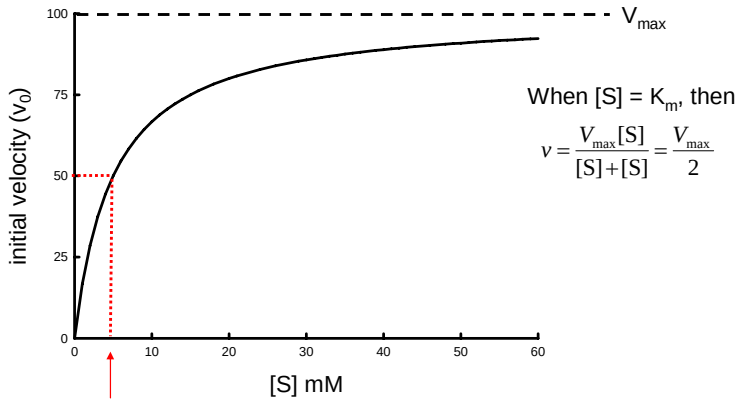
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad K_m = \frac{k_{-1} + k_2}{k_1}$$



Michaelis-Menten Equation for the single substrate case

$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$ $\quad v = \frac{V_{max}[S]}{K_m + [S]}$ $\quad K_m = \frac{k_{-1} + k_2}{k_1}$



When [S] = K_m, then

$$v = \frac{V_{max}[S]}{[S] + [S]} = \frac{V_{max}}{2}$$

K_m = concentration of substrate that results in a rate equal to ½ V_{max}

K_D vs. K_m (single substrate case)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad K_m = \frac{k_{-1} + k_2}{k_1}$$

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

K_D vs. K_m (single substrate case)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad K_d = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad K_m = \frac{k_{-1} + k_2}{k_1}$$

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

K_m approximates the affinity of an enzyme for it's substrate only when $k_{-1} >> k_2$

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

- K_m: high nM/low μM → high mM
- Physiological significance?
  - Available [S]
  - Need to regulate substrate conversion rate?
- V_{max} units: μmol/min; tells nothing about $[E]_T$ in assay (can report as μmol/min/mg)
- $V_{max} / [E]_T = k_{cat}$

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})
- $k_{cat} = V_{max} / [E]_T$ (with proper dimensional analysis)
- Calculate k_{cat} for a homodimeric (α_2) enzyme of Mr = 126,000 exhibiting V_{max}=10,000 U when 1 mg enzyme is assayed

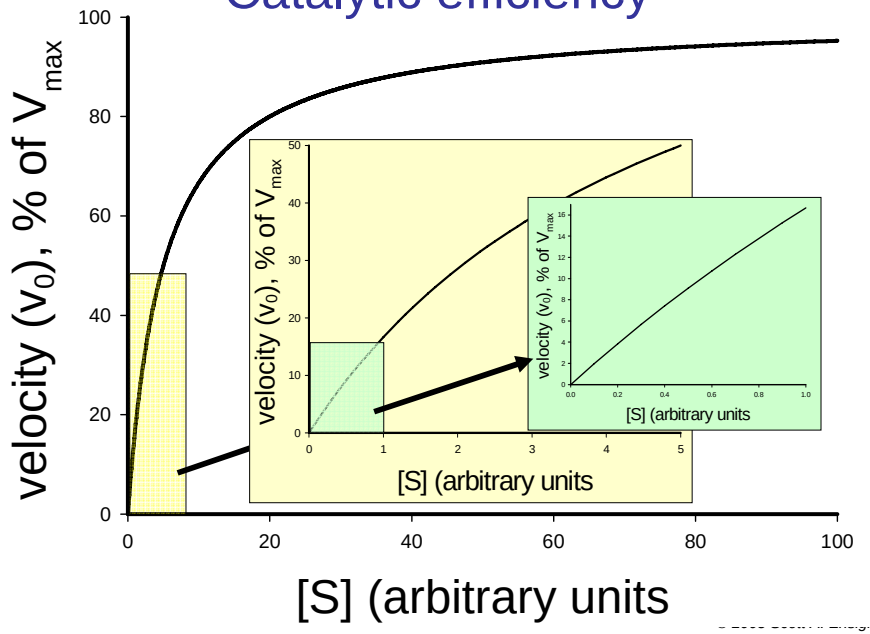
k_{cat}: turnover number

- 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})
- For the simple kinetic mechanism

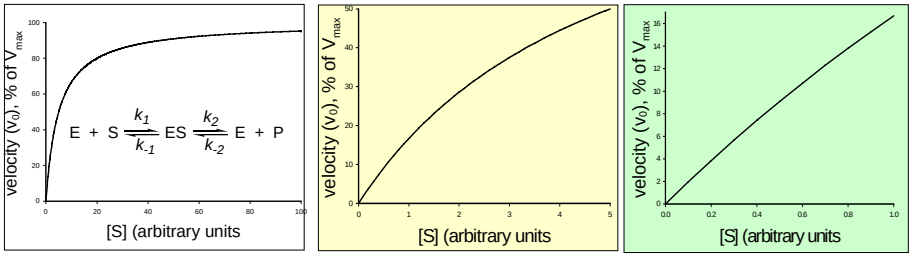
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

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

Catalytic efficiency



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



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

$[E]_T = [E] + [ES]$; when [S] << K_m, $[E] >> [ES]$ and $[E]_T \approx [E]$

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

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?

$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$ $\quad v = \frac{V_{max}[S]}{K_m + [S]}$ $\quad K_m = \frac{k_{-1} + k_2}{k_1}$

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

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 $k_2 >> k_{-1}$, reducing above expression to

$\frac{k_{cat}}{K_m} \approx k_1$ 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?

$$E + S \xrightarrow{k_1} ES \xrightarrow{fast} E + P \qquad v = k_1[E][S]$$

- 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)

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

$$v = \frac{\frac{V^f_{max}[S]}{K^S_m} - \frac{V^r_{max}[P]}{K^P_m}}{1 + \frac{[S]}{K^S_m} + \frac{[P]}{K^P_m}}$$

$$K^S_m = \frac{k_{-1} + k_2}{k_1} \qquad K^P_m = \frac{k_{-1} + k_2}{k_{-2}}$$

$$V^f_{max} = [E]_T \cdot k_2 \qquad V^r_{max} = [E]_T \cdot k_{-1}$$

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

The Haldane Relationship

$$K_{eq} = \frac{[P]}{[S]} = \frac{V^f_{max} K^P_m}{V^r_{max} K^S_m}$$

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

Models with two or more intermediates

One intermediate case: $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$

Two intermediate case: $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$

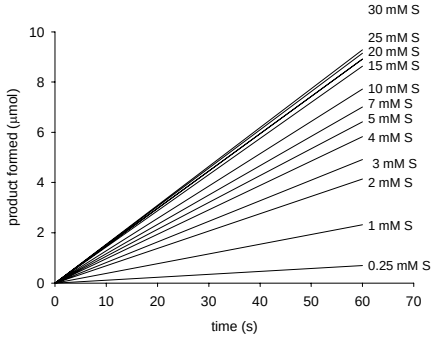
Three intermediate case: $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P$

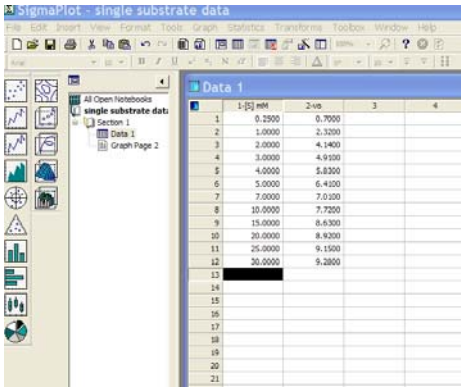
- The overall Michaelis-Menten equations are the same as for the one intermediate model
- Kinetic parameters ($V^f_{\max}, K^S_m, V^r_{\max}, K^P_m$) are defined in terms of 6 or 8 kinetic constants for the examples shown
- The RDS for product determination defines k_{cat}
- Steady state kinetics cannot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

Black box model for steady state enzyme kinetics

Analysis of kinetic data: single substrate case

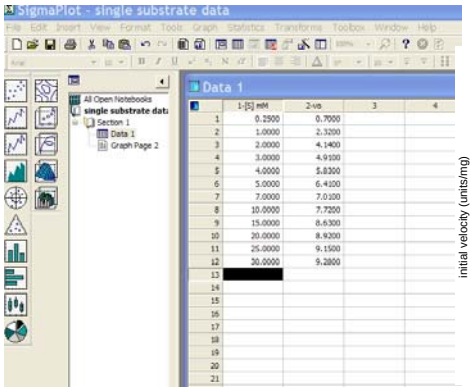
- Conduct enzyme assays with constant [E] and varying [S]
- Choose a wide range of substrate concentrations both below, around, and above estimated K_m
- For the example shown below, 1 mg of enzyme was assayed with substrate concentrations ranging from 0.25 mM to 30 mM
- Determine velocities from plots of product formed vs. time for each [S]

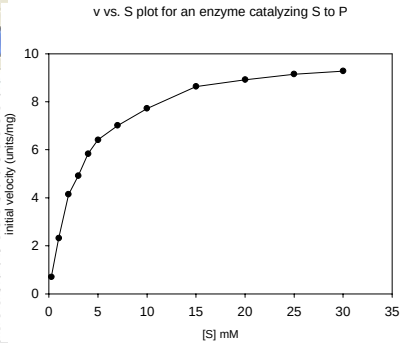




Analysis of kinetic data: single substrate case

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





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

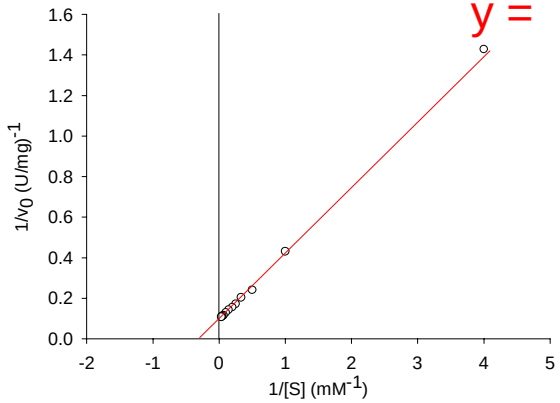
Methods used to determine K_m and V_{max}

- Estimate from v vs. S plot (qualitative at best)
- Algebraic rearrangement of Michaelis-Menten equation; replot data and estimate parameters from replots
- Nonlinear regression of v vs. S data- (beyond scope of this class)

Algebraic rearrangement of the Michaelis-Menten Equation

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

y = m x + b



- Lineweaver-Burke (double reciprocal) plot
- y-int = $1/V_{max}$
- x-int = $-1/K_m$
- slope = K_m/V_{max}

Expanding beyond S → P

•One substrate, one product	S → P
•One substrate, two products	S → P_1 + P_2
•Two substrates, one product	A + B → C
•Two substrates, two products	A + B → C + D
•Three substrates, one product	A + B + C → D

Most biochemical reactions involve two substrates

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

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

$$E + S_1 + S_2 \rightleftharpoons E + P_1 + P_2$$

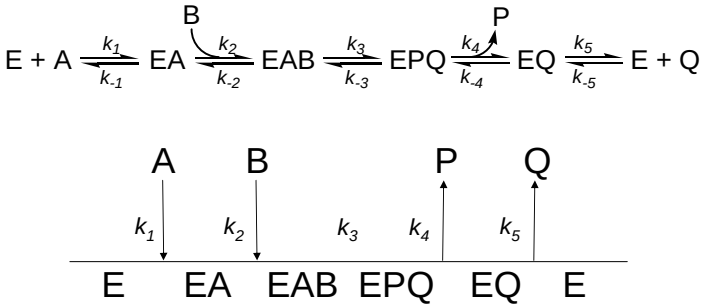
$$E + A + B \rightleftharpoons E + P + Q$$

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

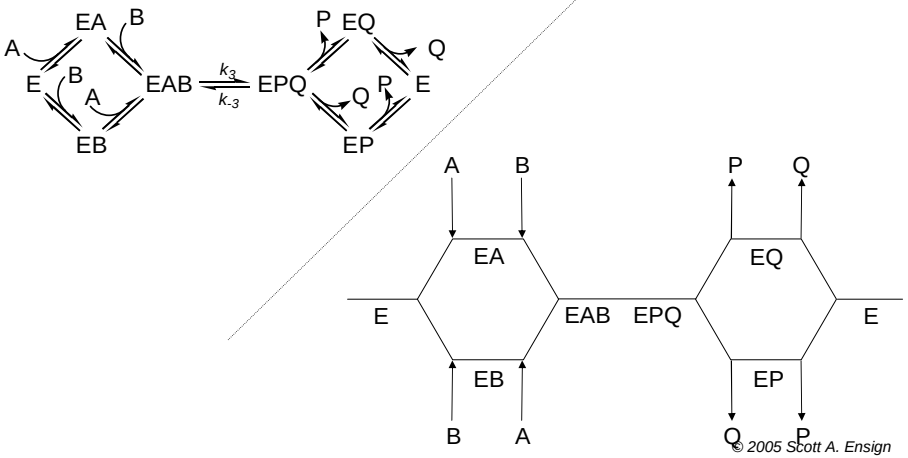
Example kinetic mechanisms for sequential reactions involving 2 substrates and 2 products

- Ordered with respect to substrate addition and product release:



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

- random with respect to substrate addition and product release:

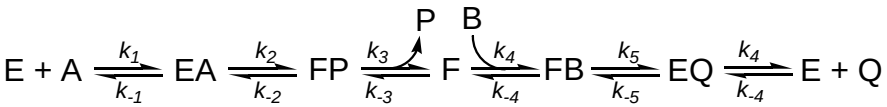


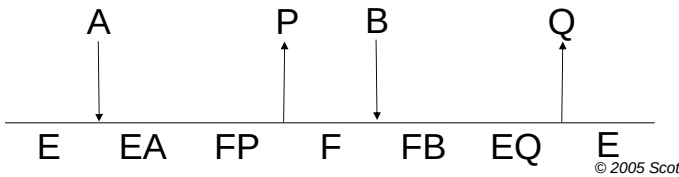
Other possible kinetic mechanisms for sequential reactions involving 2 substrates and 2 products

- 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

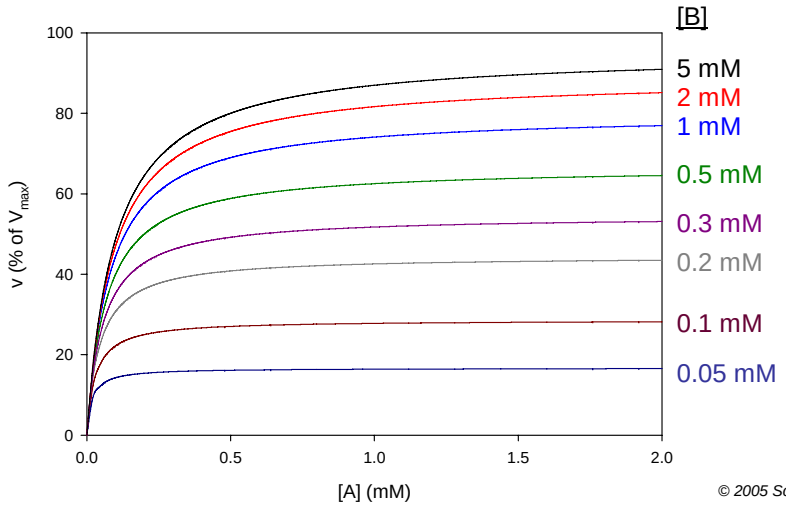
- "F" designates a modified form of the enzyme (e.g., enzyme with a covalent bond to a fragment of the first substrate)





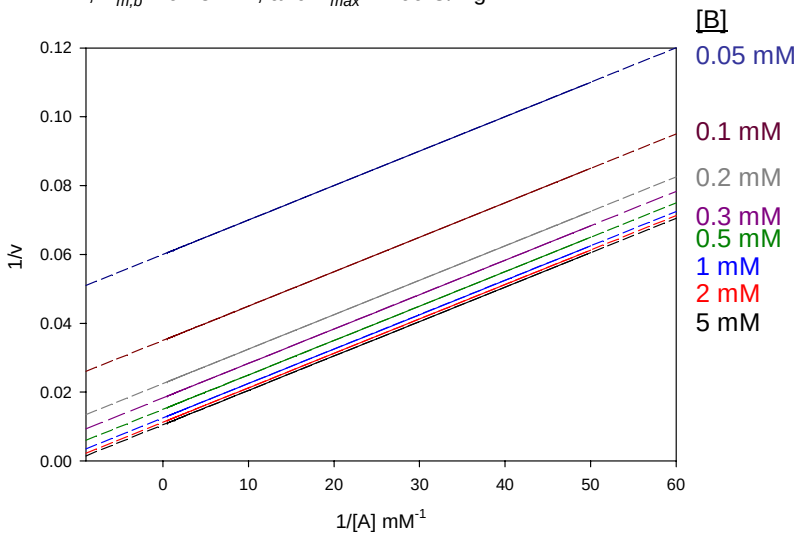
V vs. S plots for a 2 substrate ping-pong kinetic mechanism

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.



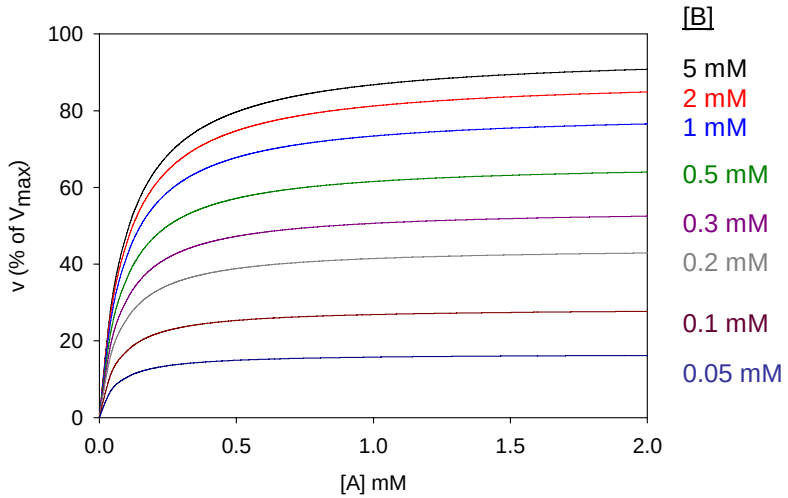
Reciprocal plots for a 2 substrate ping-pong kinetic mechanism

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.



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, $K_{i,a}$ = 0.05 mM and V_{max} = 100 U/mg.



Reciprocal plots for a 2 substrate ordered sequential kinetic mechanism

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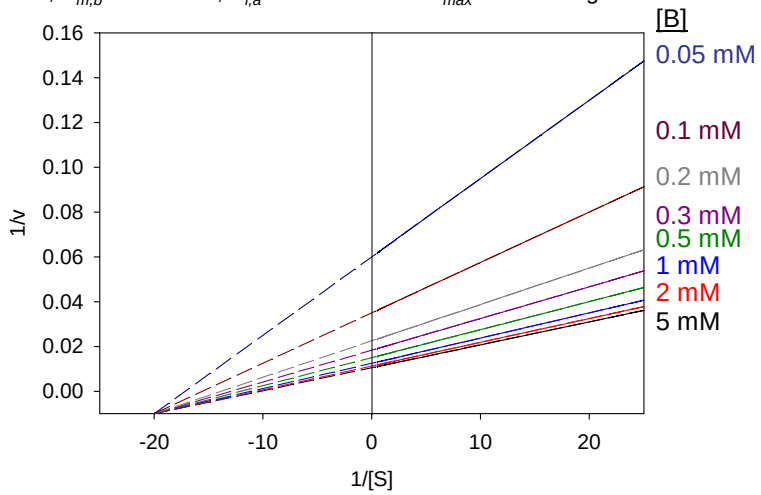


Allosteric enzymes

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

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

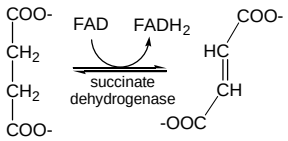
Two broad classes of “reversible” inhibitors:

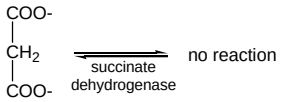
- Rapid equilibrium
  - Competitive
  - Noncompetitive
  - Uncompetitive
- Slow-(tight) binding

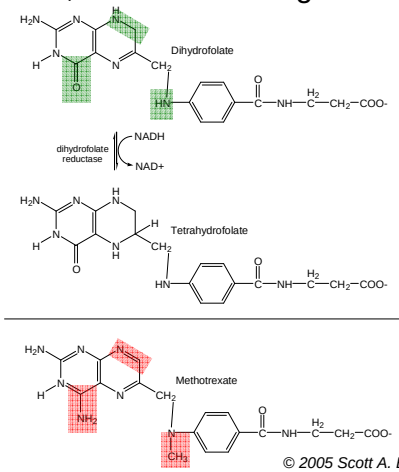
Irreversible inhibitors: inactivators (do not dissociate), include “mechanism-based inactivators (suicide substrates)

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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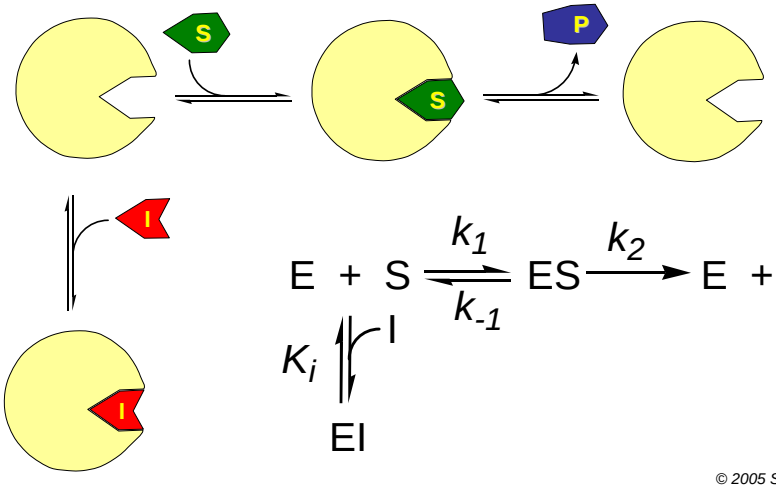


Inhibition constant K_I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$K_i \;\; I$$

$$EI$$

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

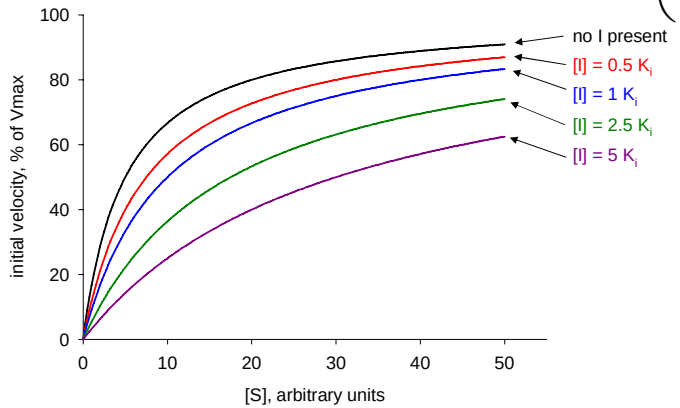
$$EI \rightleftharpoons E + I \qquad K_i = \frac{[E][I]}{[EI]}$$

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

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



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

Without inhibitor:

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

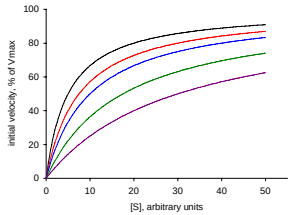
y = m x + b

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 = m x + b

Lineweaver-Burke plots in the presence of a competitive inhibitor

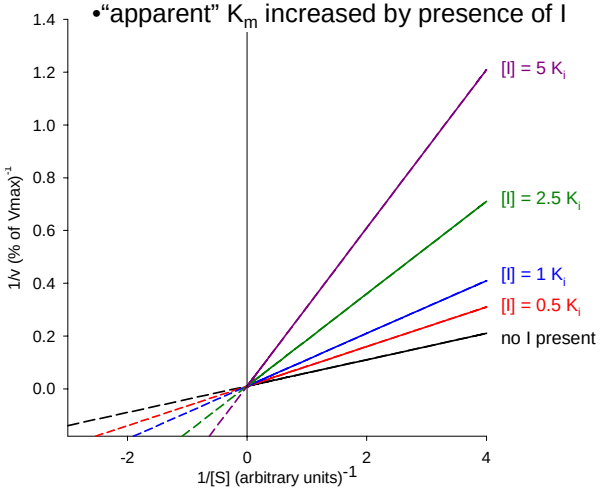


$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$K_i \uparrow\downarrow I$$

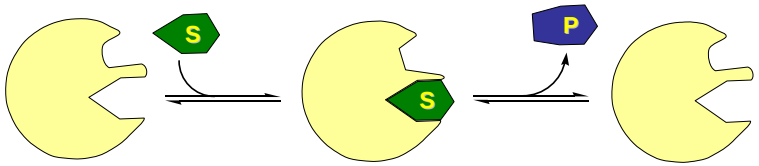
EI

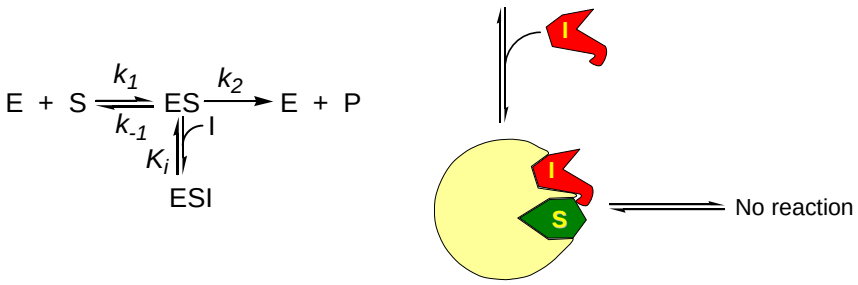
- V_{max} unaffected by presence of I
- "apparent" K_m increased by presence of I



Uncompetitive Inhibition

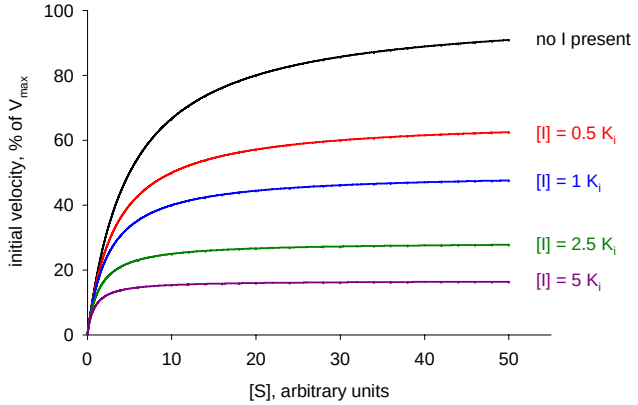
Uncompetitive inhibitor: a compound that inhibits activity by binding the ES complex but not the free enzyme E.





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

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



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

Without inhibitor:

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

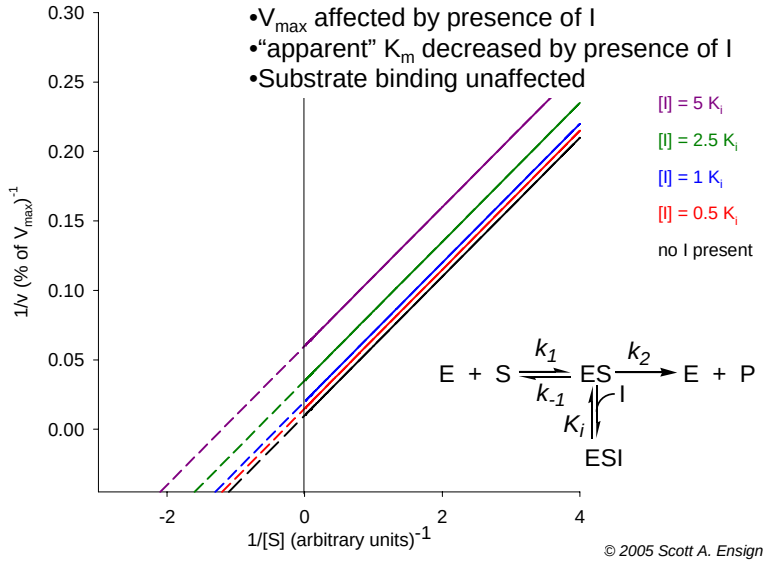
y = m x + b

With inhibitor:

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

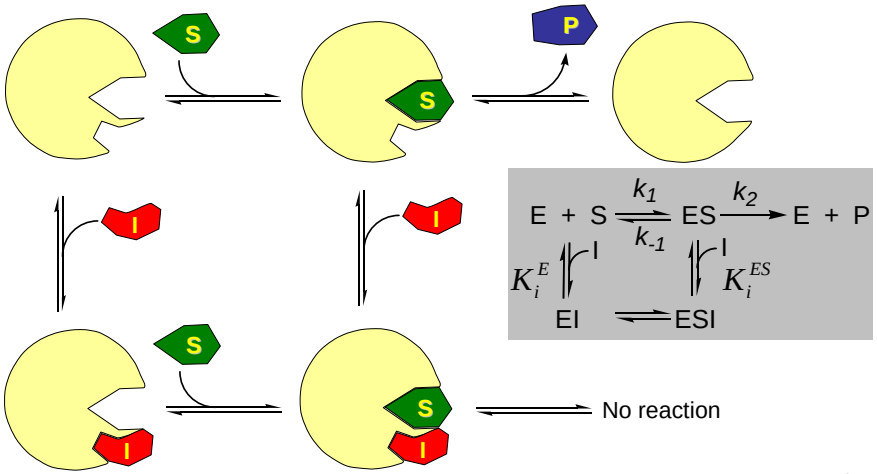
y = m x + b

Lineweaver-Burke plots in the presence of an uncompetitive inhibitor



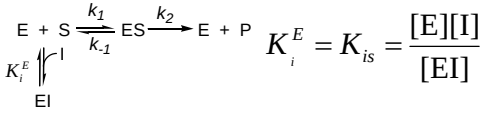
Noncompetitive Inhibition

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



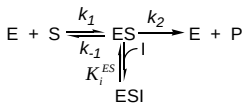
Inhibition constant nomenclature

•Competitive inhibition



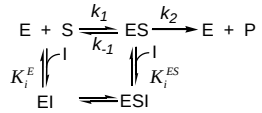
$$K_i^E = K_{is} = \frac{[E][I]}{[EI]}$$

•Uncompetitive inhibition



$$K_i^{ES} = K_{ii} = \frac{[ES][I]}{[ESI]}$$

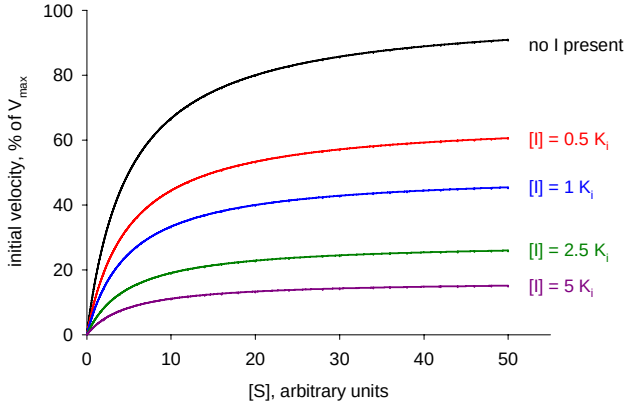
•Noncompetitive inhibition



$$K_i^E = \frac{[E][I]}{[EI]} \qquad K_i^{ES} = \frac{[ES][I]}{[ESI]}$$

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

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



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

Without inhibitor:

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

y = m x + 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 = m x + b

Lineweaver-Burke plots in the presence of a noncompetitive inhibitor