Lecture 18: Applications of K_M and k_{CAT}, Temperature effects, Inhibitors.

Goals:

- Use pH effects to support mechanism
- Predict effects of temperature on enzyme kinetics
- Distinguish between types of inhibitors (covalent, competitive, mixed/allosteric)
- Key properties of suicide & competitive inhibitors.
- Effect of competitive inhibitors on steady-state kinetics, measurement of K_I.

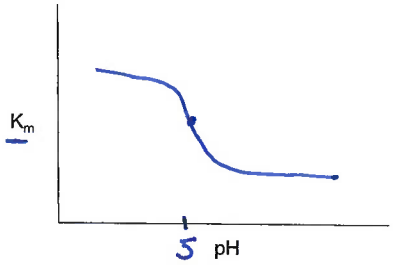
Applications of K_M and k_{CAT}:

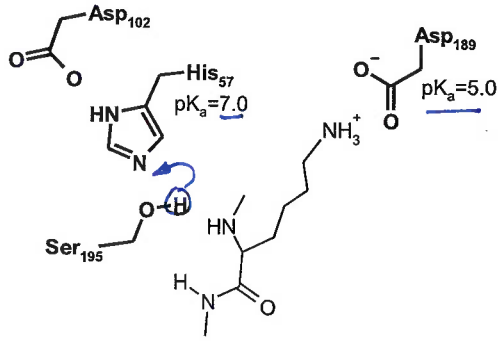
Mechanistic Information - about particular enzyme-substrate pairs.

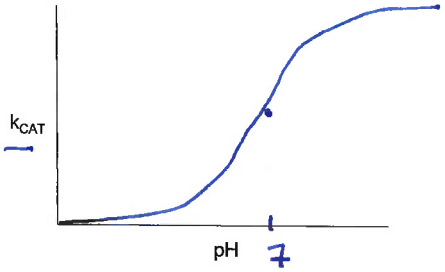
1. The active site region of Trypsin is shown below.

i) Sketch the k_{CAT} as a function of pH.

ii) Sketch the K_M as a function of pH.



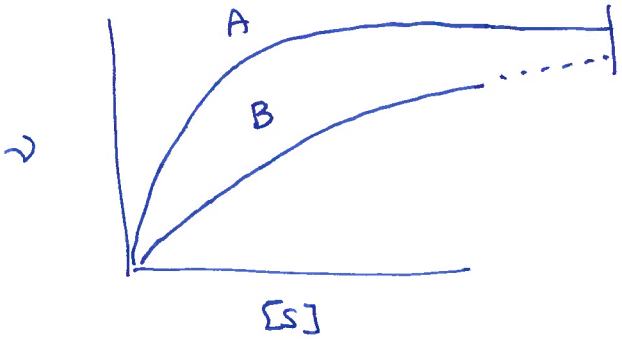


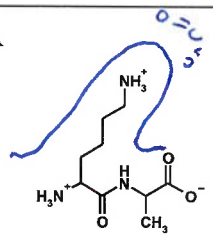
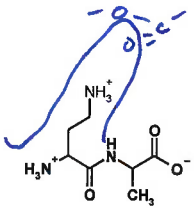


2. The two substrates shown on the right were presented to trypsin. Their structures and measured K_M and k_{CAT} values are given.

i) Explain the differences in K_M values.

ii) Why are the k_{CAT} values the same?



Substrate	K_M	k_{CAT}
A 	10 μM	1000 s^{-1}
B 	100 μM	1000 s^{-1}

better lower

No distortion of triad

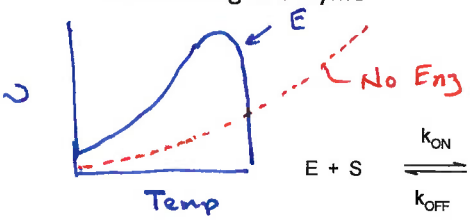
Effect of Temperature:

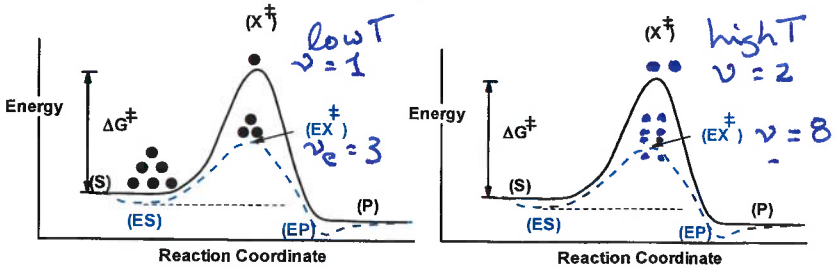
Two competing factors:

1. Increase in population of [EX]

$$\frac{n_X}{n_S} = e^{-\Delta G/RT}$$

2. Unfolding of enzyme





$$E + S \underset{k_{OFF}}{\overset{k_{ON}}{\rightleftharpoons}} (ES) \rightleftharpoons (EX) \rightleftharpoons (EP) \underset{k_{ON}}{\overset{k_{OFF}}{\rightleftharpoons}} E + P$$

(k^f_{CAT} / k^r_{CAT})

Inhibitors:

Studies on Inhibitors are useful for:

1. Mechanistic studies to learn about how enzymes interact with their substrates.
2. Understanding the role of inhibitors in enzyme regulation.
3. Drugs if they inhibit aberrant biochemical reactions:
   - penicillin, ampicillin, etc. interfere with the synthesis of bacterial cell walls
4. Understanding the role of biological toxins.
   - Amino acid analogs - useful herbicides (i.e. roundup)
   - Insecticides - chemicals targeted for insect nervous system.

Types of Inhibitors:

1. Covalent/Suicide – inhibitor *covalently* modifies enzyme, usually in active site.
2. Competitive – inhibitor blocks substrate, *reversibly*.
3. Mixed type (allosteric) – inhibitor causes allosteric change, *reversibly*.

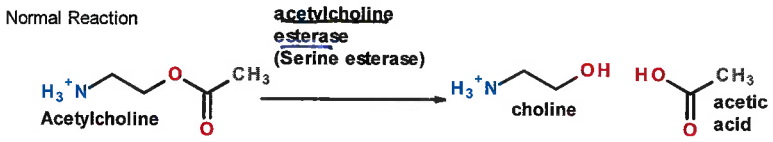
1. Suicide Inhibitors:

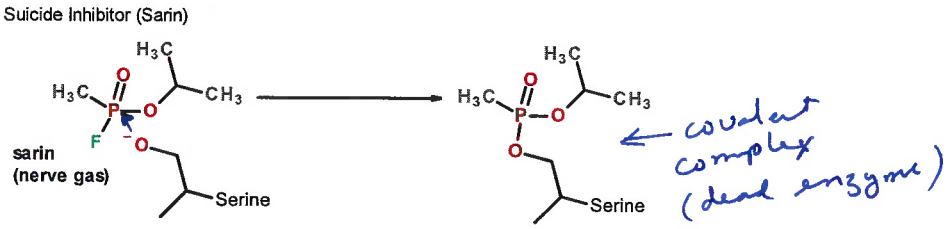
Inhibitor binds in the active site. This type of inhibitor binds *irreversibly*.

- Transition state is a reactive compound that forms a covalent bond with the enzyme, irreversibly inactivating it.

Example: Sarin nerve gas

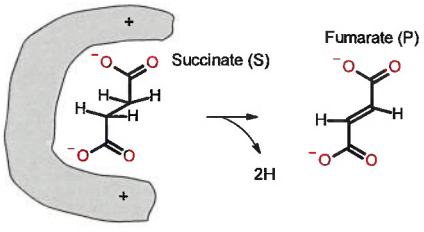
- Acetylcholine esterase is required to breakdown the neural transmitter acetylcholine in neuro-muscular junctions so that the muscle will relax.
- Esterase has an active site Serine that is activated in a similar manner as serine proteases.
- Sarin modifies the active site serine in the serine esterase acetylcholine esterase by forming a stable covalent bond with the serine that cannot be easily hydrolyzed.
- Inhibition of acetylcholine esterase results in suffocation since the diaphragm muscles no longer function properly.



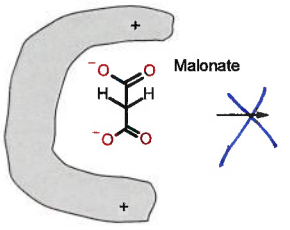


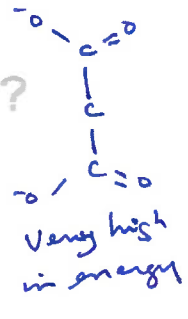
2. Competitive Inhibition:

- Succinate dehydrogenase converts succinate to fumarate by removing two hydrogens (oxidation).
- Malonate is a competitive inhibitor of succinate.



Can malonate be converted to a product using the same reaction (H removal)?





i) binds in active site (because of similarity to substrate)

ii) cannot undergo reaction "doesn't make products"

Goals: (i) Determine how well inhibitors bind $K_I = K_D$
(ii) Allosteric inhibitors / mixed type inhibitors

Complete the following statements (i-iv):

i. A competitive inhibitor binds to the Active site

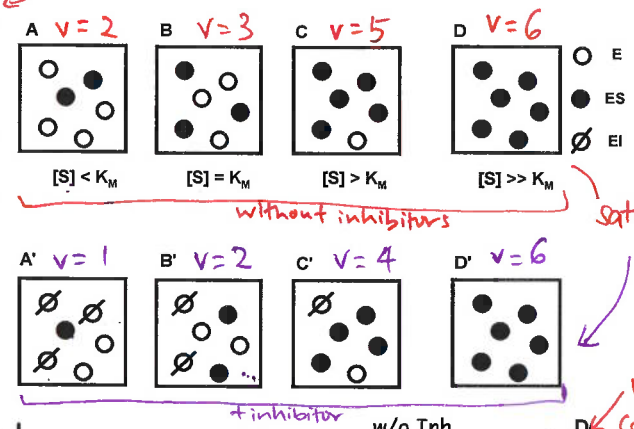
ii. A competitive inhibitor and the substrate are similar in structure.

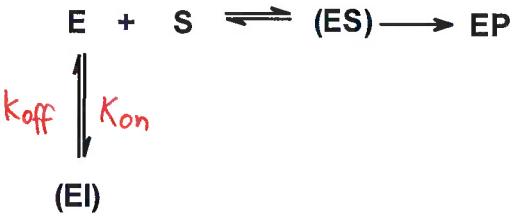
iii. A competitive inhibitor cannot undergo catalysis / a chemical reaction.

iv. A competitive inhibitor can only bind to the enzyme when the substrate is absent.

can use the effect of inhibition on kinetics to get K_I

Effect of Competitive Inhibitor on Kinetics: A competitive inhibitor reduces the amount of [E] by the formation of [EI] complex. The inhibitor cannot affect the [ES] complex after it has formed since the inhibitor can no longer bind. *How will high concentrations of substrate affect the inhibition?*

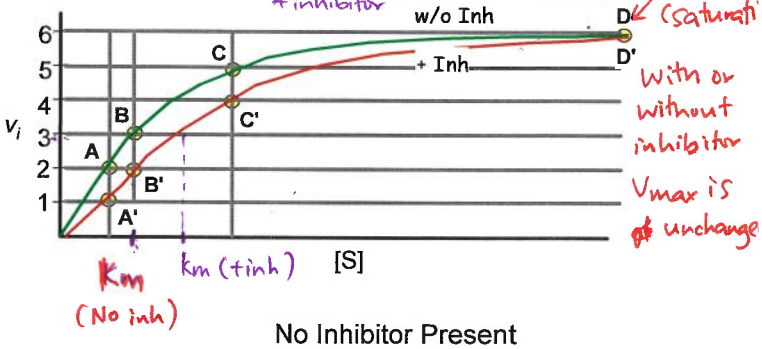




$(EI) \rightleftharpoons (E) + (I)$

$K_I = \frac{(E)(I)}{(EI)} = K_D$

to remind us that it is an inhibitor.



There are two consequences of a competitive inhibitor binding on the kinetics of the enzyme:

1. **V_{MAX} is unchanged**: At high levels of substrate all of the inhibitor can be displaced by substrate, and $[ES]=E_{TOTAL}$, $V_{MAX} = k_{CAT}[ES] = k_{CAT}[E_{TOT}]$.
2. **The *observed* K_M is increased:** It requires more substrate to reach 1/2 maximal velocity because some of the enzyme is complexed with inhibitor.

The change in K_M can be used to determine how well the inhibitor binds to the free enzyme→

No Inhibitor Present

$$v = V_{MAX}\frac{[S]}{K_M + [S]}$$ ← No inhibitor

$$\frac{1}{v} = \frac{K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$$ ← double recip

Inhibitor Present

$$\alpha = 1 + \frac{[I]}{K_I}$$

change slope

$$v = V_{MAX}\frac{[S]}{\alpha K_M + [S]}$$

$$\frac{1}{v} = \frac{\alpha K_M}{V_{MAX}}\frac{1}{[S]} + \frac{1}{V_{MAX}}$$

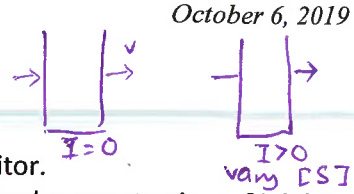
K_m is affected by the competitive inhibitor

α = ratio of slopes

(+I/no inh)

$K_I = [I]/(\alpha-1)$

α : degree of inhibition.
= ratio of slopes.



Measuring Inhibitor-Enzyme Affinity (K_I)

A. Data Collection

i) Measure initial velocity for different [S], in the *absence* of inhibitor.

ii) Measure initial velocity for different [S], in the *presence* of a fixed concentration of inhibitor (i.e. only [S] is varied, not [I]). Multiple inhibitor concentrations can be used.

B. Analysis:

1. Linearization of Data using Double-Reciprocal Plot

i) Both data sets are plotted on a double reciprocal plot.

ii) Ratio of the slopes gives α (degree of inhibition).

iii) $K_I = [I]/(\alpha-1)$.

2. Directly Fitting to Experimental Data (best method):

There are three parameters: V_{MAX}, K_M, α. The data is directly fit to theoretical equations.

i) Predict v versus [S] for I=0 [$v_{Pred}=V_{MAX}[S]/(K_M+[S])$]

ii) Predict v versus [S] for I>0 [$v_{Pred}=V_{MAX}[S]/(\alpha K_M+[S])$]

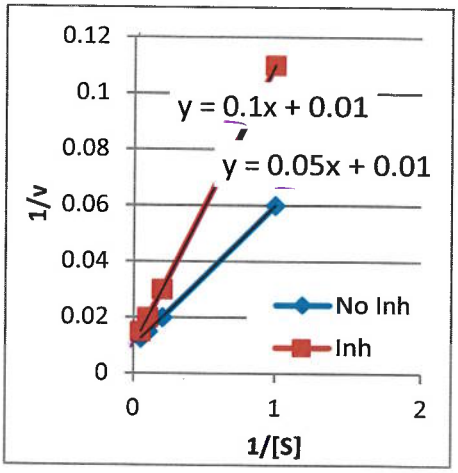
iii) Sum differences between actual and predicted velocities, $\chi^2=\sum|(v_{obs}-v_{pred})|_{I=0}+|(v_{obs}-v_{pred})|_{I>0}$

iv) Use Solver to minimize χ^2, use α to obtain K_I.

example. → **A:** Experimental Data

[S] mM	v_i [I]=0	v_i I=10μM	1/[S]	1/v [I]=0	1/v I=10μM
1	16.7	9.1	1.0	0.060	0.110
5	50.0	33.3	0.2	0.020	0.030
10	66.7	50.0	0.1	0.015	0.020

Nonh tính



B. Double Reciprocal Plots

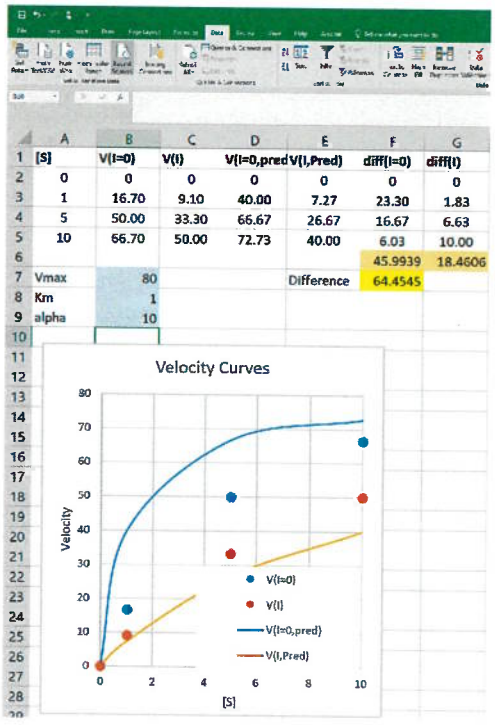
$\alpha = \frac{0.1}{0.05} = 2$

$K_I = \frac{[I]}{(\alpha-1)} = \frac{10\mu M}{2-1} = 10\mu M$

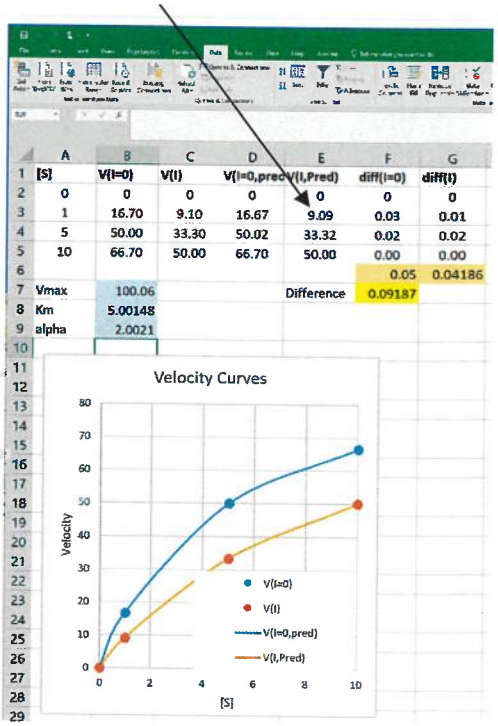
C. Fitted Parameters:

Vmax	100.06
Km	5.00
α	2.00

=B7*A3/(B9*B8+A3) = $V_{MAX}[S]/(\alpha K_M+[S])$



	A	B	C	D	E	F	G
1	[S]	V(I=0)	V(I)	V(I=0,pred)	V(I,Pred)	diff(I=0)	diff(I)
2	0	0	0	0	0	0	0
3	1	16.70	9.10	40.00	7.27	23.30	1.83
4	5	50.00	33.30	66.67	26.67	16.67	6.63
5	10	66.70	50.00	72.73	40.00	6.03	10.00
6						45.9939	18.4606
7	Vmax	80			Difference	64.4545	
8	Km	1					
9	alpha	10					



	A	B	C	D	E	F	G
1	[S]	V(I=0)	V(I)	V(I=0,pred)	V(I,Pred)	diff(I=0)	diff(I)
2	0	0	0	0	0	0	0
3	1	16.70	9.10	16.67	9.09	0.03	0.01
4	5	50.00	33.30	50.02	33.32	0.02	0.02
5	10	66.70	50.00	66.70	50.00	0.00	0.00
6						0.05	0.04186
7	Vmax	100.06			Difference	0.09187	
8	Km	5.00148					
9	alpha	2.0021					