Lecture 17: Steady-State Enzyme Kinetics

Goals:

1. Understand steady-state approximation.
2. Measure parameter (K_M) related to substrate binding.
3. Measure parameter (k_{CAT}) related to catalytic efficiency.

$M + L \rightleftharpoons ML$

$E + S \rightleftharpoons (ES) \rightleftharpoons (EX^{\ddagger}) \rightleftharpoons (EP) \rightleftharpoons (E) + P$

$K_M \approx K_D$ $\quad k_{CAT}$

$v \propto [EX^{\ddagger}]$ $\quad v = \frac{dP}{dt}$

$v \propto (ES)$

$v = k_{CAT}[ES]$

Simple Enzyme Kinetic Scheme.

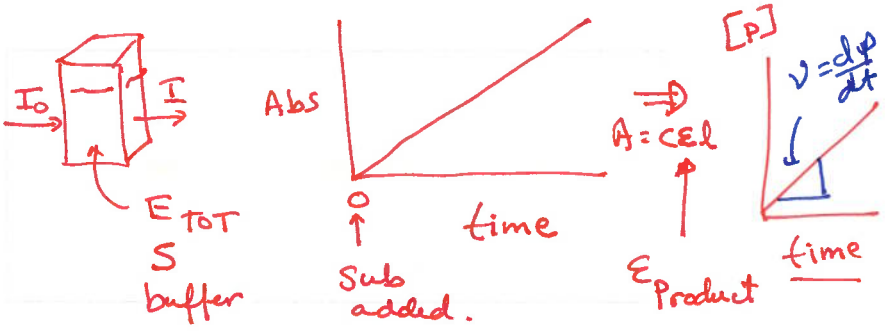
- k_{ON} (also called k_1) is the forward rate constant for substrate binding
- k_{OFF} (also called k_{-1}) is the reverse rate constant for substrate binding
- k_{CAT} (also called k_2) is the catalytic rate constant (containing terms related to the stabilization of the transition state). It is also called the **"turnover number"**, since it is the rate at which one molecule of [ES] converts to product. This will depend on particular substrate-enzyme combinations and provides information on the mechanism.
- The (ES) complex is also called the "Michaelis complex".

Enzyme Kinetics

1. Product Formation:

The rate, or *velocity*, of the enzyme catalyzed reaction can be determined by measuring the increase in the amount of product formed Δ[P] during a given period of time Δt:

$$v = \frac{\Delta[P]}{\Delta t} = \frac{d[P]}{dt}$$

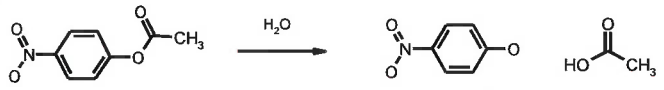


2. Experimental Measurement of Enzyme Kinetics:

i) Use chromophoric substrates

ii) Measure v = dA/dt

iii) Vary [S]



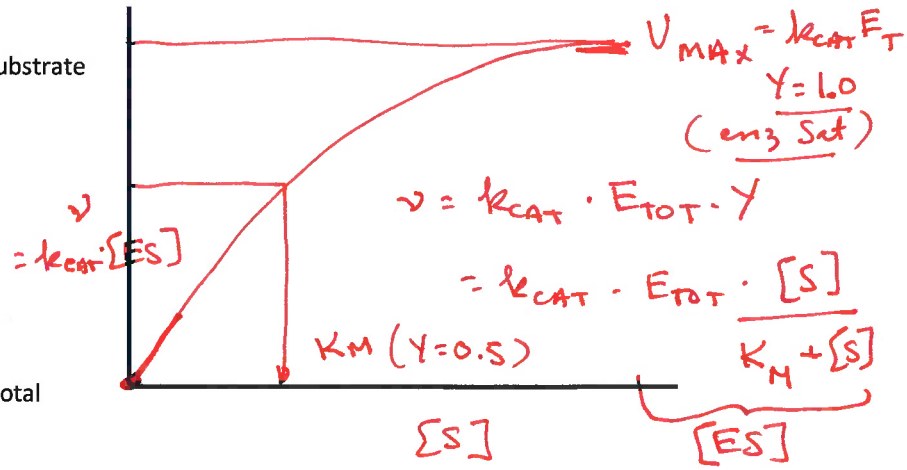
3. Empirical Derivation of Rate Law:

Assume that the rate = $k_{CAT}[ES]$

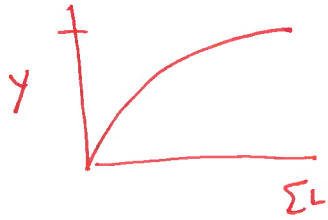
i) How does the rate depend on the substrate concentration, [S]?

low [S]: linear

high [S]: Saturated



ii) How does the rate depend on the total amount of enzyme, $[E_{TOT}]$?



$Y = \frac{[L]}{K_D + [L]}$ $\quad Y = \frac{[ML]}{[M_{TOT}]} = \frac{[ES]}{[E_{TOT}]}$

$Y \cdot E_{TOT} = ES$

4. Analytical Derivation of Rate Law - Steady-State Assumption

The goal is to relate the kinetic measurements to *readily measurable* experimental parameters:

i) The total amount of enzyme: E_{Total} = [E] + [ES]

ii) the concentration of substrate: [S]

iii) the measured velocity ($v = k_{CAT}$ [ES])

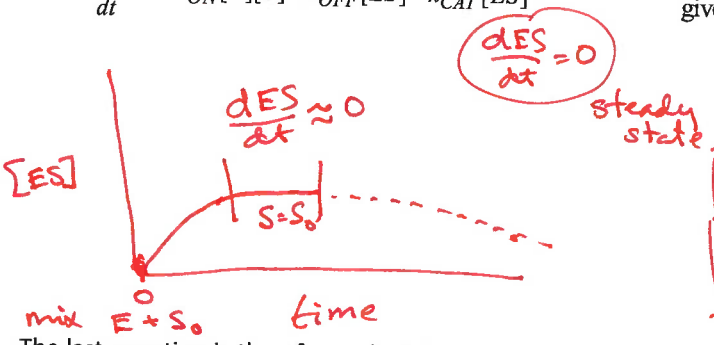
The simplest reaction scheme is:

$$E + S \underset{k_{OFF}}{\overset{k_{ON}}{\leftrightarrow}} ES \xrightarrow{k_{CAT}} EP \rightarrow E + P$$

The experimentally obtained velocity of the reaction is: $v = d[P]/dt = k_{CAT}[ES]$

The differential equation that gives the change in [ES] as a function of time is:

$$\frac{d[ES]}{dt} = +k_{ON}[E][S] - k_{OFF}[ES] - k_{CAT}[ES]$$



If we make the assumption that we are working under steady-state conditions: **d[ES]/dt = 0.**

$$0 = +k_{ON}[E][S] - k_{OFF}[ES] - k_{CAT}[ES]$$

and

$$v = k_{CAT}[ES]$$

gives :

$$v = k_{CAT}[E]_T \frac{[S]}{[S] + \frac{k_{OFF} + k_{CAT}}{k_{ON}}}$$

$$= k_{CAT} E_{Total} \frac{[S]}{[S] + K_M}$$

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

The last equation is the often called the **Michaelis-Menton** equation, named after the scientists who first derived a limited version of the equation.

i) The K_M or Michaelis constant: This is *almost* the same as the K_D ($= k_{off}/k_{on}$), the dissociation constant, except for the presence of the k_{CAT} term. Therefore, it is related to the affinity of a substrate to an enzyme. *It is a constant for any particular enzyme-substrate pair. Substrates with slow off-rates (k_{off}) bind more tightly, and possess a smaller K_M.*

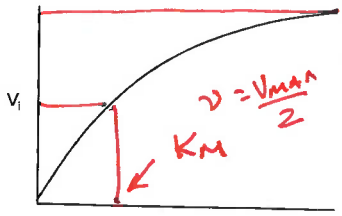
$$K_M = \frac{k_{off} + k_{CAT}}{k_{on}}$$

$K_D = k_{off}/k_{ON}$

V_{MAX}

k_{CAT} is small

$\therefore K_M \approx K_D$



When [S]=K_M the enzyme is ½ saturated with substrate: v = ½ V_{Max}

$V_{MAX} = k_{CAT} \cdot E$

$\frac{V_{MAX}}{E_{TOT}} = k_{CAT}$

ii) V_{MAX} = $k_{CAT}[E_T]$: This is the highest rate of product production possible. It is obtained at high substrate levels ([S]>>K_M). Under these conditions *all* of the enzyme is in the [ES] form (i.e. [ES]=[E_T]), the enzyme is ***saturated*** with substrate. k_{CAT} is obtained from V_{MAX} since the total amount of enzyme is known: $k_{CAT}=V_{MAX}/[E_T]$.

iii) k_{CAT} is the turn-over number – how many products are produced/sec/enzyme molecule.

$$v = E_{Total} k_{CAT} \frac{[S]}{[S] + K_M}$$

iv) Specificity constant: k_{CAT}/K_M = rate at low substrate, a measure of overall substrate specificity, often used to compare enzyme efficiency.

$$v = E_{Total} k_{CAT} \frac{[S]}{[S] + K_M} \Rightarrow \text{low } [S]$$

$v = E_{TOT}[S]\left(\frac{k_{CAT}}{K_M}\right)$ ← rate constant

Example: Two substrates are presented to trypsin.

i) Which substrate binds better to trypsin? Ala-Lys or Ala-Ser? (low K_M)

ii) Which is cleaved more quickly once bound? Ala-Lys or Ala-Ser?

iii) Which substrate is cleaved more effectively at low [S]? Ala-Lys or Ala-Ser?

Sub	K_M (uM)	k_{CAT}	k_{CAT}/K_M
Ala-Lys	0.1	10 sec^{-1}	100.0
Ala-Ser	10.0	5 sec^{-1}	0.5

higher k_{CAT}/K_M

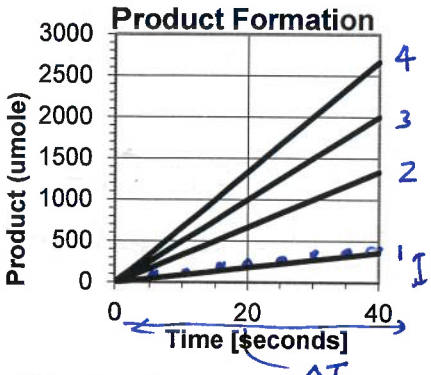
5. Measuring K_M and k_{CAT} (V_{MAX}):

Step A. Data Collection:

Measure the *initial* velocity at different substrate concentrations, usually keeping the enzyme concentration *constant*.

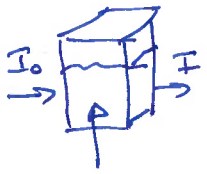
Example: The following velocity data was obtained for a number of substrate concentrations ($[E]_{Tot}$=1 nM).

Exp. #	[S] (mM)	v (umoles/sec)
1	0.1	9.0 = ΔP/ΔT
2	0.5	33.4
3	1.0	50.0
4	2.0	66.6
5	10.0	91.1
6	20.0	95.2
7	50.0	99.0



$A = [P]\varepsilon_P l$

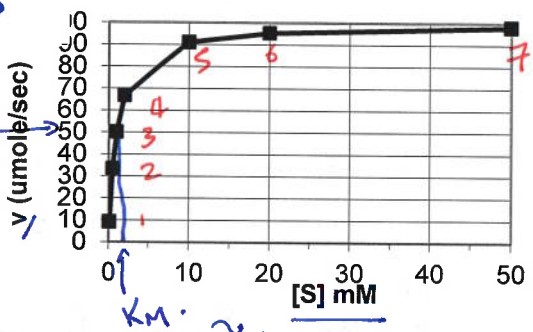
$[P] = \frac{A}{\varepsilon_P l}$



$E_{TOT} = 1nM$. Varied [S]

Step B: Analyze data

1. [S] not limiting - Velocity Curve (Least accurate):
   - i) Plot v_{OBS} versus [S].
   - ii) Obtain V_{MAX} from v at very high [S].
   - iii) K_M is the substrate concentration at gives $v=V_{MAX}/2$



$V_{MAX} \approx 100$ um/sec

$V_{MAX}/2$

$K_M \approx 1$ mM

Reflection: What are the problems with this approach?

Have to saturate.

i) too expensive.

ii) Solubility.

2. Direct fitting to kinetic equation, using Solver (this is the best).
   - i) Estimate K_M and V_{MAX}
   - ii) Calculate expected $V_{Predicted}$ for all data points.
   - iii) Adjust K_M and V_{MAX} to minimize $\sum(v_{Predicted} - v_{Observed})^2$

3. Double reciprocal plot-Useful graphical tool to detect types of inhibitors (Lineweaver-Burk Plot):
   - i) Take inverse of velocity and [S].
   - ii) Plot 1/v versus 1/[S]
   - iii) Analysis of double-reciprocal plot:

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

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

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

$y = mx + b$

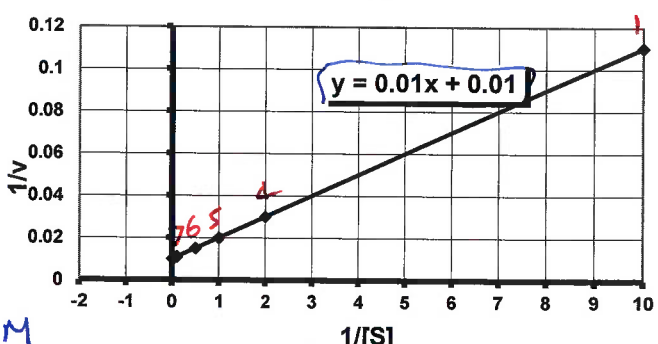
y-intercept = $1/V_{MAX}$ = 0.01

V_{MAX} = 100 um/s.

Slope = K_M/V_{MAX} = 0.01

K_M = slope × V_{MAX} = 0.01 × 100 = 1 mM

$k_{CAT} = V_{MAX}/E_T$



$$k_{CAT} = \frac{100 \times 10^{-6}\ s^{-1}\ M}{10^{-9}\ M} = 100{,}000\ s^{-1}$$