Lecture 17: Introduction to Steady-State Enzyme Kinetics

Goals:
1. Measure parameter ($K_M$) related to substrate binding.
2. Measure parameter ($K_{CAT}$) related to catalytic efficiency.

Simple Enzyme Kinetic Scheme.
- $k_{ON}$ ($k_1$) is the forward rate constant for substrate binding
- $k_{OFF}$ ($k_2$) is the reverse rate constant for substrate binding
- $k_{CAT}$ ($k_2$) 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 $\Delta [P]$ during a given period of time $\Delta t$:

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

2. Experimental Measurement of Enzyme Kinetics: How do we actually measure rates?

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 $[S]$:

$$[S] < K_M, \quad Y < 0.5$$

high $[S]$:

$$[S] > K_M, \quad Y = 1 \quad \text{all enzymes have } [S]^*$$

ii) How does the rate depend on the total amount of enzyme, $[E_{TOT}]$?
4. Analytical Derivation of Rate Law - Steady-State Conditions

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

i) The total amount of enzyme: \( E_T = [E] + [ES] \)

ii) the concentration of substrate: \([S]\)

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

We want to come up with an equation that gives us the initial velocity as a function of \([S]\), and depends on \( K_M \) and \( V_{\text{MAX}} \). We can then use our data to determine \( K_M \) and \( V_{\text{MAX}} \).

The simplest reaction scheme is:

\[
V = \frac{k_{\text{ON}}}{k_{\text{OFF}}} \frac{k_{\text{CAT}}}{[ES]} \]

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

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

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

If we make the assumption that we are working under steady-state conditions:

\[
d[ES]/dt = 0.
\]

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

and

\[
v = k_{\text{CAT}}[ES]
\]

gives:

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

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

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

The last equation is the **Michaelis-Menten equation**, named after the scientists who derived it.

**i) The** \( K_M \) **or Michaelis constant**: This is almost the same as the \( K_D \) (\( = k_{\text{off}}/k_{\text{on}} \)), the dissociation constant, except for the presence of the \( k_{\text{CAT}} \) 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 (\( k_{\text{off}} \)) bind more tightly, and possess a smaller \( K_M \).

When \([S]=K_M \) the enzyme is \( \frac{1}{2} \) saturated with substrate: \( v = \frac{1}{2} V_{\text{MAX}} \)

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

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

**iv) Specificity constant**: \( k_{\text{CAT}}/K_M \) = rate at low substrate –measure of overall specificity:

\[
v = \frac{V_{\text{MAX}}}{K_M} \frac{k_{\text{CAT}}}{[S] + K_M}
\]

\[
\Rightarrow \frac{[S]}{K_M} \ll 1
\]

\[
v = V_{\text{TOTAL}} \cdot \frac{[S]}{K_M} \]

\[
v = \frac{E_{\text{TOTAL}} \cdot [S]}{[X] + K_M}
\]

\[
v = \frac{E_{\text{TOTAL}} \cdot [S]}{[X] + K_M}
\]

\[
\Rightarrow \frac{[S]}{K_M} \ll 1
\]
Example: The following two substrates are presented to trypsin (NP=nitrophenyl, colored group released by the enzyme). The $K_M$ and $k_{CAT}$ values for each substrate are given in the table below.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (uM)</th>
<th>$k_{CAT}$ (sec$^{-1}$)</th>
<th>$k_{CAT}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-Lys-NP</td>
<td>0.1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Ala-Ser-NP</td>
<td>10.0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

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

Step A: Measure the initial velocity at different substrate concentrations, keeping the enzyme concentration constant.

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

![Graph of Product Formation vs Time](image)

Step B: Analyze data

I: [S] not limiting - Velocity Curve:

   i) Plot $v$ versus [S].
   ii) Obtain $V_{MAX}$ from $v$ at very high [S].
   iii) $K_M$ is the substrate concentration at gives $v=V_{MAX}/2$

II: [S] limiting - Double reciprocal plot

(Lineweaver-Burk Plot):

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

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

Analysis of double-reciprocal plot:

i) $y$-intercept = $1/V_{MAX}$

ii) Slope = $K_M/V_{MAX}$

$K_M$ = slope $\times$ $V_{MAX}$

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

Exp. # | [S] (mM) | v (umoles/sec) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>9.0</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>33.4</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>50.0</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
<td>66.6</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>91.1</td>
</tr>
<tr>
<td>6</td>
<td>20.0</td>
<td>95.2</td>
</tr>
<tr>
<td>7</td>
<td>50.0</td>
<td>99.0</td>
</tr>
</tbody>
</table>

$E_T = \frac{360 \text{ mmol}}{4 \text{ sec}} = 90 \text{ mmol/sec}$

$$ (ES) = E_T $$

$V_{MAX} = 100 \text{ mmol/sec}$

$K_M = 1 \text{ mM}$

$1/[S] = \frac{y}{0.1} = 0.1x + 0.1$

$y = 0.01x + 0.01$

$V_{MAX} = 100 \text{ mmol/sec}$

$[S] = \infty$, enzyme is saturated

$K_M = 0.01 \times 100 = 1 \text{ mM}$ (units of substrate concentration)

$k_{CAT} = \frac{V_{MAX}}{E_T} = \frac{10^{-4} \text{ mole/sec}}{10^{-9} \text{ mole}} = 10^5 \text{ sec}^{-1}$ (turn over)
Example: Double reciprocal plots were obtained for an enzyme reacting with three different substrates. The $V_{\text{MAX}}$ and $K_M$ values for these substrates are given in the table on the right. Which line on the double reciprocal plot corresponds to each substrate?

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{\text{MAX}}$</th>
<th>$K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

6. Why determine $K_M$ and $k_{\text{CAT}}$?

A. Mechanistic Information - about particular enzyme-substrate pairs.

1. The active site region of Trypsin is shown below.
   i) Sketch the $k_{\text{CAT}}$ 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 $k_{\text{CAT}}$ values are given.
   i) Explain the differences in $K_M$ of A versus B.
   ii) Why are the $k_{\text{CAT}}$ values the same?

B. Predictive Information: Given $K_M$, $E_{\text{TOT}}$, $k_{\text{CAT}}$, and [S], you can predict the initial velocity:

$$v = k_{\text{CAT}} [E]_T \frac{[S]}{[S] + K_M}$$

Example: An enzyme has a $k_{\text{CAT}}$ of $10^9$/sec and a $K_M$ of 0.1 $\mu$M towards a certain substrate. One (1) nmole of enzyme is mixed with 0.3 $\mu$M substrate in a volume of one ml, what is the initial rate of the reaction (dP/dt)?

$$v_i = k_{\text{CAT}} E_T \frac{[S]}{K_M + [S]} = 10^5 \frac{1}{\text{sec}} \times 1 \text{ nmole} \times 0.3 \mu M = 0.3 \mu M$$

$$= 10^5 \frac{1}{\text{sec}} \times 1 \text{ nmole} \times 0.3 \mu M + 0.3 \mu M = 1 \times 10^5 \text{ nmole} / \text{sec} (0.75)$$

$$= 10^{-4} \text{ moles} / \text{sec} \times 0.75 = 7.5 \times 10^{-5} \text{ moles} / \text{sec} = 7.5 \times 10^{-2} \text{ M} / \text{sec}$$