LECTURE 2: ENZYME KINETICS

1. A catalyst lowers energy of activation by providing a different mechanism for the reaction. Both the rates of forward and backward reaction are enhanced.

GENERAL PRINCIPLES OF CATALYSIS

2. A catalyst forms an intermediate with the reactant(s) in the initial step of the mechanism and is released in the product forming step.

3. A catalyst does not affect the enthalpies or free energies of reactants and products.

Three Types of Catalysis

Homogeneous Catalysis – reactants and catalysts are in the same phase

Heterogeneous Catalysis – reactants and catalysts are in different phases

Enzyme Catalysis – also homogeneous catalysis but catalysts are biological in origin. More complex.
Consider the reaction: $\text{H}_2\text{O}_2 \rightleftharpoons \text{H}_2\text{O} + \text{O}_2$

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Relative Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncatalyzed</td>
<td>1</td>
</tr>
<tr>
<td>Pt Black (inorganic catalyst)</td>
<td>10,000</td>
</tr>
<tr>
<td>Catalase (enzyme)</td>
<td>300,000,000,000</td>
</tr>
</tbody>
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Catalysts, in particular, enzymes are capable of astonishing rate enhancements.

How do enzymes work?

- Biological enzymes have evolved to form complex three-dimensional structures that present an “active site” surface to which reactants in a chemical reaction bind.

- These sites also position amino acid R-groups and/or reaction cofactors (such as metals) or prosthetic groups at the appropriate positions to aid in catalysis.

- Two major models for how this might work on the structural level are shown on the next slide.

Two models for the ES complex:

(a) Lock-and-key model
(b) Induced fit model

Let's take a look at a real active site!

An Active Site
ENZYME ACTIVITY MEASUREMENT

\[ E_{\text{free}} + S \underset{\text{ES}}{\overset{\text{E}}{\rightleftharpoons}} P \]

How does [enzyme] influence observed reaction velocity?

- Assumes that [E] is limiting and that the uncatalyzed reaction rate is ~0

\[ \Delta[P]/\Delta t = 1 \]
\[ 2 \times [\text{enzyme}] \quad \Delta[P]/\Delta t = 2 \]
\[ 1 \times [\text{enzyme}] \quad \Delta[P]/\Delta t = 1 \]
\[ 0.5 \times [\text{enzyme}] \quad \Delta[P]/\Delta t = 0.5 \]

ENZYME SPECIFICITY

How specific are enzymes for a given substrate?

- The answer depends upon the enzyme you’re talking about.
  Most enzymes are highly specific, acting on only a small number of substrates that are highly similar in structure. Others, such as alkaline phosphatase mentioned in your notes, are less specific.

- Specificity arises from structural and chemical complementarity between the substrate and its enzyme.

Specificity of enzymes (an example)

- Hydrogen Bonds
- Ionic Bonds
Many enzymes bind non-protein cellular components that are used as key factors in the enzyme activity. These fall into three basic categories:

1) **Metals**: Metals (e.g. Mg, Ca, Zn, Fe etc.) are thought to be bound to ~1/3 of all proteins and can play key roles in activity. An example is the Mg(2+) in the ATPase on the previous slide. These ions can confer a wider array of chemical properties to proteins over those of the 20 natural amino acids.

(2 & 3) **Coenzymes and prosthetic groups**: Low-molecular organic compounds that bind either weakly (coenzymes) or tightly (prosthetic groups) to the protein. Examples that you will see in this course include, for example, iron-sulfur clusters, heme, and coenzyme A.

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**Formula for a simple enzyme-catalyzed reaction**

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

- **E** - free enzyme
- **S** - Substrate
- **ES** - Enzyme-Substrate complex
- **P** - product

**What are we measuring?**

- Increasing [S]
- Product, mol L\(^{-1}\)
- Time
**Initial Velocity**

Measured at the very beginning of a reaction when very little P has been made.

**FOR ENZYME-CATALYZED REACTION**

\[
E + S \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} P + E
\]

- \(k_1\) is rate constant for formation of ES
- \(k_{-1}\) is rate constant for conversion of ES to E+S
- \(k_2\) is rate constant for product formation. For this reaction, \(k_2 = k_{cat}\)

Initial velocity assumption: measure activity before appreciable P accumulates: \(v_0 = k_2 [ES]\)

**ENZYME-CATALYZED REACTION EXHIBIT SATURATION KINETICS**

At high [S], the enzyme is said to be saturated with respect to substrate

**SUBSTRATE SATURATION OF AN ENZYME**

A. Low [S]  B. 50% [S] or \(K_m\)  C. High, saturating [S]
**STeady State**

The more ES present, the faster ES will dissociate into E + P or E + S. Therefore, when the reaction is started by mixing enzymes and substrates, the [ES] builds up at first, but quickly reaches a Steady State, in which [ES] remains constant. This steady state will persist until almost all of the substrate has been consumed.

**The Michaelis-Menten Equation**

\[
E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} P + E
\]

If you assume that the formation of ES equals its breakdown, making [ES] constant (steady state), then:

\[
k_1 [E][S] = k_{-1} [ES] + k_2 [ES]
\]

\[
K_M = \frac{k_{-1} + k_2}{k_1}
\]

\[
V_o = \frac{V_{max}[S]}{K_M + [S]}
\]

**Meaning of \(K_M\)**

- An important relationship that can be derived from the Michaelis-Menten equation is the following: If \(V_o\) is set equal to \(1/2\) \(V_{max}\), then the relation \(V_{max}/2 = V_{max}[S]/K_M + [S]\) can be simplified to \(K_M + [S] = 2[S]\), or \(K_M = [S]\).

- This means that at one half of the maximal velocity, the substrate concentration at this velocity will be equal to the \(K_M\).

- This relationship has been shown experimentally to be valid for many enzymes much more complex in regards to the number of substrates and catalytic steps than the simple single substrate model used to derive it.

**Meaning of \(K_m\)**

- The significance of \(K_m\) will change based on the different rate constants and which step is the slowest (also called the rate-limiting step).

- In the simplest assumption, the rate of ES breakdown to product (\(k_2\)) is the rate-determining step of the reaction, so \(k_1 \gg k_2\) and \(K_m = k_1/k_2\).

- This relation is also called a dissociation constant for the ES complex and can be used as a relative measure of the affinity of a substrate for an enzyme.

- However if \(k_2 \gg k_1\) or \(k_2\) and \(k_1\) are similar, then \(K_m\) remains more complex and cannot be used as a measure of substrate affinity.
USES OF $K_m$

- Experimentally, $K_m$ is a useful parameter for characterizing the number and/or types of substrates that a particular enzyme will utilize (an example will be discussed). It is also useful for comparing similar enzymes from different tissues or different organisms.

- Also, it is the $K_m$ of the rate-limiting enzyme in many of the biochemical metabolic pathways that determines the amount of product and overall regulation of a given pathway.

- Clinically, $K_m$ comparisons are useful for evaluating the effects mutations have on protein function for some inherited genetic diseases.

MEANING OF $V_{max}$

- The values of $V_{max}$ will vary widely for different enzymes and can be used as an indicator of an enzymes catalytic efficiency. It does not find much clinical use.

- There are some enzymes that have been shown to have the following reaction sequence:

  \[ E + S \overset{k_1}{\rightarrow} ES \overset{k_2}{\rightarrow} EP \overset{k_3}{\rightarrow} E + P \]

  - In this situation, the formation of product is dependent on the breakdown of an enzyme-product complex, and is thus the rate-limiting step defined by $k_3$.

DERIVATION OF $k_{cat}$

- A more general term has been defined, termed $k_{cat}$, to describe enzymes in which there are multiple catalytic steps and possible multiple rate-limiting steps. The Michaelis-Menten equation can be substituted with $k_{cat}$.

  \[
  V_0 = \frac{k_{cat} [E]_{tot} [S]}{K_m + [S]}
  \]

DEFINITION AND USE OF $k_{cat}$

- The constant, $k_{cat}$ (units of sec$^{-1}$), is also called the turnover number because under saturating substrate conditions, it represents the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule.

- In practice, $k_{cat}$ values (not $V_{max}$) are most often used for comparing the catalytic efficiencies of related enzyme classes or among different mutant forms of an enzyme.
Important Conclusions of Michaels - Menten Kinetics

- when $[S] = K_M$, the equation reduces to
  \[ v = \frac{V_{\text{max}}[S]}{K_M + [S]} = \frac{V_{\text{max}}}{2} \]
- when $[S] >> K_M$, the equation reduces to
  \[ v = \frac{V_{\text{max}}[S]}{K_M + [S]} = \frac{V_{\text{max}}}{K_M} \]
- when $[S] << K_M$, the equation reduces to
  \[ v = \frac{V_{\text{max}}[S]}{K_M + [S]} = \frac{V_{\text{max}}[S]}{[S]} \]

ENZYME KINETICS – PROBLEM SOLVING - $K_m$

- $K_m$ is the $[S]$ at $1/2 V_{\text{max}}$
- $K_m$ is a constant for a given enzyme
- $K_m$ is an estimate of the equilibrium constant for S binding to E
- Small $K_m$ means tight binding; high $K_m$ means weak binding
- $K_m$ is a measure of [S] required for effective catalysis to occur

ENZYME KINETICS – PROBLEM SOLVING - $V_{\text{max}}$

- $V_{\text{max}}$ is a constant for a given enzyme
- $V_{\text{max}}$ is the theoretical maximal rate of the reaction - but it is NEVER achieved
- To reach $V_{\text{max}}$ would require that ALL enzyme molecules have tightly bound substrate
MEASURING Km and $V_{\text{max}}$ - LINEWEAVER-BURKE EQ

- Curve-fitting algorithms can be used to determine $K_m$ and $V_{\text{max}}$ from $v$ vs. $[S]$ plots.
- Michaelis-Menten equation can be rearranged to the “double reciprocal” plot and $K_m$ and $V_{\text{max}}$ can be graphically determined.

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

ENZYME KINETICS – SAMPLE PROBLEM

The following data were obtained from an enzyme kinetics experiment. Graph the data using a Lineweaver-Burk plot and determine, by inspection of the graph, the values for $K_m$ and $V_{\text{max}}$.

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<tr>
<th>[S] (µM)</th>
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</tr>
<tr>
<td>0.33</td>
<td>2.08</td>
</tr>
<tr>
<td>1.00</td>
<td>3.33</td>
</tr>
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</table>

ENZYME KINETICS – SAMPLE PROBLEM

An enzymatic assay was carried under two different sets of conditions out using a pure substrate S. The results are tabulated below.

<table>
<thead>
<tr>
<th>[S]/10-5 M</th>
<th>Condition A</th>
<th>Condition B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>0.21</td>
<td>0.08</td>
</tr>
<tr>
<td>2.0</td>
<td>0.25</td>
<td>0.1</td>
</tr>
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<td>3.0</td>
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<tr>
<td>8.0</td>
<td>0.44</td>
<td>0.16</td>
</tr>
<tr>
<td>16.0</td>
<td>0.40</td>
<td>0.18</td>
</tr>
</tbody>
</table>

- a. Plot the data using the Lineweaver-Burk plot.
- b. Calculate the values of $V_{\text{max}}$ and $K_m$ for both sets of conditions.
- c. Suggest possible reasons why the two sets of results might be different.

ENZYME KINETICS – Catalytic EFFICIENCY

TURNOVER NUMBER
- The $k_{\text{cat}}$ is a direct measure of the catalytic conversion of product under saturating substrate conditions.
- $k_{\text{cat}}$, the turnover number, is the maximum number of substrate molecules converted to product per enzymemolecule per unit of time. Values of $k_{\text{cat}}$ range from less than 1/sec to many millions per sec.

CATALYTIC EFFICIENCY
- It shows what the enzyme can accomplish when abundant enzyme sites are available.
- It is the $k_{\text{cat}}/K_m$ value that allows direct comparison of the effectiveness of an enzyme toward different substrates.
ENZYME KINETICS – SAMPLE PROBLEM

Calculate the specificity constant for an enzyme if its $k_{cat} = 1.4 \times 10^4 \text{ s}^{-1}$ $K_m = 90 \mu M$.

BI-SUBSTRATE REACTIONS

- The Michaelis–Menten model of enzyme kinetics was derived for single substrate reactions
- The majority of enzymatic reactions have multiple substrates and products
- Bi-substrate reactions account for ~60% of the known enzymatic reactions.

\[
A + B \leftrightarrow E \rightarrow P + Q
\]

SUBSTRATE ADDITION / PRODUCT RELEASE

- The order of substrate addition and product release in most enzymatic reactions follow two reaction mechanisms
  - Sequential reaction - all substrates must bind to the enzyme before the reaction occurs and products are released
    - Ordered sequential
    - Random sequential
  - Ping-pong reaction - one or more products are released before all substrates have been added and an alternate stable enzyme form, F, is produced in the half reaction

1) Sequential Reaction

- Ordered sequential

- Random sequential
2) Ping-pong Reaction

Initial Velocity Plots

- sequential reaction exhibits an intersecting pattern of lines
- Order and random substrate additions cannot be distinguished in this type of plot

Influence of enzyme concentration

\[ v = \frac{K_s[E][S]}{K_m + [S]} \]

\[ v = k_3[E], \text{ as } [S] \gg [E] \]

Influence of temperature

Optimum temperature, most of them are in the range from 35 to 45°C.
Enzyme Inhibition

Enzyme inhibitors are important for a variety of reasons

1) they can be used to gain information about the shape on the enzyme active site and the amino acid residues in the active site.
2) they can be used to gain information about the chemical mechanism.
3) they can be used to gain information about the regulation or control of a metabolic pathway.
4) they can be very important in drug design.

Inhibitors

\[
\begin{align*}
\text{Irreversible inhibition} \\
\text{Reversible inhibition} \\
\text{competitive inhibition} \\
\text{non-competitive inhibition} \\
\text{uncompetitive inhibition}
\end{align*}
\]

Enzyme Inhibition

- **Reversible inhibitor**: a substance that binds to an enzyme to inhibit it, but can be released
  - usually involves formation of non-covalent bonds
  - Generally two types
    - Dead end
    - Product
- **Irreversible inhibitor**: a substance that causes inhibition that cannot be reversed
  - usually involves formation or breaking of covalent bonds to or on the enzyme

Influence of pH

<table>
<thead>
<tr>
<th>pH</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td>4</td>
<td>High</td>
</tr>
<tr>
<td>6</td>
<td>Low</td>
</tr>
<tr>
<td>8</td>
<td>High</td>
</tr>
<tr>
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Optimum pH

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- Inhibitors
- Irreversible inhibition
- Reversible inhibition
  - competitive inhibition
  - non-competitive inhibition
  - uncompetitive inhibition
Irreversible inhibition

• Irreversible inhibition:
The inhibitor combines with the essential group of enzyme active center by covalent bond, resulting in enzymatic activity loss.

Inhibition Patterns

Inhibitors act in a variety of mechanisms

• An inhibitor may bind at the same site as one of the substrates
  – these inhibitors structurally resemble the substrate
• An inhibitor may bind at an alternate site affecting catalytic activity without affecting substrate binding
• Many inhibitors do both
• Most common types
  – Competitive
  – Mixed or Non-competitive
  – Uncompetitive

Competitive Inhibition

• Competitive inhibitor competes with a substrate for the enzyme - substrate binding site

Malonate is a competitive inhibitor of succinate for succinate dehydrogenase

Competitive Inhibition

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

\[ K_i \int \]

• A competitive inhibitor reduces the amount of free enzyme available for substrate binding thus increasing the Km for the substrate

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

\[ K_i \int \]

• The effect of a competitive inhibitor can be overcome with high concentrations of the substrate
**Competitive Inhibition**

- **Unimolecular Reaction**
- **Bimolecular Reaction**

**Uncompetitive Inhibition**

- An uncompetitive inhibitor binds to the enzyme-substrate complex but not to free enzyme.
- The result is a decrease in Vmax and Km.
- The effect of an uncompetitive inhibitor cannot be overcome by high concentrations of the substrate.
Uncompetitive

Mixed or Non-Competitive Inhibition

- The inhibitor can bind to both free enzyme and the ES complex
- The affinity of the inhibitor to the two complexes might be different
  - If binding of inhibitor changes the affinity for the substrate, $K_m$ will be changed and called mixed inhibition
  - If only $V_{max}$ affected called Non-competitive inhibitor

Mixed Inhibition

- The result will be decrease in $V_{max}$ and either an increase or decrease in $K_m$
- The effect of a non-competitive inhibitor can only be partially overcome by high concentrations of the substrate
Non-Competitive

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ENZYME KINETICS – PROBLEM SOLVING - $V_{max}$

THEORETICAL MAXIMUM VELOCITY

- $V_{max}$ is a constant for a given enzyme
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MEASURING $K_m$ and $V_{max}$ - LINEWEAVER-BURKE EQ

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Lineweaver-Burk equation:

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right) \frac{1}{[S]} + \frac{1}{V_{max}}$$
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ENZYME KINETICS – SAMPLE PROBLEM

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Competitive Inhibition

(a) Competitive inhibition

\[ E + S \overset{K_i}{\rightleftharpoons} ES \rightarrow E + P \]

\[ I \]

Typically, I is a substrate analog.

Effects of Competitive Inhibitor on Enzyme Kinetics

\[ K_i \text{ (inhibitor dissociation constant)} \]

\[ K_{\text{app}} = \frac{k_{\text{off}}}{k_{\text{on}}} \]

\[ K_{\text{Mapp}} = K_M(1 + [I]/K_i) > K_M \]

\[ V_{\text{appmax}} = V_{\text{max}} \]

A Substrate and Its Competitive Inhibitor

Some HIV Protease Inhibitors
Mark (Noncompetitive) Inhibition

\[ K_{\text{app}} M = K_M \]

\[ V_{\text{app}} = V_{\max}/(1 + [I]/K_I) < V_{\max} \]

Uncompetitive Inhibition

\[ K_{\text{app}} = K_M(1 + [I]/K_I) < K_M \]

\[ V_{\text{app}} = V_{\max}/(1 + [I]/K_I) < V_{\max} \]
Irreversible Inhibition

E + I $\xrightarrow{k_{1}}$ k$_{2}$ $\xrightarrow{k_{3}}$ E·I $\xrightarrow{k_{4}}$ E

Plot: ln(residual enzyme activity) vs. time

If [I]>>[E], conditions are pseudo-first order and slope is $-k_{\text{obs}}$ (pseudo-first order inactivation rate constant)

$k_{\text{inact}}$ (second-order inactivation constant) = $k_{1}k_{2}/k_{3} = k_{\text{obs}}/[I]$

Irreversible Inhibition by Adduct Formation

Irreversible Inhibition of Chymotrypsin by TPCK

Enzyme Kinetics – Sample Problem

A chemist measured the initial rate of enzyme catalyzed reaction in the absence and presence of inhibitor A and, in a separate procedure inhibitor B. In each case, the inhibitors’ concentration was 8.0 mM. The data are shown below.

<table>
<thead>
<tr>
<th>[S] /M</th>
<th>V (M/s) No Inhibitor</th>
<th>V (M/s) Inhibitor A</th>
<th>V (M/s) Inhibitor B</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 x 10$^{-4}$</td>
<td>1.25 x 10$^{-6}$</td>
<td>5.8 x 10$^{-7}$</td>
<td>3.8 x 10$^{-7}$</td>
</tr>
<tr>
<td>1.0 x 10$^{-3}$</td>
<td>2.0 x 10$^{-6}$</td>
<td>1.04 x 10$^{-6}$</td>
<td>6.3 x 10$^{-7}$</td>
</tr>
<tr>
<td>2.5 x 10$^{-3}$</td>
<td>3.13 x 10$^{-6}$</td>
<td>2.00 x 10$^{-6}$</td>
<td>1.00 x 10$^{-6}$</td>
</tr>
<tr>
<td>5.0 x 10$^{-3}$</td>
<td>3.85 x 10$^{-6}$</td>
<td>2.78 x 10$^{-6}$</td>
<td>1.25 x 10$^{-6}$</td>
</tr>
<tr>
<td>1.0 x 10$^{-2}$</td>
<td>4.55 x 10$^{-6}$</td>
<td>3.57 x 10$^{-6}$</td>
<td>1.43 x 10$^{-6}$</td>
</tr>
</tbody>
</table>
The effect of an inhibitor on an enzyme was tested and the experiment gave the results below. Plot the data and determine, by inspection of the graph, what type of inhibition is involved.

<table>
<thead>
<tr>
<th>[S] μM</th>
<th>V (μmol/min) with 0.0 nM Inhibitor</th>
<th>V (μmol/min) with 25 nM Inhibitor</th>
<th>V (μmol/min) with 50 nM Inhibitor</th>
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