Chapter 12
Properties of enzymes

1. Reaction kinetics
2. Enzyme inhibition
3. Control of enzyme activity
4. Drug design
Properties of enzymes

1. Early enzymologist did work with crude preparation, i.e. cell homogenate from yeast or liver, but could characterize their activity by measuring the rate of the reaction under varying conditions, i.e. competing substrate or enzyme inhibitor.

2. Study of enzyme reaction rates = enzyme kinetics can reveal path followed by reactants, which can be indicative of the reaction mechanism.
1) Reaction Kinetics

A) Chemical kinetics is described by rate equations

A $\rightarrow$ P

Sequence of elementary reactions, with intermediates

A $\rightarrow$ I₁ $\rightarrow$ I₂ $\rightarrow$ P

Description of each elementary reaction collectively constitute the mechanistic description of the overall reaction process
Reaction order indicates the number of molecules participating in an elementary reaction

At a constant temperature, the rate of an elementary reaction is proportional to the frequency with which the reacting molecules come together.

The proportionality constant is the rate constant, $k$.

For $A \rightarrow P$, rate of appearance of $P$ and disappearance of $A$ is the velocity ($v$) of the reaction:

$$v = \frac{d[P]}{dt} = -\frac{d[A]}{dt} = k[A]$$

Velocity at any time point is proportional to the concentration of the reactant $A$, first-order reaction; units (Mol/sec)
The reaction order of an elementary reaction corresponds to the molecularity of the reaction, which is the number of molecules that must simultaneously collide to generate a product.

First-order reaction is a unimolecular reaction, i.e. radioactive decay.

Bimolecular reaction: $2A \rightarrow P$ is a second-order react.

$$v = \frac{d[A]}{dt} = k[A]^2$$

units ($\text{Mol}^{-1} \text{ sec}^{-1}$)
Bimolecular reaction: \( A + B \rightarrow P \) is also a second-order reaction

\[
\nu = -\frac{d[A]}{dt} = -\frac{d[B]}{dt} = k [A][B]
\]

Unimolecular and bimolecular reactions are common.

Termolecular reactions are unusual, because simultaneous collision of three molecules is rare. Fourth and higher-order reactions are unknown.
SAMPLE CALCULATION 12-1

Determine the velocity of the elementary reaction $X + Y \rightarrow Z$ when the sample contains 3 $\mu$M $X$ and 5 $\mu$M $Y$ and $k$ for the reaction is 400 $M^{-1} \cdot s^{-1}$.

Use Equation 12-3 and make sure that all units are consistent:

$$
\begin{align*}
    v &= k[X][Y] \\
    &= (400 \, M^{-1} \cdot s^{-1})(3 \, \mu M)(5 \, \mu M) \\
    &= (400 \, M^{-1} \cdot s^{-1})(3 \times 10^{-6} \, M)(5 \times 10^{-6} \, M) \\
    &= 6 \times 10^{-9} \, M \cdot s^{-1} \\
    &= 6 \, nM \cdot s^{-1}
\end{align*}
$$
A rate equation indicates the progress of a reaction as a function of time

$$\ln [A] = \ln [A]_0 -kt$$

$$[A] = [A]_0 \ e^{-kt}$$

Linear equation in form of $y = mx + b$; can be blotted
A plot of a first-order rate equation

\[ \ln [A] = \ln [A]_o - k t \]
Hallmark of first-order reaction is that its half-time or half-life, $t_{1/2}$, is a constant and thus independent of the initial concentration of the reactants

$$t_{1/2} = \ln 2 / k = 0.693 / k$$

i.e. radioactive decay
Half-time for a second-order reaction
2A $\rightarrow$ P is:

$$\frac{1}{[A]} = \frac{1}{[A]_0} + kt$$

$$t_{1/2} = \frac{1}{k [A]_0}$$

And therefore, in contrast to a first-order reaction, depends on the concentration of the initial reactant.

These equations may be used to distinguish first-order from second-order reactions by blotting $\ln[A]$ versus $t$ and $1/[A]$ versus $t$ and observing which of these blots is linear.
To experimentally determine the rate constant of a second-order reaction $A + B \rightarrow P$, it is often convenient to increase the concentration of one reactant relative to the other, $[B] \gg [A]$; under these conditions, $[B]$ does not change significantly over the course of the reaction. The reaction rate therefore depends only on $[A]$. The reaction appears to be first order with respect to $[A] = \text{pseudo-first-order reaction}$
The decay of a hypothetical radioisotope has a rate constant of $0.01 \text{ s}^{-1}$. How much time is required for half of a 1-g sample of the isotope to decay?

The units of the rate constant indicate a first-order process. Thus, the half-life is independent of concentration. The half-life of the isotope (the half-time for its decay) is given by Eq. 12-9:

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{0.01 \text{ s}^{-1}} = 69.3 \text{ s}$$
All enzymes can be analyzed such that their reaction rates as well as their overall efficiency can be quantified.

1902 \( \beta \)-fructofuranosidase:

\[
\text{sucrose} + \text{H}_2\text{O} \rightarrow \text{glucose} + \text{fructose}
\]

If \([\text{sucrose}] >> [\text{enzyme}]\) then the reaction rate becomes \textit{zeroth order} with respect to sucrose.

Proposition: two elementary reactions:

\[
\begin{align*}
E + S & \rightleftharpoons ES \\
& \rightarrow P + E
\end{align*}
\]

\(K_2\) is rate limiting; and we assume that it is irreversible.
The Michaelis-Menten equation assumes that ES maintains a steady state

Michaelis-Menten equation describes the rate of an enzymatic reaction as a function of substrate concentration.

Formation of product from ES is a first-order process

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

2. Assumption of steady-state, \([S] \gg [E] \rightarrow [S], [E]\) and \( [ES] \) remain constant, except for beginning and end of the reaction, hence \([ES]\) maintains steady-state.
The progress curves for a simple enzyme-catalyzed reaction.

\[ [E]_T = [E] + [ES] \]

\[ \frac{d[ES]}{dt} \approx 0 \]

Steady state
The Michaelis-Menten equation assumes that ES maintains a steady state.

The **Michelis constant** $K_M$ is defined as:

$$K_M = \frac{k_1 + k_2}{k_1}$$

The maximal velocity is:

$$V_{max} = k_2 [E]_T$$

The initial velocity (before 10% of substrate has been converted):

$$v_o = \frac{V_{max} [S]}{K_M + [S]}$$
The Michaelis-Menten equation

The Michaelis-Menten equation is the basic equation of enzyme kinetics:

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

It describes a rectangular hyperbola.
A blot of the initial velocity $v_o$ of a simple enzymatic reaction versus the substrate concentration $[S]$. A graph shows the relationship between the initial velocity and substrate concentration, with $V_{max}$, $v_o$, and $K_M$ labels.
The Michaelis constant has a simple operational definition

At the substrate concentration at which \([S] = K_M\);
\[ v_o = \frac{V_{max}}{2} \]

So that \(K_M\) is the substrate concentration at which the reaction velocity is half-maximal

Therefore, if an enzyme has a small \(K_M\) value, it achieves maximal catalytic efficiency at low substrate concentration

\(K_M\) depends on pH, temperature, is also a measure for affinity of E to substrate
SAMPLE CALCULATION 12-3

An enzyme-catalyzed reaction has a $K_M$ of 1 mM and a $V_{\text{max}}$ of 5 nM $\cdot$ s$^{-1}$. What is the reaction velocity when the substrate concentration is (a) 0.25 mM, (b) 1.5 mM, or (c) 10 mM?

Use the Michaelis–Menten equation (Eq. 12-25):

(a) $v_o = \frac{(5 \text{ nM} \cdot \text{s}^{-1})(0.25 \text{ mM})}{(1 \text{ mM}) + (0.25 \text{ mM})}$

$\quad = \frac{1.25 \text{ nM} \cdot \text{s}^{-1}}{1.25}$

$\quad = 1 \text{ nM} \cdot \text{s}^{-1}$

(b) $v_o = \frac{(5 \text{ nM} \cdot \text{s}^{-1})(1.5 \text{ mM})}{(1 \text{ mM}) + (1.5 \text{ mM})}$

$\quad = \frac{7.5 \text{ nM} \cdot \text{s}^{-1}}{2.5}$

$\quad = 3 \text{ nM} \cdot \text{s}^{-1}$

(c) $v_o = \frac{(5 \text{ nM} \cdot \text{s}^{-1})(10 \text{ mM})}{(1 \text{ mM}) + (10 \text{ mM})}$

$\quad = \frac{50 \text{ nM} \cdot \text{s}^{-1}}{11}$

$\quad = 4.5 \text{ nM} \cdot \text{s}^{-1}$

Note: When units in the numerator and denominator cancel it is unnecessary to convert them to standard units before performing the calculation.
\( K_{cat}/K_M \) is a measure of catalytic efficiency

The catalytic constant of an enzyme is defined as:

\[
k_{cat} = \frac{V_{max}}{[E]_T}
\]

This is also known as the turnover number of an enzyme, because it corresponds to the number of reactions that one active site catalyzes per unit time, see table.

For simple enzymes/reactions, \( k_{cat} = k_2 \)

Note: \( k_{cat} \) is a constant, but \( V_{max} \) depends on the amount of enzyme in the reaction.
$K_{cat}/K_M$ is a measure of catalytic efficiency

When $S \ll K_M$, very little ES is formed and hence $[E] \sim [E]_T$

Then $K_{cat}/K_M$ is the apparent second-order rate constant of the enzymatic reaction

Then the rate of the reaction varies directly with how often enzyme and substrate encounter one another in solution

The most efficient enzymes have $K_{cat}/K_M$ values near the diffusion-controlled limit of $10^8-10^9$ M$^{-1}$ sec$^{-1}$

That is: the enzyme catalyzes the reaction almost every time it encounters a substrate. It is catalytically perfect!
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_M$ (M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ · s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholinesterase</td>
<td>Acetylcholine</td>
<td>$9.5 \times 10^{-5}$</td>
<td>$1.4 \times 10^4$</td>
<td>$1.5 \times 10^8$</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>CO$_2$</td>
<td>$1.2 \times 10^{-2}$</td>
<td>$1.0 \times 10^6$</td>
<td>$8.3 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td>HCO$_3^-$</td>
<td>$2.6 \times 10^{-2}$</td>
<td>$4.0 \times 10^5$</td>
<td>$1.5 \times 10^7$</td>
</tr>
<tr>
<td>Catalase</td>
<td>H$_2$O$_2$</td>
<td>$2.5 \times 10^{-2}$</td>
<td>$1.0 \times 10^7$</td>
<td>$4.0 \times 10^8$</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>$N$-Acetylglycine ethyl ester</td>
<td>$4.4 \times 10^{-1}$</td>
<td>$5.1 \times 10^{-2}$</td>
<td>$1.2 \times 10^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$N$-Acetylvaline ethyl ester</td>
<td>$8.8 \times 10^{-2}$</td>
<td>$1.7 \times 10^{-1}$</td>
<td>$1.9$</td>
</tr>
<tr>
<td></td>
<td>$N$-Acetyltirosine ethyl ester</td>
<td>$6.6 \times 10^{-4}$</td>
<td>$1.9 \times 10^2$</td>
<td>$2.9 \times 10^5$</td>
</tr>
<tr>
<td>Fumarase</td>
<td>Fumarate</td>
<td>$5.0 \times 10^{-6}$</td>
<td>$8.0 \times 10^2$</td>
<td>$1.6 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Malate</td>
<td>$2.5 \times 10^{-5}$</td>
<td>$9.0 \times 10^2$</td>
<td>$3.6 \times 10^7$</td>
</tr>
<tr>
<td>Urease</td>
<td>Urea</td>
<td>$2.5 \times 10^{-2}$</td>
<td>$1.0 \times 10^4$</td>
<td>$4.0 \times 10^5$</td>
</tr>
</tbody>
</table>
Isotopic Labeling

Laborytory useful to label molecules with isotopes (stable/unstable) to detect them later on. Frequently replaced by fluorescent tags.

Radioactive isotopes, radionuclides,

Nucleic acid frequently labeled by $^{32}$P,

Cell growth by incorporation of $^3$H-tymidine

Proteins by incorporation of $^{35}$S-Met
# Commonly used radionuclides

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life</th>
<th>Type of Radiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H</td>
<td>12 years</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{14}$C</td>
<td>5715 years</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{24}$Na</td>
<td>15 hours</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{32}$P</td>
<td>14 days</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{35}$S</td>
<td>87 days</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{40}$K</td>
<td>$1.25 \times 10^9$ years</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{45}$Ca</td>
<td>163 days</td>
<td>$\beta$</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>59 days</td>
<td>$\gamma$</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>8 days</td>
<td>$\beta, \gamma$</td>
</tr>
</tbody>
</table>

$^a\beta$ particles are emitted electrons and $\gamma$ rays are emitted photons.
Detection of Isotopes

Geiger counter measure radiation-induced ionization of a gas is not sensitive enough to detect \(^3\)H or \(^{14}\)C.

Liquid scintillation is more sensitive, radiation induces fluorescence, light emission is measured by PMT.

Autoradiography, immobilized radioactive substance (gel, membrane) is exposed to X-ray film, dark staining, replaced by phosphorimagers.

Microradiography, cover sample with photographic emulsion and examining under microscope.
RNA probe hybridized to Drosophila polytene chromosomes for Gene mapping
C) Kinetic data can provide values of $V_{\text{max}}$ and $K_M$

$V_{\text{max}}$ is difficult to determine from the classical $v_o$ versus [S] blot because $v_o$ only asymptotically reaches $V_{\text{max}}$

In practice the values for $V_{\text{max}}$ and $K_M$ are determined from a double-reciprocal blot (Lineweaver-Burk), which uses the reciprocal of the Michaelis-Menten equation

$$\frac{1}{v_o} = \left( \frac{K_M}{V_{\text{max}}} \right) \frac{1}{[S]} + \frac{1}{V_{\text{max}}}$$

This is a linear equation
A double-reciprocal (Lineweaver-Burk) plot

\[ \frac{1}{v_o} \]

\[ \frac{1}{V_{\text{max}}} \]

\[ \frac{1}{[S]} \]

Slope = \( \frac{K_M}{V_{\text{max}}} \)

\[ [S] = 0.5 \, K_M \]

\[ [S] = 5 \, K_M \]
SAMPLE CALCULATION 12-4

Determine $K_M$ and $V_{\text{max}}$ for an enzyme from the following data using Eq. 12-29:

<table>
<thead>
<tr>
<th>[S] (mM)</th>
<th>$v_o$ (μM · s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
</tr>
<tr>
<td>5</td>
<td>6.3</td>
</tr>
<tr>
<td>10</td>
<td>7.6</td>
</tr>
<tr>
<td>20</td>
<td>9.0</td>
</tr>
</tbody>
</table>

First, convert the data to reciprocal form ($1/[S]$ in units of mM$^{-1}$, and $1/v_o$ in units of μM$^{-1} · s$). Next, make a plot of $1/v_o$ versus $1/[S]$. The $x$- and $y$-intercepts can be estimated by extrapolation of the straight line or can be calculated by linear regression. According to Eq. 12-29 and Fig. 12-4, the $y$-intercept, which has a value of $\sim 0.1$ μM$^{-1} · s$, is equivalent to $1/V_{\text{max}}$, so $V_{\text{max}}$ (the reciprocal of the $y$-intercept) is 10 μM · s$^{-1}$. The $x$-intercept, $-0.33$ mM$^{-1}$, is equivalent to $-1/K_M$, so $K_M$ (the negative reciprocal of the $x$-intercept) is equal to 3.0 mM.
Steady state kinetics cannot unambiguously establish a reaction mechanism

Complex enzymatic reaction mechanisms are not revealed by steady state kinetics

\[ E + S \rightleftharpoons ES \rightleftharpoons EX \rightleftharpoons EP \rightleftharpoons E + P \]
Steady state measurements in this example mean that all the pipes are filled with water and changes in Input pressure are measured against changes in Output pressure.
D) Bisubstrate reactions follow one of several rate equations

Enzymatic reactions involving two substrates are very common (~60%):

\[
\begin{align*}
A + B & \iff E \\
P + Q & \iff \text{E}
\end{align*}
\]

For example transfer reactions:

\[
\begin{align*}
P \rightarrow X + B & \iff \text{E} \\
P + B & \iff B \rightarrow X
\end{align*}
\]

Or oxidation-reduction reactions: i.e. alcohol dehydrogenase
Some bisubstrate reactions

(a)

\[ R_1-C-NH-R_2 + H_2O \overset{\text{trypsin}}{\longrightarrow} R_1-C-O^- + H_3N-R_2 \]

Polypeptide

(b)

\[ CH_3-C-OH + NAD^+ \overset{\text{alcohol dehydrogenase}}{\longrightarrow} CH_3-CH + NADH \]
Sequential reactions occur via single displacements

Reactions in which all substrates must combine with the enzyme before a reaction can occur and products can be released are known as sequential reactions (= single displacement reactions)

Can be subclassified into ordered mechanism, with defined order of substrate binding, i.e. bdg of first substrate only allows binding of the next

Or random mechanism with random substrate bdg, i.e. two independent substrate bdg sites
Cleland notation: substrates: A, B, etc. products: P, Q, etc.

A is leading, B is following substrate; many NAD+ requiring dehydrogenases follow an ordered bisubstrate mechanism
Example of a random bisubstrate reactions, i.e. some dehydrogenases and kinases (transfer phosphate group from ATP to protein)
Ping pong reactions occur via double displacements

Group transfer reactions in which one or more products are released before all substrates have been added are known as ping pong reactions = double displacement reactions.

Note that the two substrates A, and B do not encounter each other on the enzyme.
Bisubstrate mechanisms can be distinguished by kinetic measurements.

Rate equations for bisubstrate reactions are more complicated than those of single substrate reactions.

Contain as many as 4 kinetic constant versus 2 for Michaelis-Menten ($V_{\text{max}}$ and $K_M$).
2) Enzyme Inhibition

Many substances alter the activity of an enzyme by combining with it to alter (i) substrate binding or (ii) turnover number.

Substances that reduce the activity of an enzyme are inhibitors.

Pharmaceutically important, i.e. AIDS drugs inhibit viral enzymes (DNA/RNA Polymerase).
Enzyme Inhibition

Irreversible inhibitors, inactivate the enzyme, for example chemicals that modify amino acids in active site of enzyme (Aspirin)

Reversible inhibitors, may structurally resemble substrate but do not react
A) Competitive inhibition involves inhibitor binding at an enzyme’s substrate binding site.

A substance that directly competes with a normal substrate for the enzymes binding site is known as a competitive inhibitor.

Competitive inhibitor resembles normal substrate but does not react or reacts only slowly.
Example succinate dehydrogenase is inhibited by malate

\[
\begin{align*}
\text{Succinate} & \quad \overset{\text{succinate dehydrogenase}}{\rightarrow} \quad \text{Fumarate} \\
\text{Malonate} & \quad \overset{\text{succinate dehydrogenase}}{\rightarrow} \quad \text{NO REACTION}
\end{align*}
\]
Product inhibition

The product of a reaction, which is necessarily able to bind to the enzyme’s active site, may accumulate and compete with substrate for binding to the enzyme.

One mechanism through which the cell controls activity of its enzymes.
Transition state analogs

These are particularly effective inhibitors, compound that mimics the transition state and thus may bind stronger than the substrate

Example adenosine deaminase, $K_M$ is $3 \times 10^{-5} \text{ M}$

inosine acts as inhibitor with an inhibition constant of $K_I$ $3 \times 10^{-4} \text{ M}$ (dissociation constant for the enzyme-inhibitor binding)

But the transition state analog 1,6 Dihydroinosine has a $K_I$ of $1.5 \times 10^{-13} \text{ M}$
Adenosine deaminase

Adenosine

$K_M$ is $3 \times 10^{-5}$ M

Inosine

$K_I$ is $3 \times 10^{-4}$ M

transition state analog $K_I$ of $1.5 \times 10^{-13}$ M

1,6-Dihydroinosine
The degree of competitive inhibition varies with the fraction of enzyme that has bound inhibitor.

General model for competitive inhibition

\[
\begin{align*}
E + S & \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} ES \\
& \overset{k_2}{\rightarrow} P + E \\
E + I & \rightleftharpoons EI \\
\end{align*}
\]

\[
K_I = \frac{[E][I]}{[EI]}
\]

NO REACTION
The degree of competitive inhibition varies with the fraction of enzyme that has bound inhibitor.

A competitive inhibition therefore reduces the concentration of free enzyme available for substrate binding: \([E]_T = [E] + [ES] + [EI]\)

Thus the Michaelis-Menten equation is modified by a factor \(\alpha\):

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

\(\alpha\) is a function of the inhibitors concentration and its affinity for the enzyme, cannot be less than 1:

\[
\alpha = 1 + \frac{[I]}{K_I}
\]
A plot of $v_o$ versus $[S]$ in the presence of different concentrations of a competitive inhibitor.

$$v_o = \frac{V_{max}}{1 + \frac{[I]}{K_i}}$$

Where $\alpha = 1 + \frac{[I]}{K_i}$.
The degree of competitive inhibition varies with the fraction of enzyme that has bound inhibitor.

The presence of $[I]$ makes $[S]$ appear to be less than what it really is.

$\alpha$ is a factor by which $[S]$ must be increased to overcome a competitive inhibitor.

As $[S]$ reaches infinity, $v_o$ approaches $V_{max}$ for any concentration of inhibitor.

Thus the inhibitor does not affect the enzymes turnover number!
Methanol poisoning

Is treated by ethanol

Methanol is converted by liver alcohol dehydrogenase to formaldehyde, which is highly toxic (blindness, death)

Ethanol competes with methanol for binding to alcohol dehydrogenase, slowing production of formaldehyde,

Large proportion of methanol will be excreted in urine, same principle for antifreeze poisoning
Alcohol dehydrogenase

\[
\begin{align*}
\text{Methanol} & \quad \text{Formaldehyde} \\
\text{Ethanol} & \quad \text{Acetaldehyde}
\end{align*}
\]
**$K_I$ can be measured**

Double reciprocal:

$$v_o = \frac{V_{\text{max}} [S]}{\alpha K_M + [S]}$$

$$\frac{1}{v_o} = \frac{\alpha K_M}{V_{\text{max}}} \frac{1}{[S]} + \frac{1}{V_{\text{max}}}$$

A plot of this equation is linear and has a slope of $\alpha K_M / V_{\text{max}}$, a $1/[S]$ interception of $-1/\alpha K_M$.

This double-reciprocal plot for varies concentrations of I intersect at $1/V_{\text{max}}$, a property that is diagnostic of competitive inhibition!
Lineweaver-Burk plot of competitively inhibited enzyme

Note intersection at $1/V_{\text{max}}$

Slope = $\alpha K_M/V_{\text{max}}$

$\alpha = 1 + \frac{[I]}{K_i}$
SAMPLE CALCULATION 12-5

An enzyme has a $K_M$ of 8 $\mu$M in the absence of a competitive inhibitor and a $K_M^{\text{app}}$ of 12 $\mu$M in the presence of 3 $\mu$M of the inhibitor. Calculate $K_1$.

First calculate the value of $\alpha$ when $K_M = 8 \mu$M and $K_M^{\text{app}} = 12 \mu$M:

\[ K^\text{app}_M = \alpha K_M \]

\[ \alpha = \frac{K^\text{app}_M}{K_M} \]

\[ \alpha = \frac{12 \mu\text{M}}{8 \mu\text{M}} = 1.5 \]

Next, calculate $K_1$ from Eq. 12-32:

\[ \alpha = 1 + \frac{[I]}{K_1} \]

\[ K_1 = \frac{[I]}{\alpha - 1} \]

\[ K_1 = \frac{3 \mu\text{M}}{1.5 - 1} = 6 \mu\text{M} \]
B) **Uncompetitive inhibition** involves inhibitor binding to the enzyme-substrate complex and not to the substrate binding site as with competitive inhibitor.

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

\[
K'_{i} = \frac{[ES][I]}{[ESI]} \\
\]

ESI \longrightarrow NO REACTION
<table>
<thead>
<tr>
<th>Type of Inhibition</th>
<th>Michaelis–Menten Equation</th>
<th>Lineweaver–Burk Equation</th>
<th>Effect of Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>$v_o = \frac{V_{\text{max}}[S]}{K_M + [S]}$</td>
<td>$\frac{1}{v_o} = \frac{K_M}{V_{\text{max}} [S]} + \frac{1}{V_{\text{max}}}$</td>
<td>None</td>
</tr>
<tr>
<td>Competitive</td>
<td>$v_o = \frac{V_{\text{max}}[S]}{\alpha K_M + [S]}$</td>
<td>$\frac{1}{v_o} = \frac{\alpha K_M}{V_{\text{max}} [S]} + \frac{1}{V_{\text{max}}}$</td>
<td>Increases $K_M^{\text{app}}$</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>$v_o = \frac{V_{\text{max}}[S]}{K_M + \alpha'[S]} = \frac{(V_{\text{max}}/\alpha') [S]}{K_M/\alpha' + [S]}$</td>
<td>$\frac{1}{v_o} = \frac{K_M}{V_{\text{max}} [S]} + \frac{\alpha'}{V_{\text{max}}}$</td>
<td>Decreases $K_M^{\text{app}}$ and $V_{\text{max}}^{\text{app}}$</td>
</tr>
<tr>
<td>Mixed (noncompetitive)</td>
<td>$v_o = \frac{V_{\text{max}}[S]}{\alpha K_M + \alpha'[S]} = \frac{(V_{\text{max}}/\alpha') [S]}{(\alpha/\alpha')K_M + [S]}$</td>
<td>$\frac{1}{v_o} = \frac{\alpha K_M}{V_{\text{max}} [S]} + \frac{\alpha'}{V_{\text{max}}}$</td>
<td>Decreases $V_{\text{max}}^{\text{app}}$, may increase or decrease $K_M^{\text{app}}$</td>
</tr>
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$\alpha = 1 + \frac{[I]}{K_i}$ and $\alpha' = 1 + \frac{[I]}{K_i'}$
Lineweaver-Burk plot of uncompetitively inhibited enzyme

Note that all lines have identical slope of $\frac{K_M}{V_{\text{max}}}$.
Uncompetitive inhibition

Note that adding more substrate does not reverse the effect of the uncompetitive inhibitor, in contrast to what is observed with a competitive inhibitor.

Uncompetitive inhibitors need not to resemble the substrate but distort the active site, thereby rendering the enzyme catalytically inactive.
C) Mixed inhibition involves inhibitor binding to both the free enzyme and the enzyme-substrate complex

\[
\begin{align*}
E + S & \xrightleftharpoons[k_{-1}]{k_1} ES \\
+ & + \\
I & I
\end{align*}
\]

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

Mixed inhibition = noncompetitive inhibition
Lineweaver-Burk plot of a mixed inhibition

\[
\frac{1}{v_o} = \frac{1 - \frac{\alpha'}{(\alpha - 1)K_M}}{\alpha - \frac{\alpha'}{(\alpha - 1)V_{max}}} 
\]

- \( \frac{\alpha'}{\alpha K_M} \)

Increasing [I]

\( \alpha = 2.0 \)  
\( \alpha' = 1.5 \)

\( \alpha = 1.5 \)  
\( \alpha' = 1.25 \)

\( \alpha = \alpha' = 1 \) (no inhibitor)

Slope = \( \alpha K_M / V_{max} \)

\( \alpha = 1 + \frac{[I]}{K_i} \)  
\( \alpha' = 1 + \frac{[I]}{K_i} \)
Irreversible Inhibition

The kinetics of an inactivator (irreversible inhibitor) resembles that of a pure noncompetitive inhibitor because the inactivator reduces the concentration of functional enzyme at all substrate concentrations. $V_{\max}$ decreases and $K_M$ is unchanged.
HIV Enzyme Inhibitors

The human immunodeficiency virus causes acquired immunodeficiency syndrome (AIDS) by infecting and destroying the host’s immune system.

Viral RNA genome is transcribed into DNA by reverse transcriptase, DNA is the integrated into host genome.

Viral proteins are synthesized as large precursors: polyproteins, processed by HIV protease.

No effective vaccine! => inhibitors
Inhibitors of reverse transcriptase

Archetype is AZT (3’-azido-3’-deoxythymididine; Zidovudine): taken up by cells, phosphorylated and incorporated into DNA but does not support chain elongation because it lacks 3’ OH.

Cellular polymerases have low affinity for this drug but reverse transcriptase has high affinity!

3’-Azido-3’-deoxythymididine (AZT; Zidovudine)  2’,3’-Dideoxycytididine (ddC, Zalcitabine)
HIV particles budding from a lymphocyte
Inhibitors of HIV protease

HIV protease is a homodimer of 99-residue subunits. Mechanistically belongs to aspartic protease (as pepsin does [gastric protease operates at low pH]).

Cleaves Phe-Pro or Tyr-Pro, peptidomimetic drugs, ritonavir, saquinavir contain bulky groups that bind to active site, mimic tetrahedral transition state.
Inhibitors of HIV protease

HIV protease substrate

Saquinavir

Ritonavir

$K_I = 0.40 \text{ nM}$

$K_I = 0.015 \text{ nM}$
HIV Inhibitors

However, both classes of drugs have side-effects: they need to be taken several times a day over many years.

Nucleoside analogs for example interfere with rapidly proliferating cells (replication) such as bone marrow cells that give rise to erythrocytes.

Acquired resistance also limits effectiveness of these drugs, rapid occurrence of mutated forms of the virus.

No magic bullet drug, combinatorial therapy.
3) Control of Enzyme Activity

Metabolic processes must be coordinated by regulation of enzyme activities. Same for changes in environmental conditions. Two ways:

1. **Control of enzyme availability:** Amount of enzyme depends on its rate of synthesis and degradation, can be altered within minutes (bacteria) to hours (eukaryotes)

2. **Control of enzyme activity:** can be controlled through allosteric effectors (see hemoglobin for example) and covalent modifications (phosphorylation)
A) Allosteric control involves binding at a site other than the active site

Allosteric control of aspartate transcarbamoylase (ATCase) from E. coli.

First and unique step in pyrimidine synthesis

Both substrates bind cooperatively to enzyme
The feedback inhibition of ATCase regulates pyrimidine synthesis

**Allosteric inhibition** of ATCase by cytidine triphosphate (CTP), a pyrimidine (feedback inhibition)

**Allosteric activation** by adenosine triphosphate (ATP), a purine nucleotide
Kinetics of the ACTase reaction

Sigmoidal rather than hyperbolic curve -> cooperative substrate binding

Allosteric effectors shift curve to the right or left
Regulation of ACTase activity

Through this feedback inhibition and allosteric activation, ACTase coordinates the rates of purine and pyrimidine nucleotides, which are required in roughly equal amounts (important also to keep error rate of DNA polymerase low!)
Allosteric changes alter ACTase's substrate-binding site

ACTase (300 kD), subunit composition $c_6r_6$, catalytic and regulatory subunits

Catalytic subunits arranged as two sets of trimers ($c_3$) in complex with three sets of regulatory dimers ($r_2$)

Each regulatory dimer contacts two catalytic subunits in different $C_3$ trimers

Isolated catalytic trimer are active and not affected by allosteric regulators (ATP or CTP)

Regulatory subunits reduce activity of cat. subunits
Structure of ACTase

- 2x Catalytic $c_3$ trimers
- 3x Regulatory $r_2$ dimers

CTP binding to $r_2$ dimers
Allosteric changes alter ACTase’s substrate-binding site

ATP preferentially binds to ATCase’s active (R or high substrate affinity) state -> increasing substrate af.

CTP as inhibitor binds to the inactive (T or low substrate affinity) state -> decreasing substrate af.

Similarly, the unreactive bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA) binds tightly to the R-state but not to T-state

Large conformational changes, mostly quarternary shifts associated with T -> R transition

Substrate binding to one subunit thus increases affinity in the other subunits
$N$-(Phosphonacetyl)-L-aspartate (PALA)

Carbamoyl phosphate + Aspartate
Tertiary and quarternary conformational changes in ACTase

(a) T state

(b) Substrate binding induces T -> R transition

(c) R state

No substrate
Low affinity
T state

Substrate binding induces T -> R transition
Allosteric transitions in other enzymes often resemble those of hemoglobin and ATCase

Allosteric enzymes are widely distributed and occupy key regulatory positions

Are almost always symmetrical containing at least two subunits in which quaternary changes communicate binding and catalytic effects among all active sites

Quaternary shifts are mostly rotations of subunits relative to one another

Secondary structures are largely preserved in T -> R transitions
B) Control by covalent modification usually involves protein phosphorylation

In addition to allosteric interactions, many enzymes are regulated through covalent modification.

Most common in eukaryotes: phosphorylation and dephosphorylation, phosphate group on Thr, Ser, Tyr (about 30% of human proteins).

By protein kinases, protein phosphatase.
A phosphorylation / dephosphorylation cycle
Example: glycogen phosphorylase

Catalyzes **phosphorolysis** (bond cleavage by substitution of a phosphate group) of glycogen $\alpha$ (1->4)-linked glucose to **glucose-1-phosphate** (G1P)

\[
\text{Glycogen} + \text{Pi} \leftrightarrow \text{glycogen} + \text{G1P}
\]

(n residues) \hspace{1cm} (n-1 residues)

Rate determining step in glycolysis

Mammals have three isoenzymes muscle, brain and liver
Structure of rabbit muscle phosphorylase

Muscle glycogen phosphorylase is a homo-dimer of 842 Aa
Phosphorylated form, phosphorylase a (ser14-P)
Dephospho form, phosphorylase b
Phosphorylation and dephosphorylation can alter enzymatic activity in a manner that resembles allosteric control.

The glycogen phosphorylase has two conformational states R (active), T (inactive, substrate access blocked).

Phosphorylation of Ser 14 promotes T → R transition, the R form, now responds to allosteric effectors such as AMP.

ATP and glucose-6-phosphate (G6P) bind to T state of phosphorylase b and inactivate the enzyme.
Conformational changes in glycogen phosphorylase

Phosphorylase b

Ser 14, AMP

T state

R state

Phosphorylase a
The control of glycogen phosphorylase activity

- T form (inactive)
- R form (active)

- ATP and/or G6P
- AMP
- Glucose

2 ATP \rightarrow 2 P_i 
2 ADP \rightarrow 2 H_2O 

phosphorylase kinase 
phosphoprotein phosphatase
Cascades of protein kinases enable sensitive responses to metabolic needs

Who controls the regulators?

That is the activity of the protein kinases and phosphatases?

Signal amplification by cascades of kinases, small change, stimuli -> large response
4) Drug Design

Most of the drugs in today's use were developed over the last 30 years, exceptions: digitalis (heart stimulants), quinine (malaria), mercury (syphilis)

How are new drugs discovered?

Mostly by screening of compound libraries, using an in vitro assay with the purified enzyme -> determining $K_I$

Results in lead compound (good candidate has a dissociation constant of 1 $\mu$M)

Chemical modification of lead compound to improve its pharmacological properties (5-10'000)
Quinine and chloroquine

Antimalaria drugs, share quinoline ring system

Pass through cell membranes and inhibit heme crystallization/storage in parasite (Plasmodium)
Structure-based drug design accelerates drug discovery

Since mid 1980s, rational drug design, based on protein structure

Model hydrogen bonding donors and acceptors, cavities etc.

Used to develop analgesics (pain relievers) Celebrex and Vioxx, HIV inhibitors
Combinatorial chemistry and high-throughput screening are useful drug discovery tools.

Rapid and cheap synthesis of large numbers of related compound that can then be used for robotic high throughput screening (but be aware: bullshit in will result in bullshit out!)

The combinatorial synthesis of arylidene diamides: if ten different variants of each R group are used in the synthesis, then 1000 different derivatives will be synthesized.
B) A drug's bioavailability depends on how it is absorbed and transported in the body.

The in vitro assay to uncover lead compound is only the first step in the drug discovery process.

Besides causing the desired response in its isolated target protein, a useful drug must be delivered in sufficiently high concentration to this protein where it resides in the human body.
Bioavailability / Pharmacokinetics

For example, orally delivered drugs:

1) pass through the stomach, must be chemically stable
2) must be absorbed from gastrointestinal tract, from bloodstream must bass through cell membrane etc.
3) Should not bind to other substances, lipophilic substances for example are absorbed by plasma proteins and fat tissue
4) Must survive detoxification reactions for xenobiotics by liver enzymes
5) Avoid rapid excretion by the kidney
6) Must pass from capillaries to target tissue
7) May cross blood-brain barrier
8) Pass through the plasma membrane

These are Pharmacokinetic parameters that define bioavailability
C) Clinical trials test for efficacy and safety

Successful drug must be safe and efficacious in humans
Test first in animals
Test in humans through clinical trials, monitored by FDA (food and drug administration, USA)
Three increasingly detailed and expensive phases:

Phase I, designed to test safety of drug candidate and dosage range, method, and frequency
20-100 volunteers, or volunteer patients with target disease
Clinical trials test for efficacy and safety

**Phase II**, test drug efficacy against target disease,
100-500 volunteer patients, refine dosage, check for side effects
single bind tests against placebo

**Phase III**, monitors adverse reactions from long-term use and confirms efficacy, 1000-5000 patients
double blind test

Only 5 out of 5000 drug candidates that enter preclinical trials (3 years) reach clinical trials (7-10 years), of these, only 1 will be approved (cost 300 Mio $), majority fail in Phase II,
Clinical trials test for efficacy and safety

Phase IV, post-marketing surveillance, if 1 in 10'000 individuals show life-threatening side effects, it will be taken from market (e.g. Vioxx in 2004, etc.)
Cytochromes P450 are often implicated in adverse drug reactions.

Why can a drug be tolerated well by many but show dangerous effects in few?

Individual differences in drug tolerance due to differences in disease stage, other drugs taken, age, sex and environmental factors.

Cytochromes P450 function in large part to detoxify xenobiotics, participate in metabolic clearance of majority of drugs.
Cytochromes P450 are often implicated in adverse drug reactions

Cytochromes P450 constitute a superfamily of heme-containing enzymes present in nearly all organisms

57 isoenzymes in human genome, eg. CYP2D6
-> + polymorphic variants !

Monooxygenases embedded in the ER

\[ \text{RH} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O} \]

more water soluble

R: steroids, PCB, drugs, tobacco smoke, broiled meat etc...
Cytochromes P450 are often implicated in adverse drug reactions

Cytochromes P450 can also convert non-harmful drugs into highly toxic compounds

Example acetaminophen (antipyretic /fever reducer) at low dose (1.2g/day) but at > 10g/day is highly toxic
The metabolic reactions of acetaminophen

Acetaminophen (N-acetyl-p-aminophenol) is oxidized by cytochrome P450 to acetimidoquinone. 95% of the acetaminophen is conjugated to glucuronide or sulfate, while 5% is toxic and converted into the acetaminophen-glutathione conjugate. Glutathione (γ-L-Glutamyl-L-cysteinyl-glycine) is used to detoxify the acetaminophen-glutathione conjugate.