Enzymes

Objectives
I. Define the function and properties of enzymes
   A. Substrate specificity
      1. Absolute specificity
      2. Group specificity
      3. Linkage specificity
      4. Stereo specificity
   B. Product specificity
   C. Rate
      1. Best man made catalyst versus best enzyme.
II. Describe the classification of enzymes based on reaction type as defined by The Enzyme Commission of The International Union of Biochemistry and Molecular Biology.
   A. The six major classes.
      1. The important subclasses
III. Enzyme Nomenclature:
   A. Most are named by naming the substrate of the reaction and the type of reaction catalyzed.
   B. Synthases and Synthetases named by naming the product followed by Synthase or Synthetase.
   C. Some enzymes still go by historically old common names.
   D. Recognize the correlation between the name of an enzyme and the function of the enzyme.
IV. Enzymes as conjugated proteins:
   A. Compare and contrast the roles of cofactors, coenzymes, and cosubstrates on enzyme activity.
   B. Apoprotein / Apoenzyme versus Holoprotein / Holoenzyme.
   C. Nature / Source of the prosthetic group.
V. The “Sites”
   A. Discuss the role of the Substrate Site and its importance to enzyme specificity/activity.
   B. Discuss the role of the Active Site and its importance to enzyme specificity/activity.
   C. What are they?
   D. Where are they?
VI. Describe the effect that an enzyme has on the activation energy of a reaction.
VII. Compare and contrast the Lock and Key theory of enzyme action with the Binding Energy / Induced Fit theory.
   A. Where does the Lock and Key theory break down?
VIII. Possible mechanisms of enzyme action.
   A. Proximity Effect
   B. Nucleophilic
   C. Electrophilic
   D. Acids - Base Catalysis
   E. Covalent Intermediates
IX. How does pH and temperature affect the rate of an enzyme catalyzed reaction.
X. Understand the effect of substrate concentration on enzyme catalyzed reactions.
   A. Saturation Kinetics; Enzyme is saturated with substrate
   B. Maximum Velocity (Maximum Rate) $V_{\text{Max}}$
XI. Equations fit to Enzyme Kinetics and how they describe the action of an enzyme at a cellular level.
A. Michaelis-Menton Equation
B. Lineweaver-Burke Plot
C. Turnover Number - $V_{\text{Max}}/\text{[ET]}$ or $K_{\text{cat}}$
D. Specificity Constant - $K_{\text{cat}}/K_m$

XII. Discuss the mechanisms by which certain substances inhibit enzyme activity.
A. Describe and distinguish between irreversible and reversible inhibition
   1. Explain the role of acetylcholinesterase in nerve transmission
   2. The effects of diisopropylfluorophosphate on acetylcholinesterase.
   3. The effect of aspirin on the role of cyclooxygenase.
B. Describe and distinguish between competitive, uncompetitive, mixed type, and noncompetitive reversible inhibition.
   1. Where on the enzyme does the reversible inhibitor bind?
   2. How does this binding interaction bring about enzyme inhibition (mechanism of action)?
   3. Effect the inhibitor has on the $V_{\text{Max}}$ and $K_m$ of the enzyme.

XIII. Understand the regulatory mechanisms of enzyme activity.
A. Substrate availability
B. Equilibrium considerations
C. Product inhibition
   1. Feed back inhibition
D. Protein / enzyme synthesis
E. Irreversible covalent modification
   1. Zymogen formation and activation
   2. Mechanism of activation
F. Reversible covalent modification
   1. The actions of protein kinases
   2. The actions of phosphoprotein phosphohydrolases (protein phosphatases)
G. Allosteric enzymes (allosterism)
   1. Structure of an allosteric enzyme
      a) Subunit structure
      b) Binding sites
   2. Kinetics of an allosteric enzyme
      a) Tense (T) state versus the relaxed (R) state
   3. Positive allosteric effectors versus negative allosteric effectors
      a) Their effects on the T and/or R state
   4. Models of allosterism
      a) Concerted Model versus Sequential Model
   5. Hemoglobin as a model allosteric protein
      a) Subunit structure
      b) Oxygen binding sites
      c) Allosteric effectors and how they modulate the oxygen carrying activity of the molecule.

General Considerations

Enzyme comes from the Greek “enzymos” meaning “leavened”. Most enzymes are proteins. All enzymes
are catalysts. A Catalyst accelerates the approach of a chemical reaction toward equilibrium without changing the equilibrium position. Enzymes provide cells with the ability to exert kinetic control over thermodynamic potentiality.

A bit of nomenclature. Chemical reactions in general, organic, inorganic, and/or physical chemistry convert reactants to products. Enzyme catalyzed reactions convert a SUBSTRATE or SUBSTRATES to products.

When compared to man made catalysts, enzymes have several unique properties.

1. They are extremely Specific. Enzymes will catalyze reactions involving only one substrate or a very small group of structurally related substrates. Enzymes show
   1. Absolute Specificity - working upon only a single substrate
   2. Group Specificity - working upon a related group of molecules containing a specific functional group.
   3. Linkage Specificity - working on molecules that contain a specific type of chemical bond.

2. Enzymes are Stereospecific. If a molecule exists as a pair of enantiomers, the enzyme will use only one of the pair as substrate and produce only one of the pair as the product. For example, the enzymes that are involved in amino acid metabolism and/or protein synthesis will only utilize the L-amino acids as substrates.

3. Reactions catalyzed by enzymes produces only the desired product(s). Wasteful side reactions do not occur during enzyme catalyzed reactions.

4. Enzymes are very much faster than man made catalysts. The best man made catalysts increase reaction rates about $10^7$ fold. On average, man made catalysts increase reaction rates $10^2$ to $10^4$ fold. Enzymes can enhance the rate from $10^{17}$ to $10^{20}$ fold when compared to the uncatalyzed reaction.

The Enzyme Commission of The International Union of Biochemistry and Molecular Biology has divided enzymes into six major classes based upon the type of reaction catalyzed. Within each major class there are subclasses and within each subclass there are sub-subclasses. The commission assigns each individual enzyme a series of four numbers to uniquely identify a particular enzyme.

The six major classes are:

1. OXIDOREDUCTASES catalyze oxidation reduction reactions. Subclasses within this class include: DEHYDROGENASES, REDUCTASES, OXYGENASES, OXIDASES, and PEROXIDASES.

2. TRANSFERASES catalyze group transfer reactions. They transfer an atom or functional group from a donor molecule to an acceptor molecule. Functional groups transferred include amino, carbonyl, methyl, phosphoryl, and acyl. For example KINASES, a subclass of transferases, catalyze the transfer of a phosphoryl ($–PO_3^{–2}$) group from a phosphate donor (usually ATP) to a phosphate acceptor molecule. A phosphoester is formed between the phosphoryl group and a –OH group on the acceptor. AMINOTRANSFERASES transfer amino groups, METHYLTRANSFERASES transfer methyl groups, etc.
3. **HYDROLASES** catalyze hydrolysis reactions. This class of enzymes catalyze the hydrolytic cleavage of ester, amide, or some other water susceptible bond. They catalyze the addition of water (H–OH) across the bond. Subclasses include ESTERASES, PHOSPHATASES, and PROTEASES.

4. **LYASES** catalyze nonhydrolytic and nonoxidative elimination reactions to form double bonds (C=C, C=O, or C=N) or they catalyze the addition of a group (XY) across a double bond. **DECARBOXYLASES**, **DEHYDRASES**, and **DEAMINASES** are subclasses that catalyze the removal of a group to form a double bond. The **SYNTHASES** are a subclass of lyases that add substrates across a double bond.

5. **ISOMERASES** catalyze isomerization reactions, the rearrangement of groups around a central atom. They change the configuration around some central atom in the molecule. **MUTASES** and **EPIMERASES** are two important subclasses.

6. **LIGASES** catalyze ligation reactions, the joining of two small molecules into one larger molecule. **SYNTHETASES** and **CARBOXYLASES** are subclasses. Reactions catalyzed by synthetases use an outside energy source, usually ATP, to drive the formation of the new chemical bond to completion.

Enzymes named using the Enzyme Commission of The International Union of Biochemistry and Molecular Biology conventions all end in the suffix “ase”. Enzymes have a unique four digit identification number and a two part **SYSTEMATIC NAME**. The Enzyme Commission also suggests a **RECOMMENDED NAME**, a shorter version of the systematic name, for common usage. Enzymes are named in one of two ways. The majority of enzymes are named by naming the substrate(s) of the reaction followed by the type of reaction catalyzed. For example:

- **E.C. 2.7.1.1**  
  **SYSTEMATIC NAME** - ATP:Hexose-6-phosphotransferase  
  **RECOMMENDED NAME** - Hexokinase

- **E.C. 2.7.1.2**  
  **SYSTEMATIC NAME** - ATP:Glucose-6-phosphotransferase  
  **RECOMMENDED NAME** - Glucokinase

- **E.C. 3.1.3.16**  
  **SYSTEMATIC NAME** - Protein-serine/threonine phosphatase  
  **RECOMMENDED NAME** - Protein Phosphatase

- **E.C. 6.5.1.1**  
  **SYSTEMATIC NAME** - DNA:ATP Ligase  
  **RECOMMENDED NAME** - DNA Ligase

Recommended names for some Transferase, Synthase, and Synthetase enzymes are the product followed by synthase or synthetase. For example:

- **E.C. 2.3.3.1**  
  **SYSTEMATIC NAME** - Acetyl-CoA:Oxaloacetate C-acetyltransferase  
  **RECOMMENDED NAME** - Citrate Synthase

catalyzes the synthesis of citrate from two smaller molecules by the addition of one molecule across a double bond present on the second molecule.

A few enzymes are named using archaic nonsystematic common names - For example:

- Chymotrypsin (E.C. 3.4.21.1)
- Trypsin (E.C. 3.4.21.4)
- Thrombin (Fibrinogenase E.C. 3.4.21.5), etc.
Cofactors, Cosubstrates, and Coenzymes

Some enzymes are conjugated proteins, they require a nonprotein prosthetic group for proper function. These prosthetic groups can be metal ions or small organic molecules. If the nonprotein part is a metal ion it is called a COFACTOR. Common cofactors include Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), Fe\(^{3+}\), Ca\(^{2+}\), Zn\(^{2+}\). If the nonprotein molecule necessary for enzymatic activity is a small organic molecule it is either a COENZYME or COSUBSTRATE. Coenzymes and Cosubstrates are often the metabolically active forms of the Vitamins. Coenzymes are covalently attached or very tightly bound to the protein. Cosubstrates are diffusible, moving around within the cell, associating with the enzyme when needed and diffusing away when not needed. Cofactors, Coenzymes, and Cosubstrates are all considered PROSTHETIC GROUPS. The protein without its necessary prosthetic group is called an APOENZYME. The active enzyme with its needed prosthetic group bound or covalently attached is called the HOLOENZYME.

Binding Sites

Enzymes are globular proteins. Within the three dimensional structure of the enzyme there are two important regions. The SUBSTRATE BINDING SITE is a noncontiguous subset of amino acid side chains within the protein that interacts with and binds the substrate. The amino acid side chains that comprise the substrate binding site are usually well separated along the 1\(^{\circ}\) structure of the protein. Substrate(s) bind to the amino acids in this site by the weak intermolecular interactions. The ACTIVE SITE is likewise a noncontiguous subset of amino acid side chains within the three dimensional protein structure. The side chains that comprise the active site are necessary participants in the catalytic process. Protein folding (2\(^{\circ}\) and 3\(^{\circ}\) structure) brings and holds these distant amino acid side chains in the proper location and conformation to form these sites.

General Mechanisms of Enzyme Action

Using thermodynamics the progress of a chemical reaction can be plotted on an energy diagram. The pool of reactants have an average kinetic energy. At any given instant there is a small percentage of molecules that will collide with sufficient energy to climb the energy barrier and reach the TRANSITION STATE (X\(^{*}\)). At the transition state there is a high probability that product will form. The energy required to reach the transition state is the FREE ENERGY OF ACTIVATION, \(\Delta G^\ddagger\). The rate of a chemical reaction, its kinetics, is
proportional to the concentration of molecules with sufficient energy to reach the transition state. Catalysts increase the rate of chemical reactions by lowering the free energy of activation. With the decreased activation energy more molecules have sufficient energy to climb the energy barrier and the rate of the reaction is increased in proportion to the activation energy decrease.

An enzyme, like all catalysts, increases the rate of a chemical reaction by lowering the activation energy. An enzyme lowers the activation energy by breaking the reaction into smaller steps. Thermodynamic calculations are made using the starting conditions of a system and the final conditions, the steps required (the path taken) to accomplish the task matters not at all. An enzyme catalyzed reaction can be broken up into a minimum of four steps.

\[
E + S \xrightarrow{1} ES \xrightarrow{2} EX^* \xrightarrow{3} EP \xrightarrow{4} E + P
\]

1. Substrate binds to the enzyme.
2. The substrate is brought to the transition state.
3. Product, bound to the enzyme, is formed.
4. The product dissociates from the enzyme.

Each of these “reaction steps” has a small activation energy. The sum of the activation energies for these steps is very much less than the activation energy of the uncatalyzed reaction.

Early studies with enzymes led (Hermann) Emil Fischer in 1890 to postulate that the three dimensional structure of the substrate binding site / active site is a special pocket or cleft with a three dimensional structure exactly complementary to the three dimensional structure of the substrate. Substrates fit into enzymes like a key fits into a lock or a hand fits into a glove. The LOCK and KEY analogy was a good first approximation. It did explain how enzymes recognize their substrate molecules. However, it did not explain two important facets of enzyme activity. If the substrate is exactly complementary to the substrate binding site where does the energy for the conversion of substrate to product come from. This complex would be an extremely stable low energy state and from an energetic point of view the enzyme would not have the internal energy to convert substrate to product. Second, how does the enzyme catalyze the reverse reaction? If the substrate is exactly complementary to the enzyme substrate/active site, the product could not be since it is a different molecule and has a different shape. So how does the product bind to the enzyme for the reverse reaction. *(Hermann) Emil Fischer in 1891 devised the Fischer Projection; a two dimensional representation of the three dimensional asymmetric organic molecule.*

The modern accepted notion of how enzymes work was first proposed by Polanyi (1921) and Haldane (1930). Pauling (1946) and Jencks (1970’s) subsequently modified and elaborated upon the hypothesis. These individuals independently hypothesized that the empty substrate binding site has a three dimensional structure complementary to the structure of the transition state and maximal binding between substrate and
enzyme occurs only when the substrate is in the transition state. Substrate initially binds to the substrate/active site by one or two weak intermolecular interactions; hydrogen bonds, salt bridges, and/or hydrophobic interactions. These initial interactions brings the substrate closer to other groups on the enzyme with which it can interact. These additional interactions between substrate and enzyme begin to force the substrate into a configuration that begins to resemble the transition state. Each additional binding interaction brings the substrate closer to other groups in the substrate/active site that can bind and brings the substrate closer to the transition state. When a maximal number of binding interactions have occurred the substrate has been contorted into the transition state.

The energy required to lower the activation energy comes from the weak intermolecular interactions between enzyme and substrate. Each weak interaction between enzyme and substrate liberates a small amount of energy that stabilizes the interaction. The Binding Energy, $\Delta G_B$, is the energy derived from the enzyme/substrate interaction. Binding Energy is the major source of free energy used by enzymes to lower the activation of the reaction. Two fundamental principles explain how enzymes use non-covalent binding energy:

1. Much of the catalytic power of enzymes is derived from the free energy released in forming numerous weak non-covalent interactions between an enzyme and its substrate. The binding energy also contributes to specificity.
2. Weak interactions are optimized. The maximum number have formed between the enzyme and the substrate in the transition state. Enzyme active sites are complementary to the transition states through which the substrates must pass as they are converted to products during the enzyme catalyzed reaction.

Daniel Koshland recognized that enzymes are conformationally dynamic molecules. The native state is not a single low energy conformation, the enzyme can adjust its shape to accommodate binding of other molecules. Koshland hypothesized that substrate binding by an enzyme is an interactive process and that the shape of the substrate site/active site of the enzyme is modified during the substrate binding process. In this hypothesis, the Induced Fit Hypothesis, the conformational changes can be as small as the movement of an amino acid side chain closer to the substrate or as large as the movement of entire domains within the enzyme molecule. Each of the numerous interactions between enzyme and substrate is energetically favorable. Induced fit aligns the amino acid residues that make up the substrate binding site and active site so that they coordinate with the transition state precisely and interact with the substrate and product less effectively.

The conformational change that the enzyme undergoes during substrate binding brings reactive groups on the substrate(s) close to one another and it brings the catalytic amino acid side chains of the active site into close proximity with the reacting species. This proper alignment of reacting groups in the substrate and
active site is termed the **PROXIMITY EFFECT**.

Once substrate has bound to the enzyme the amino acid side chains of the active site catalyze the reaction. In biochemistry, as in organic chemistry, there are two general reaction types:

1. **NUCLEOPHILIC** in which an electron rich group attacks an electron poor molecule.
2. **ELECTROPHILIC** in which an electron poor group attacks an electron rich molecule.

Nucleophiles usually have an unshared pair of electrons or a negative charge. Certain amino acid side chains fit the role of nucleophiles. These amino acids include Glu, Asp, Ser, His, Thr, Tyr, and Cys. On the other hand electrophiles usually have a positive charge. The side chains of the amino acids Lys, Arg, and His can fit the role of electrophiles, as do metal ion cofactors.

During the course of nucleophilic or electrophilic enzyme catalyzed reactions a **COVALENT INTERMEDIATE** is often transiently formed. These transient covalent intermediates form when the substrate molecule or some fragment of the substrate is covalently linked to the enzyme. This covalent complex is then broken down to the products by the attack of a second nucleophile, H₂O or OH⁻, or a second electrophile, H⁺. Formation of a covalent intermediate between the enzyme (catalyst) and some or all of the substrate splits the reaction up into several steps, and is one of the major types of catalysis - **COVALENT CATALYSIS**. Group transfer reactions catalyzed by the transferases, synthetases, synthases, hydrolases, and isomerases often use covalent catalysis.
A second major type of catalysis is **ACID - BASE CATALYSIS**, in which the rate acceleration is achieved by the transfer of a proton, $\text{H}^+$. The side chains of the amino acids, Glu, Asp, His, Lys, Arg, and to some extent Ser, Tyr, and Cys can act as acid-base catalysts donating a proton to or accepting a proton from the substrate molecule. Histidine side chains are very often involved in acid-base catalysis since the $\text{pK}_a$ of this side chain is near to the pH of biochemical systems. Metal ion cofactors near or in the active site stabilize the intermediates formed during acid-base catalysis by forming salt bridges with negatively charged intermediates that transiently form within the active site.

![Enzyme Kinetics](image)

**Enzyme Kinetics**

In typical kinetic experiments the appearance of product versus time is monitored. Because enzymes are such efficient catalysts, products appear extremely rapidly. The most valuable data is obtained during the initial phases of the reaction when the substrate concentration is high and very little, if any, product has formed. During the initial phase of the reaction, the initial rate or initial velocity ($v_0$) is measured.

The initial rate of an enzyme catalyzed reaction is dependent upon the enzyme concentration. Increasing enzyme concentrations results in a more rapid initial rate, decreasing enzyme concentrations results in slower initial rates. When the kinetics of a particular enzyme are studied a series of experiments are performed with the parameter to be tested varied. The initial velocity, $v_0$, of these reactions is then plotted against the varied parameter.

**Rate vs. Temperature & Rate vs. pH**

Central to the study of enzyme mechanisms is measuring the rate of the enzyme catalyzed reaction or Enzyme Kinetics. For an uncatalyzed chemical reactions the rate approximately doubles for every 10°C increase in temperature. With enzyme catalyzed reactions the rate increases with temperature up to a point. Beyond this point the rate decreases. Above a certain temperature the enzyme begins to denature. As the enzyme denatures it loses its native conformation and as the native structure is lost enzyme activity is lost. Structure and function are intimately related.
For uncatalyzed chemical reactions pH may affect the rate of the reaction or it may have no effect. Reactions with rates that vary with pH are those reactions that require an acid or base as a catalyst. With enzyme catalyzed reactions there is a sharp pH optimum. Above or below this pH optimum the enzyme loses activity. The change in activity on the up slope and down slope of these sharp pH optima indicate that a specific amino acid side change must be protonated or deprotonated in the native state for catalysis to occur. At the extremes of pH, very acidic or very basic, the enzyme loses activity because it is denatured.

Rate vs. Substrate Concentration

When the rate of an enzyme catalyzed reaction versus the substrate concentration is studied at low substrate concentrations the rate of the reaction increases rapidly as the substrate concentration increases. With the continued increase of substrate concentration, there comes a point where adding more substrate results in very little if any increase in the initial rate. At this point the enzyme is said to be SATURATED with substrate. At saturation every enzyme molecule is bound with substrate and there is “excess” substrate waiting to bind to the enzyme and be converted to product. At saturation the enzyme has attained its maximal velocity, or $V_{\text{max}}$.

Saturation kinetics is consistent with physical binding of substrate to enzyme as one of the initial steps of the reaction. A simplified equation for an enzyme catalyzed reaction can be written as:

$$E + S \underset{k^{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_2}{\overset{k_2}{\rightleftharpoons}} E + P$$

Biochemists assume that during the initial part of the reaction the concentration of ES is at a steady state, i.e., the concentration of ES is not changing over the course of the time studied. They also assume that very little product (P) has formed so the rate of the reverse reaction, $k_2$, can be ignored.
Numerous equations have been derived to describe the rectangular hyperbolic relationship between the initial rate, $v_0$, and substrate concentration $[S]$. The Michaelis-Menton equation was one of the first to adequately describe this relationship. The Michaelis-Menton equation is:

$$v_0 = \frac{V_{\text{max}} [S]}{[S] + \frac{k_{-1} + k_2}{k_1}}$$

Where $v_0 = \text{initial velocity}$

$[S] = \text{substrate concentration}$

$V_{\text{max}} = \text{maximal velocity at saturation; the rate of reaction at infinite [substrate]}$

$\frac{k_{-1} + k_2}{k_1} = \text{a group of constants (the rate constants of the individual reaction steps)}$.

These constants can be combined and a new constant can be defined. $K_m$, the Michaelis-Menton constant, is precisely defined as the rate constants of the individual reaction steps.

The Michaelis-Menton equation then simplifies to:

$$v_0 = \frac{V_{\text{max}} [S]}{[S] + K_m}$$

Experimentally, $K_m$ is equal to the substrate concentration that results in an initial velocity, $v_0$, equal to one half of $V_{\text{max}}$. $K_m$ has units of concentration, moles/liter or M.

Conceptually, the $K_m$ of an enzyme is a rough approximation of enzyme affinity for substrate. The smaller the numerical value for $K_m$ the higher the affinity of enzyme for substrate.

The Michaelis-Menton equation describes the relationship between $v_0$ and $[S]$ very well. $V_{\text{max}}$ and $K_m$ are useful constants for describing the properties of an enzyme. However, the Michaelis-Menton equation is not the most useful for obtaining these values precisely. $K_m$ and $V_{\text{max}}$ values must be extrapolated from a plot of the experimental data or from a graph of the equation.

A more useful equation for obtaining precise values of $K_m$ and $V_{\text{max}}$ is the Lineweaver-Burke or Double Reciprocal Plot. These investigators performed a linear transformation of the Michaelis-Menton equation and came up with the equation:

$$\frac{1}{v_0} = \left( \frac{K_m}{V_{\text{max}}} \right) \frac{1}{[S]} + \frac{1}{V_{\text{max}}}$$
When $1/v_0$ is plotted against $1/[S]$ a straight line results. This straight line has a slope equal to $K_m/V_{max}$; a Y-intercept of $1/V_{max}$; and an X-intercept of $-1/K_m$. The values for $K_m$ and $V_{max}$ can be directly and accurately obtained from the Lineweaver-Burke plot.

$V_{max}$ and $K_m$ are constants for a particular enzyme. They are not the best constants for describing or comparing enzymes because they are dependent upon the enzyme concentration employed during their determination. A change in enzyme concentration results in a corresponding change in the values of $V_{max}$ and $K_m$. There are two constants that are independent of enzyme concentration that are used extensively to describe and compare enzymes. The first of these constants is the TURNOVER NUMBER or $K_{cat}$.

$$K_{cat} = \frac{V_{max}}{[E_T]}$$

where $[E_T]$ = the total concentration of enzyme present in the experimental mixture. The TURNOVER NUMBER is the number of catalytic events per unit of time – units s$^{-1}$ or min$^{-1}$. The TURNOVER NUMBER is the number of product molecules formed per enzyme active site per unit of time.

The second important constant used to describe the properties of an enzyme is the SPECIFICITY CONSTANT.

$$\text{Specificity Constant} = \frac{K_{cat}}{K_m}$$

This constant measures the rate of an enzyme catalyzed reaction at low substrate concentrations and has units s$^{-1}$M$^{-1}$ or min$^{-1}$M$^{-1}$. In the cell the substrate concentration for most enzymes is at or below the $K_m$ of the enzyme. This constant measures the efficiency of an enzyme at cellular substrate concentrations. The higher the specificity constant, the more the enzyme "prefers" the substrate.

Enzyme Inhibitors

An INHIBITOR is a chemical compound that interacts with an enzyme and decreases its activity, decreases the rate of product formation. The chemical compound added to the enzyme can be a naturally occurring molecule or a XENOBIOTIC. Enzyme inhibitors are used in biochemistry to determine enzyme mechanisms. In pharmacology many of the modern drugs are enzyme inhibitors.

There are two types of enzyme inhibitors - IRREVERSIBLE and REVERSIBLE.

An IRREVERSIBLE INHIBITOR either binds exceedingly tightly to the enzyme or forms a covalent bond with one of the amino acid side chains necessary for catalysis. The classic example of an irreversible inhibitor is Diisopropyfluorophosphate. This compound irreversibly inhibits enzymes.
that contain serine residues in the active site by forming a covalent bond with the hydroxyl group of the serine side chain. Many Hydrolase enzymes (proteases & esterases) contain serine in their active sites and are inhibited by this compound. Diisopropylfluorophosphate is a deadly poison that kills by inhibiting Acetylcholine Esterase. Acetylcholine is a neurotransmitter. Acetylcholine Esterase is present in the synaptic space where it hydrolyzes acetylcholine into acetate and choline thereby stopping stimulation of the post synaptic neuron or muscle cell. When this enzyme is inhibited, acetylcholine is not destroyed and the post synaptic neuron continues to fire or the muscle cell continues to contract.

Aspirin (acetylsalicylic acid) is another example of an irreversible inhibitor. Aspirin irreversibly inhibits the enzyme Cyclooxygenase. Cyclooxygenase is a key enzyme in the synthesis of the eicosanoids. Among the many functions of the eicosanoids is the mediation of the inflammatory response; pain, swelling, redness, & heat. When aspirin inhibits Cyclooxygenase, the acetate group on the molecule is transferred to the hydroxyl group of the active site serine side chain. When the acetate group is attached to Cyclooxygenase the enzyme is inactivated, the eicosanoids are not synthesized, and the pain goes away.

Reversible inhibitors bind to enzymes by weak non-covalent interactions; H-bonds, salt bridges, hydrophobic interactions, and/or London forces. Since the binding is by weak forces the inhibitors are in equilibrium between the free form and a form bound to the enzyme.

\[
\text{Enzyme} + \text{Inhibitor} \rightleftharpoons \text{Enzyme}\cdot\text{Inhibitor Complex}
\]

\[
E + I \rightleftharpoons EI
\]

\(K_i\) is the dissociation constant for the EI complex. It is a measure of the affinity of the enzyme for the inhibitor. As the numerical value for \(K_i\) decreases the affinity of the enzyme for the inhibitor increases, similar to \(K_m\).

Reversible inhibitors come in three main types - Competitive, Uncompetitive, and Mixed Type. Noncompetitive inhibitors are a special type of Mixed Type inhibitors.

Competitive Inhibitors — \(EI \rightleftharpoons I + E + S \rightleftharpoons ES\)
COMPETITIVE INHIBITORS have a three dimensional structure similar to the normal substrate and/or to the transition state. These inhibitors bind to the enzyme at the substrate binding site. Once bound the enzyme cannot convert this molecule to “product” and the enzyme is inhibited. Since these inhibitors look like the substrate and bind at the same site as the substrate, they compete with the normal substrate for interaction with the enzyme. High concentrations of the normal substrate can over come the action of the inhibitor. High concentrations of substrate can out compete for binding to the substrate site, can get to the substrate site more often than the inhibitor. Examining the kinetic parameters of an enzyme treated with a competitive inhibitor reveals an apparent increase in the value of $K_m$ but no change in $V_{max}$. Apparently an increase in substrate is needed to over come the competition between inhibitor and substrate.

UNCOMPETITIVE, MIXED TYPE, and NONCOMPETITIVE INHIBITORS do not resemble the substrate and they do not bind to the substrate binding site. They bind to sites on the enzyme away from the substrate binding site. Increasing the concentration of the normal substrate will not over come the actions of these inhibitors. Their actions can be reversed only by diluting or removing the inhibitor. The distinction between these types of inhibitors are made on the basis of where they bind to the enzyme, when in the reaction sequence they bind to the enzyme, and the kinetics of the reaction in the presence of the inhibitor.

Uncompetitive Inhibitors — $E + S \rightleftharpoons ES + I \overset{K_i}{\underset{K_{i}}{\rightleftharpoons}} ESI$

UNCOMPETITIVE INHIBITORS bind only to the enzyme-substrate complex (ES) at a site away from the substrate site. This type of inhibitor either significantly slows or prevents the conformational changes in the enzyme required for induced fit and catalysis. Uncompetitive inhibitors result in a decrease in $V_{max}$ and an apparent decrease in the $K_m$. With some of the enzyme bound by both substrate and inhibitor, the amount of functional enzyme is reduced and the amount of substrate to half saturate is likewise reduced. The Lineweaver-Burke plots show a series of parallel lines shifted left when compared to the uninhibited reaction.

Mixed Type Inhibitors — $E + I \overset{K_i}{\underset{K_{i}}{\rightleftharpoons}} EI + S \rightleftharpoons EIS \text{ or } E + S \rightleftharpoons ES + I \overset{K_i}{\underset{K_{i}}{\rightleftharpoons}} ESI$
MIXED TYPE INHIBITORS also bind at a site away from the substrate site, but they can bind to either the free enzyme (E) or to the enzyme-substrate complex (ES). Mixed Type inhibitors have two $K_i$’s. $K_{ia}$ is measured when the inhibitor binds to the free enzyme whereas $K_{ib}$ is for the binding of the inhibitor to the ES complex. In Mixed Type inhibition $K_{ia}$ is numerically different from $K_{ib}$. It is postulated that Mixed Type inhibitors significantly slows substrate binding and once the substrate is bound they slow or prevent the conformational changes in the enzyme required for induced fit and catalysis. Mixed Type inhibitors result in a decrease in the measured $V_{max}$ and an apparent increase in the $K_m$. A higher substrate concentration is needed to “force” the substrate onto the EI complex. Graphically, the Lineweaver-Burke plots show a series of lines intersecting at a point above the negative X-axis.

NONCOMPETITIVE INHIBITORS are a special type of mixed type inhibitors. Like the mixed type, noncompetitive inhibitors bind either to the free enzyme (E) or to the enzyme-substrate complex (ES) at a site away from the substrate site. What makes Noncompetitive inhibitors special is that in this case $K_{ia}$ equals $K_{ib}$. Kinetically Noncompetitive inhibitors result in a decrease in $V_{max}$ with no change in the $K_m$. Graphically, the Lineweaver-Burke plots show a series of lines intersecting at $-1/K_m$.

<table>
<thead>
<tr>
<th>Inhibitor Type</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
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</thead>
<tbody>
<tr>
<td>Competitive</td>
<td>Increase</td>
<td>No Change</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>Decrease</td>
<td>Decrease</td>
</tr>
<tr>
<td>Mixed Type</td>
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</tr>
<tr>
<td>Noncompetitive</td>
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</tbody>
</table>
Control of Enzyme Activity

Enzymes catalyze the vast majority of the chemical reactions that occur within an organism. The activity of these enzymes must be regulated as a means for controlling the overall metabolic process. The cell cannot waste raw materials by overproducing an unnecessary product. Likewise, the cell cannot survive if some needed compound is underproduced.

Enzyme activity in the cell is regulated by several different factors and mechanisms. Enzyme activity can be controlled by manipulating the concentrations of substrate and/or product and it can be controlled by modifying the activity of the protein.

**SUBSTRATE AVAILABILITY.** The availability of substrates, cofactors, coenzymes, and/or cosubstrates will determine the rate of cellular enzymatic reactions. If the concentration of substrate, cofactor, coenzyme, and/or cosubstrate is low the rate of the reaction will be slow. An increase in their concentration will increase the rate of the reaction. When adequate product is present the enzyme can be sequestered away from the substrate or the transport of substrate into the cell can be inhibited.

**EQUILIBRIUM CONSIDERATIONS.** The ratio of product to substrate concentration affects the rate of enzyme catalyzed reactions since many of the reactions in the cell are reversible reactions. *In vivo* these reactions will reach an equilibrium and an equilibrium constant can be calculated. In the cell the rate of reversible reactions can be controlled by adjusting the ratio of product to substrate. Removing product or increasing substrate concentration increases the rate of the reaction. Allowing the concentration of product in the cell to approach the equilibrium concentration slows the reaction rate.

**PRODUCT INHIBITION.** Some enzymes are inhibited by their products. As the concentration of product increases it acts as a competitive inhibitor toward the enzyme that produced it. Product inhibition is one type of **FEEDBACK INHIBITION.**

**ENZYME SYNTHESIS.** There are genetic controls over the synthesis and cellular concentrations of certain key enzymes. By controlling the synthesis of an enzyme the cell can activate or terminate its activity. When a particular substrate becomes available the cell synthesizes the enzymes necessary for its utilization. The lack of a substrate can “turn off” the synthesis of the enzymes that utilize it. **INDUCTION** is the stimulation of enzyme synthesis. **REPRESSION** is the inhibition of enzyme synthesis. Induction or repression of enzyme synthesis is slow to respond to cellular changes, minutes to hours are required before a change can be noted within a eukaryotic cell.

**IRREVERSIBLE COVALENT MODIFICATION.** Some enzymes, especially those involved in an irreversible process, are synthesized in inactive forms called **ZYMGENS** or **PROENZYMES.** When needed, zymogens are activated by the enzymatic removal of a small peptide or several small peptides from the larger protein molecule. The peptide(s) removed is(are) called **ACTIVATION PEPTIDE(S).** **Protein hydrolases (Proteases)** catalyze the removal of the activation peptide(s). After the piece or pieces of the zymogen molecule have been removed, the protein undergoes a conformational change, it refolds, bringing the enzyme into a new native, active conformation.

The digestive enzymes are synthesized as zymogens to protect the cell that synthesizes them from auto-
digestion. Many of the enzymes of the Hemostasis Cascade (Blood Clotting) are synthesized as Zymogens as are many of the enzymes of the Complement Cascade. The Hemostasis Cascade and the Complement Cascade remain inactive until needed to stem blood loss or destroy invading bacteria, respectively. This method for controlling enzyme activity is irreversible, once activated the enzyme must be destroyed to be inactivated, to be “turned off”. It takes on the order of seconds to minutes to activate zymogens.

**Reversible Covalent Modification.** When the activity of an enzyme is regulated by reversible covalent modification, some group is added to or removed from a specific site or sites on the enzyme. The addition or removal of this functional group increases or decreases the activity of the enzyme. Common modifying functional groups include phosphoryl (–PO$_3^{-2}$), acetyl, adenyllyl, methyl, amide, carboxyl, myristoyl, palmitoyl, prenyl, sulfate, and adenosine diphosphate ribosyl (ADP) groups. A phosphoryl group, –PO$_3^{-2}$, (common usage phosphate group PO$_4^{3-}$) is the group most commonly added to or removed from an enzyme to modulate its activity. It is usually attached to the protein by an ester linkage involving the hydroxyl group(s) of specific serine, threonine, or tyrosine residues. Adenosine triphosphate (ATP) is the usual phosphate donor. Protein kinases catalyze the transfer of phosphate from ATP to the protein. Phosphoprotein phosphohydrolases (Phosphoprotein phosphatases, Protein phosphatases, or Phosphatases) catalyze the hydrolytic removal of phosphate; i.e., the enzyme catalyzes the addition of water across the phosphoester bond releasing the phosphate. Reversible Covalent Modification is a rapid form of enzyme control taking fractions of a second to evoke a cellular response.

**Allosteric Control.** Allosteric Control requires an Allosteric Enzyme. This subset of enzymes share several common features. Allosteric enzymes are always multimeric proteins. The subunits of the allosteric enzyme can be identical, in which case each subunit contains at least two binding sites. One site is the substrate binding/active site, the site where the substrate binds and catalysis occurs. The second binding site(s) is(are) the allosteric site(s). A variety of small molecules called Allosteric Effectors or Allosteric Modulators bind at these sites. Alternatively, the subunits can be different, in this case one subunit, the Catalytic Subunit, carries the substrate/active site and the other subunit, the Regulatory Subunit, contains the allosteric sites.

The kinetics of an allosteric enzyme do not follow simple Michaelis-Menton kinetics. With an allosteric
enzyme a plot of $v_0$ versus $[S]$ results in a sigmoidal (S) shaped curve, rather than a rectangular hyperbolic curve. At low substrate concentrations the enzyme has little activity because it has low affinity for its substrate. The enzyme is said to be in its low affinity TENSE CONFORMATION, or the low affinity T STATE. As the substrate concentration increases, more substrate molecules bind to the subunits of the enzyme and increased substrate binding shifts the enzyme from the T state to its high affinity conformation, its RELAXED CONFORMATION, or the R STATE. The shift from T to R is the result of a change in the conformation of the enzyme protein and results in a dramatic increase in enzyme activity. Substrate binding to an allosteric enzyme is a COOPERATIVE EVENT. Binding of the first substrate molecule to one of the subunits of the enzyme makes the binding of additional substrate molecules to the remaining subunits easier.

A variety of EFFECTOR MOLECULES bind to allosteric enzymes and cause a change in enzyme activity. Most allosteric enzymes have several very different allosteric effectors and each effector molecule has its own unique allosteric binding site. Effector molecules can be the product of the allosteric enzyme, the product of a different enzyme, or some “signal molecule”. They can cause a decrease in enzyme activity or they can cause an increase in activity. NEGATIVE ALLOSTERIC EFFECTORS (NEGATIVE ALLOSTERIC MODULATORS) bind to their allosteric sites, shift the enzyme conformation more toward the T state, and thereby decreases the activity of the enzyme since substrate binds with less affinity. POSITIVE ALLOSTERIC EFFECTORS (POSITIVE ALLOSTERIC MODULATORS) bind to their allosteric sites on the enzyme and shift the enzyme more toward the active R conformation. This shift is accompanied by increased affinity for the substrate, increased substrate binding, and increased enzymatic activity. Allosteric control is the most rapid method for controlling enzyme activity. It can fine tune enzymatic activity to the ever changing conditions within the cell. Negative effectors and positive effectors are always present in the cell in ever varying concentrations. As conditions within the cell change the ratio of positive effectors to negative effectors change. The rate of an allosteric enzyme is controlled by the concentration ratio of positive effectors to negative. Since each modulator has its own allosteric binding site, both types of effectors can bind to the enzyme. Even though they bind to different sites, they are in competition with each other. If the concentration of positive effectors is higher than that of negative effectors, the positive effectors bind more often to their allosteric site and an increase in enzyme activity is observed. Conversely, if the concentration of negative effectors is higher than that of positive effectors, the negative effectors bind more often and a decrease in enzyme activity is noted.
Two models have been presented in an attempt to describe the cooperative substrate binding by the subunits of an allosteric enzyme.

The first is the **Concerted Theory**. In this theory the protein exists in an equilibrium between the T and R states. The substrate can only bind to the R form. When substrate binds the equilibrium is shifted from the T to the R state. With more protein in the R form, more substrate can bind and the activity of the enzyme is increased. Negative allosteric effectors bind only to the T state pulling the equilibrium more toward the T state. Positive modulators bind only to the R form shifting the equilibrium toward the R form.

The second theory is the **Sequential Theory**. In this model, in the absence of substrate all of the enzyme molecules are in the T state. When the first substrate binds, one of the subunits of the protein shifts to the R form. This shift is communicated to the other subunits of the protein making it easier for subsequent substrate molecules to bind. As more substrate binds to the enzyme more of the subunits switch to the R conformation. With more substrate bound there is an increase in catalytic activity. In this model, the allosteric modulators bind only to the T state, and their binding influences the affinity of enzyme for substrate. Negative effectors decrease the affinity making it more difficult for the first substrate to bind and begin the shift to the R state. Positive effectors increase the affinity making substrate binding easier.

**Hemoglobin - The Model for an Allosteric Protein**

Although hemoglobin is not an enzyme it does display allosteric properties. The oxygen binding curve for hemoglobin is not hyperbolic, rather it is sigmoidal (S) in shape. At low partial pressures of oxygen, the hemoglobin molecule has little affinity for oxygen. At a critical concentration of oxygen the affinity of the hemoglobin (Hb) molecule switches from the low affinity T state to high the affinity, R state, and oxygen
rapidly binds. This switch from a low affinity state to a high affinity state is identical to the switch observed with allosteric enzymes.

When Hb is deoxygenated (no oxygen bound) it is in the T or low affinity state. As the partial pressure of oxygen is increased a partial pressure is reached at which the first oxygen molecule binds to Hb. The binding of the first oxygen causes the subunit that binds it to switch from the T to the R state. This switch from T to R in one subunit is communicated to the other subunits of the molecule via intersubunit contacts. This communication between subunits makes it easier for the other subunits to bind oxygen since they are more toward the R conformation. After 2 or 3 molecules of oxygen have bound, the entire Hb molecule is in the R state and the last remaining oxygen binds easily.

MAX PERUTZ determined the X-ray structure of deoxygenated hemoglobin (deoxy Hb) and oxygenated hemoglobin (oxy Hb). When Hb switches from the deoxy/T state to the oxy/R state the \( \alpha_1\beta_1 \) subunit pair shifts 15° relative to the \( \alpha_2\beta_2 \) subunit pair, closing the central cavity of the Hb molecule. This shift is accompanied by a change in 30 contacts (mostly hydrophobic interactions, but some important salt bridges and H bonds) along the interface between the \( \alpha_1\beta_1 \) pair and the \( \alpha_2\beta_2 \) pair. A shift in 19 contacts along the \( \alpha_1\beta_2 \) and the \( \alpha_2\beta_1 \) interface also occurs.

Hemoglobin has three physiologically important negative allosteric effectors - molecules that shift the equilibrium between the T and R states toward the T state and decrease oxygen affinity. The first negative allosteric effector is the hydrogen ion, H\(^+\). In actively metabolizing tissues CO\(_2\) is produced in high concentrations. The CO\(_2\) can spontaneously react with water to form H\(_2\)CO\(_3\), (carbonic acid), that immediately ionizes to H\(^+\) and HCO\(_3^-\).
CO₂ + H₂O ⇌ H₂CO₃ ⇌ H⁺ + HCO₃⁻

CO₂ produced in the tissues rapidly diffuses into the erythrocyte (down the concentration gradient). Erythrocytes contain the enzyme Carbonic anhydrase that catalyzes and increases the rate of carbonic acid formation. The H⁺ produced in the red cell from the CO₂ in the tissues binds to acidic side chains on the Hb molecule, disrupting contacts that stabilize the R form and shifting the molecule from R to T. This shift from R to T assures the complete delivery of oxygen from Hb to the tissues.

The second negative allosteric effector is the bicarbonate ion (HCO₃⁻). Bicarbonate binds to the four amino termini of the hemoglobin subunit chains forming carbamates. In the absence of HCO₃⁻ the four amino termini donate positive charges that stabilize the R conformation; carbamate formation removes these four charges shifting the molecule away from R and more towards T. The effect of H⁺ and HCO₃⁻ on the R to T transition is called the BOHR EFFECT.

The third major negative allosteric effector is 2,3-Bisphosphoglycerate (2,3-BPG). This molecule binds in the central cavity of the Hb molecule. It binds to the positive charges on the amino termini of the Hb β subunits (amino acids 1 or 2 of each β chain) and to four other basic amino acid side chains of the β subunits present in the central cavity of the molecule (amino acid 82 & 143 of each β chain). 2,3-BPG binds to the T state and stabilizes this conformation. 2,3-BPG cannot bind to the oxygenated form of Hb because the central cavity is too narrow to accommodate it. 2,3-BPG shifts the Hb binding curve to the right, it decreases the oxygen affinity of hemoglobin.

How Does Hemoglobin Perform Its Function?

In the lungs the partial pressure of oxygen is high (100 mm Hg) and the partial pressure of CO₂ is low. The high partial pressure of O₂ forces the first O₂ to bind to Hb, beginning the T to R transition. HCO₃⁻ is carried from the tissues to the lungs bound to Hb, in solution in the cytoplasm of the red cell and dissolved in the blood plasma. Since CO₂ is low in the lungs, the

\[
\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- 
\]
equilibrium is shifted toward CO₂ production. Carbonic anhydrase in the red cell catalyzes the back reaction and increases the rate of CO₂ formation. The H⁺ and HCO₃⁻ is consumed and CO₂ is produced and exhaled. The decrease in [H⁺] and [HCO₃⁻] induces an increased shift toward the R conformation. The increased shift toward the R form forces 2,3-BPG out of the central cavity of the molecule completing the shift to R.

In the tissues the partial pressure of O₂ is low (35 to 40 mm Hg under resting conditions) and the [CO₂] is high. The
low oxygen concentrations starts the shift from R to T as oxygen begins to dissociate from the Hb molecule. The high CO₂ concentrations in the tissues and the low CO₂ concentration is the RBC drives the diffusion of CO₂ into the RBC. Carbonic anhydrase in the red cell catalyzes the formation of H₂CO₃ which immediately ionizes to H⁺ and HCO₃⁻, increasing their concentration. The H⁺ and HCO₃⁻ binds to Hb further shifting the equilibrium to the T form and causing the release of more oxygen. As Hb becomes more and more deoxygenated, when the molecule is almost completely in the T state, 2,3-BPG binds and completes the shift to the T conformation releasing more of the bound oxygen. About 40% of the oxygen carried by Hb would be delivered to the tissues under resting conditions. Rapidly metabolizing tissues, e.g., rapidly contracting muscle, have low partial O₂ pressures and very high concentrations of carbon dioxide (high [H⁺] and [HCO₃⁻]). Under these conditions greater than 95% of the oxygen carried by the Hb can be delivered to the tissues.