Irreversible Enzyme Inhibition

- Irreversible inhibitors form stable covalent bonds with the enzyme (e.g., alkylation or acylation of an active site side chain)
- There are many naturally-occurring and synthetic irreversible inhibitors
- These inhibitors can be used to identify the amino acid residues at enzyme active sites
- Incubation of I with enzyme results in loss of activity

Affinity labels for studying enzyme active sites

- **Affinity labels** are active-site directed reagents
- They are irreversible inhibitors
- Affinity labels resemble substrates, but contain reactive groups to interact covalently with the enzyme

Covalent complex with lysine residues

- Reduction of a Schiff base forms a stable substituted enzyme

\[
\text{Lys} \quad (\text{CH}_2)_{4} \quad \text{NH}_2 \\
\text{O} \\
\text{R} \quad \text{C} \quad \text{H} \\
\text{H}_2\text{O} \quad \text{H}_2\text{O} \\
\text{Lys} \quad (\text{CH}_2)_{4} \quad \text{N} \\
\text{R} \quad \text{C} \quad \text{H} \\
\text{Schiff base} \quad \text{NaBH}_4 \\
\text{Lys} \quad (\text{CH}_2)_{4} \quad \text{NH} \\
\text{R} \quad \text{C} \quad \text{H}_2 \\
\]

Inhibition of serine protease with DFP

- Diisopropyl fluorophosphate (DFP) is an organic phosphate that inactivates serine proteases
- DFP reacts with the active site serine (Ser-195) of chymotrypsin to form DFP-chymotrypsin
- Such organophosphorous inhibitors are used as insecticides or for enzyme research
- These inhibitors are toxic because they inhibit acetylcholinesterase (a serine protease that hydrolyzes the neurotransmitter acetylcholine)
Kinetics of Multisubstrate Reactions

Single Displacement

(a) Sequential (ordered or random)

\[ \frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_v}{K_{v} K_{A}} \left( \frac{1}{[A]} - \frac{1}{[B]} \right) \]

- Ordered
- Random

\[ v \]

\[ \frac{1}{[B]} \]

a) When [B] is variable and [A] is constant.

b) When [A] is variable and [B] is constant.

The data are plotted on a double-reciprocal plot:

\[ \frac{1}{v} \]

\[ \frac{1}{[A]} \]

\[ rac{1}{[B]} \]

\[ v \]

\[ \frac{1}{[A]} \]

\[ \frac{1}{[B]} \]

\[ v \]

\[ \frac{1}{[A]} \]

\[ \frac{1}{[B]} \]

\[ v \]

\[ \frac{1}{[A]} \]

\[ \frac{1}{[B]} \]

\[ v \]

The slopes from the double reciprocal plot are then plotted against 1/[A]₀:

\[ \text{Slope} = \frac{K_v}{V_{\text{max}}} \]

The y-intercepts from the double-reciprocal plot are then plotted against 1/[A]₀:

\[ \text{Intercept} = \frac{V_{\text{max}}}{K_v} \]

\[ \frac{1}{[A]_0} \]

\[ v \]

\[ \frac{1}{[A]_0} \]

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**Regulatory Enzymes**  
*group of enzymes which do not exhibit MM Kinetics*

- Often $E_1$ is a regulatory enzyme (1\textsuperscript{st} committed step in a metabolic sequence of reactions)
- Often multimeric
- Often bind some metabolite other than S at a place other than the active site which will affect activity (allosterism)
- Often exhibit sigmodial kinetics
The best way to understand the difference between hyperbolic (M-M) and sigmoidal (non M-M) is to study the difference between Myoglobin and Hemoglobin.

Myoglobin vs Hemoglobin

- Single polypeptide chain
- 153 amino acids
- Typical globular protein
- In muscle
- Contains One Heme
- Stores oxygen
- Follows M-M

- 4 polypeptide chains 2 α 141 AA; 2 β 145 AA
- In Red Blood Cells
- Contains Four Heme Groups
- Carries oxygen
- Does not follow M-M

Oxygen Binding to Mb and Hb

A. Oxygen Binds Reversibly to Heme

- **Oxymyoglobin** - oxygen bearing myoglobin
- **Deoxymyoglobin** - oxygen-free myoglobin

- In oxymyoglobin, six ligands are coordinated to the ferrous ion in octahedral symmetry
- Oxygen is coordinated between the iron and the imidazole sidechain of His-64
**Oxygen-binding site of whale oxymyoglobin**

- Octahedral geometry of coordination complex (six ligands around iron)
- His-93 (proximal histidine) liganded to Fe
- His-64 (distal histidine)

**Oxygen-binding in myoglobin**

(a) OxyMb,  (b) DeoxyMb

- $O_2$ (green), Fe(II) orange

**Conformational changes in a hemoglobin chain induced by oxygenation**

- Oxygen binding to Fe pulls the His toward ring plane
- Helix with His shifts position, disrupting some ion pairs between subunits (blue to red position)

**Oxygen-Binding Curves of Myoglobin and Hemoglobin**

- Curves show reversible binding of $O_2$ to Mb and Hb
- Fractional saturation ($Y$) is plotted versus the partial pressure of oxygen, $pO_2$ (oxygen concentration)
- The shape of the Hb curve shows a **positive cooperativity** in the binding of 4 $O_2$ molecules (i.e. the $O_2$ affinity of Hb increases as each $O_2$ molecule is bound)
**O₂ binding curves (continued)**

- Mb-O₂ binding curve is **hyperbolic**, indicating a single equilibrium constant for binding O₂.
- Hb-O₂ binding curve is **sigmoidal**, and reflects the binding of 4 molecules of O₂, one per each heme group.

---

**Easy to Explain Hyperbolic Binding Curve of Mb**

Simple Equilibrium Binding

\[
\text{Mb} + \text{O}_2 \leftrightarrow \text{MbO}_2 \quad K_f = \frac{[\text{MbO}_2]}{[\text{Mb}][\text{O}_2]}
\]

\[
Y = \frac{[\text{MbO}_2]}{[\text{Mb}] + [\text{MbO}_2]}
\]

\[
Y = \frac{K_f [\text{Mb}][\text{O}_2]}{[\text{Mb}] + K_f [\text{Mb}][\text{O}_2]}
\]

\[
Y = \frac{K_f [\text{O}_2]}{1 + K_f [\text{O}_2]} \quad Y = \frac{[\text{O}_2]}{K_f + [\text{O}_2]} \quad \text{This is the form for a hyperbolic curve}
\]

\[
K_f = P_{50} \quad Y = \frac{[\text{O}_2]}{P_{50} + [\text{O}_2]}
\]

---

**Hemoglobin unlike Myoglobin has Sequential Interaction**

This means that the binding sites depend on each other through some kind of interaction.

\[
A = \text{Hb}; \quad B = \text{O}_2
\]

\[
A + B \leftrightarrow AB \quad K_f
\]

\[
AB + O_2 \leftrightarrow AB_2 \quad aK_f \quad a > 1
\]

\[
AB_2 + O_2 \leftrightarrow AB_3 \quad abK_f \quad b > 1
\]

\[
AB_3 + O_2 \leftrightarrow AB_4 \quad abcK_f \quad c > 1
\]

Take this model to its logical conclusion which gives an equation useful for analyzing sigmoidal binding data.

*All or nothing!*

\[
A + 4B \leftrightarrow AB_4 \quad K' = \frac{[AB_4]}{[A][B]^4}
\]
\[ A = Hb; B = O_2 \]

\[ Y = \frac{[AB]}{[A] + [AB]} \]

\[ Y = \frac{[B]}{K_d + [B]} \quad \text{(This is the form for a sigmoidal curve)} \]

### Oxygen-binding curves

(a) Comparison of O₂-binding to Mb and Hb

### Hill Equation

\[ Y = \frac{[B]^n}{K_d + [B]^n} \]

Again, \(K_d = P_{50}\)

\[ \ln(Y) = -n \ln(P_{50}) + n \ln[B] \]

\[ \frac{1-Y}{Y} \]

### Hill Plot

- \(n = 2.8\)
- \(K_d = 158\)
- \(P_{50}, n = 1\)
- \(K_d = 0.3\)
- \(P_{50}, n = 1\)
The Hill Coefficient

\[ n_H = \text{maximum slope} \]

- \( n_H = 1 \) hyperbolic binding (noncooperative)
- \( n_H > 1 \) positive cooperativity (filling one binding site increases the affinity of other binding sites)
- \( n_H < 1 \) negative cooperativity
- \( n_H = n \) (where \( n = 4 \)) complete cooperativity for Hb

For hemoglobin, \( n_H = 2.8 \)

How does positive cooperativity work?
Conformational change on binding

Regulation of Enzyme Activity

- **Regulatory enzymes** - activity can be reversibly modulated by effectors
  - Such enzymes are usually found at the first unique step in a metabolic pathway (the first “committed” step)
  - Regulation at this step conserves material and energy and prevents accumulation of intermediates

Two Methods of Regulation

1. **Noncovalent allosteric regulation**
2. **Covalent modification**

- **Allosteric enzymes** have a second **regulatory site** (allosteric site) distinct from the active site
- **Allosteric inhibitors or activators** bind to this site and regulate enzyme activity via conformational changes

Hemoglobin is an Allosteric Protein

- Oxygen binding and release from Hb are regulated by **allosteric interactions**
- **Allosteric effectors (modulators)** bind to a protein at a site separate from the functional binding site (may be activators or inhibitors)
- The activity of an **allosteric protein** is regulated by allosteric effectors
**Two conformations of hemoglobin: T and R**

- **Active (R state)** and **inactive (T state)** forms are in rapid equilibrium in allosteric proteins
- Binding of **substrates** and allosteric **activators** stabilize the R state and shift the equilibrium in the R direction
- Allosteric **inhibitors** stabilize the T state and shift the equilibrium in the T direction

**Equilibrium between different conformations of protein in aqueous solution**

\[ R \leftrightarrow T \]

- **High Affinity**
  - **Relaxed** (T state)
  - **Tense** (R state)
- **Low Affinity**
  - Positively cooperative - \( \text{O}_2 \) binds preferentially to R form and shifts the equilibrium to R side

**Two models**

- **Concerted model:**
  - Subunits either all T state or all R state
- **Sequential model:**
  - Mixture of T subunits and R subunits is possible
  - Binding of S converts only that subunit from T to R

**Oxygen-binding curves**

- (b) Binding of the R (high-affinity) and T (low affinity) forms of Hb
Both Models are Used to Discuss Regulatory Enzymes

Which is Better?

Concerted Theory

• Substrate binds to shift equilibrium to R form
• Inhibitors preferentially bind to the lower affinity form (T state) and shifts equilibrium to T form
• Activators preferentially bind to the higher affinity form (R state) and shifts equilibrium to R form

Symmetry-driven Theory

The enzyme has two forms—all R or T form

The binding to inhibitors and activators will bind to either the T or R state, respectively.

Role of cooperativity of binding in regulation

• Addition of modulators alters enzyme activity
• Activators can lower $K_m$, inhibitors can raise $K_m$
Sequential Theory

Ligand-induced Theory

The enzyme has two forms – R form and T form but it differs from concerted theory in allowing the existence of both high- and low-affinity subunits in an oligomeric molecule with fractional saturation.

Hill Equation – Quantify the cooperativity

Allosteric Effects on Hemoglobin

• Oxygen Binding
• 2,3-Bisphosphoglycerate binding
• pH (Bohr Effect)

Conformational changes during O₂ binding to hemoglobin

- Oxygen binding to Hb has aspects of both the sequential and concerted models

2,3-Bisphosphoglycerate Binding

- BPG binds to deoxy form of Hb
- Binds to a site other than where O₂ binds
- BPG causes the T form to predominate
- Therefore, lowers oxygen affinity
2,3-Bisphosphoglycerate Binding

This is an adaptive response, requiring several days at high altitude. The production of excess BPG, although it reduces the oxygen affinity, it makes the protein more efficient at delivering oxygen to the tissues at high altitudes.

The Bohr Effect

Lower pH → decrease Hb affinity for O₂

Thus more oxygen is delivered to tissues at a lower pH even when the pO₂ remains unchanged

\[
\text{Hb-}4\text{O}_2 + \text{nH}^+ \leftrightarrow \text{Hb-nH}^+ + 4\text{O}_2
\]

When a tissue is more active, the amount of carbon dioxide produced will be increased (PₐCO₂ is higher). Carbon dioxide reacts with water as shown in the following equation:

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-
\]