Lecture 14: Cooperative Binding

Goals:
- Relate distribution of bound ligands to degree of cooperativity
- Understand approximation that gives the Hill equation.
- Generate Hill Plot, obtain $K_D$ and $n_H$ from Hill plot.
- Interpret $n_H$ in terms of type of cooperativity.

Review of Binding Types:
Non-cooperative: No interaction between sites. A protein with a single site must show non-cooperative binding.

Allosteric control - non-cooperative binding:
- **Heterotropic activator** increases R-state. Binding affinity of ligand for one or more non-interacting sites increases.
- **Heterotropic inhibitor** increases T-state. Binding affinity of ligand for one or more non-interacting sites decreases.

Co-operative binding: When binding of the ligand to one site affects the binding of the ligand on another site on the protein.

Homotropic positive cooperativity:
Multiple interacting ligand binding sites required. Binding at one increases affinity at another by increasing R state.

Homotropic negative cooperativity:
Multiple interacting ligand binding sites required. Binding at one decreases affinity at another by increasing T state.

Allosteric control with cooperative binding:
- **Heterotropic activator** increases R-state, increasing average affinity.
- **Heterotropic inhibitor** increases T-state, reducing average affinity.

Ligand binds to multiple interacting states (homotropic) with some form of cooperative binding, neg or positive (positive cooperativity for the ligand is shown here).
Characterization of Degree of Cooperativity

Hill Coefficient:

It is possible to quantify the degree of cooperativity by analysis of the binding data using a Hill plot. The outcome of this analysis is the Hill coefficient, which has the characteristics summarized to the right.

Consider a two-step binding:

\[ M + [L] \xrightarrow{k_{D1}} [ML] \xrightarrow{k_{D2}} [ML_2] \]

\[ M + [L] \xrightarrow{k_{D1}} [ML] \]

\[ K_{D1} = \frac{[ML][L]}{[M][ML]} \]

\[ K_{D2} = \frac{[ML][L]}{[ML_2]} \]

i) Non-cooperative. The binding constant remains the same for both binding events. @Y=0.5

6 molecules x 2 binding sites

\[ y = 0.5 \]

\# of occupied sites = 6

II) Negative cooperativity if \( k_{D2} > k_{D1} \) (or \( k_{A2} < k_{A1} \)) i.e. the second binding is lower in affinity.

\[ KA_2 \ll KA_1 \]

\[ K_{D2} > K_{D1} \]

iii) Positive cooperativity if \( k_{D2} < k_{D1} \) (or \( k_{A2} > k_{A1} \)) i.e. the second binding is higher in affinity.

\[ KA_2 \gg KA_1 \]

\[ K_{D2} < K_{D1} \]

Infinitely Positive Cooperativity

Two Binding Sites:

Consider an infinitely positive cooperative system such that the second dissociation constant (\( K_{D2} \)) is much lower than the dissociation constant for binding the first ligand (\( K_{D1} \)).

Then the only species present in solution are \([M]\) and \([ML_2]\).

For an infinitely positive cooperative system the fractional saturation is:

\[ Y = \frac{[ML_2]}{[M]+[ML_2]} \]

\[ Y = \frac{1}{K_{D2}} \frac{[ML][L]}{[M][ML][L]} \]

\[ Y = \frac{1}{K_{D2}} + \frac{1}{K_{D2}} \frac{[ML][L]}{[M][ML][L]} \]

What is the observed \( K_{D2} \)? (What [L] gives \( Y = 0.5 \))

\[ y = 0.5 \]

\[ 2 \]

\[ 0.5 \]

\[ \frac{[L]^2}{K_{D1} \cdot K_{D2} + [L]^2} \]

\[ \sqrt{K_{D1} \cdot K_{D2}} \]

\[ @ \ Y = 0.5 \]
Three Binding Sites:

\[ Y = \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} = \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} \]

What is the observed \( K_D \)? (What \([L]\) gives \( Y = 0.5 \)?)

\[ y = 0.5 \implies \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} = \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} \]

\[ \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} = \frac{[L]^3}{3\sqrt[3]{K_{D1}K_{D2}K_{D3}}} \]

\[ \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} = \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} \]

\[ \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} = \frac{[L]^3}{K_{D1}K_{D2}K_{D3} + [L]^3} \]

\[ n \text{-ligands - Infinitely Positive Cooperativity:} \]

\[ Y = \frac{[L]^{n}}{K_{D1}K_{D2} \cdots K_{Dn} + [L]^{n}} \]

What is the observed \( K_D \)? (What \([L]\) gives \( Y = 0.5 \)?)

\[ Y = 0.5 \implies \frac{[L]^{n}}{K_{D1}K_{D2} \cdots K_{Dn} + [L]^{n}} \]

\[ n \text{-ligands – General Cooperative Binding:} \]

\[ \Rightarrow \text{For less cooperative systems, the fractional saturation can be approximated as:} \]

\[ Y = \frac{[L]^{n}}{K_{D \text{ave}}^{n} + [L]^{n}} \]

- Where \( n_h \) is the Hill coefficient. This is a measure of the degree of cooperativity.
- \( K_{D \text{ave}} \) is the "average" \( K_D \); when \([L]=K_{D \text{ave}},\ Y=1/2.\)
- The \( K_{D \text{ave}} \) is usually a complex polynomial of the individual dissociation constants.
- Exception: for two binding sites \( K_{D \text{ave}} = \sqrt{K_{D1}K_{D2}}, \) regardless of the degree of cooperativity.

<table>
<thead>
<tr>
<th>Type</th>
<th>Formula</th>
<th>Condition</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Site - Non-cooperative (( n_h = 1 ))</td>
<td>[ Y = \frac{[L]}{K_D + [L]} ]</td>
<td>( Y=0.5 ) when ( [L]=K_D )</td>
<td></td>
</tr>
<tr>
<td>Two sites - infinitely positive cooperative (( n_h = 3 ))</td>
<td>[ Y = \frac{[L]^2}{K_{D1}K_{D2} + [L]^2} ]</td>
<td>( Y=0.5 ) when ( [L]=\frac{2}{3}K_{D1}K_{D2} )</td>
<td>( [ML] = 0 )</td>
</tr>
<tr>
<td>N-sites - infinitely positive cooperative (( n_h = N ))</td>
<td>[ Y = \frac{[L]^N}{K_{D1}K_{D2} \cdots K_{Dn} + [L]^N} ]</td>
<td>( Y=0.5 ) when ( [L]=\sqrt[2N]{K_{D1}K_{D2} \cdots K_{Dn}} )</td>
<td>( [ML] \cdots [ML_{N-1}] = 0 )</td>
</tr>
<tr>
<td>N-sites - just plain pos. cooperative (( n_h ))</td>
<td>[ Y = \frac{[L]^{n_h}}{K_{D-\text{ave}}^{n_h} + [L]^{n_h}} ]</td>
<td>( Y=0.5 ) when ( [L]=K_{D-\text{ave}} )</td>
<td>( [ML] \cdots [ML_{N-1}] &gt; 0 )</td>
</tr>
</tbody>
</table>
Hill Equation and Plot:
The Hill coefficient, and the "average" $K_D$ can be obtained from a Hill Plot. The Hill plot is based on the following transformation of the above binding equation:
- Plot of $\log [Y/(1 - Y)]$ versus $\log[L]$
- The Hill coefficient, $n_h$, is the slope as the line crosses the x-axis.
- The $\log K_{D,ave}$ is the intersection of the Hill curve with the x-axis.

Non-Cooperative Systems ($n=1$):

$$ y = \frac{[L]^n}{K_D^{n_h} + [L]^n} $$

This is a straight line with a unit (1) slope.
- Intersection with x-axis ($Y = 0.5$) gives $\log(K_D)$.

Cooperative Systems.

Intermediate Ligand Concentration ($@Y=0.5$):
- Slope: Hill coefficient ($0 \leftrightarrow 1 \leftrightarrow n$)
- Intercept: Ligand concentration to give $Y = 0.5 = \log(K_{D,ave})$.

Low ligand:
At very low ligand concentration, the binding appears non-cooperative because most of the macromolecule is in the [M] form. Therefore the Hill plot is initially linear, with a slope = 1, intersecting x-axis at $\log K_D$.

High ligand: At very high ligand concentration, the binding also appears non-cooperative because most of the macromolecule is in the [ML] form.

Therefore the Hill plot is again linear, with a slope = 1, intersecting the x-axis at $\log K_{D,ave}$. 

Association constants

**Microscopic** $K_A$ ($K_{A'}$): $K_{A'}$ reflects the intrinsic affinity between the protein and the ligand.
- $K_A = K_{ON}/K_{OFF}$
- This is the association constant for a single binding site.
- This is what would be measured for a monomeric protein.
- $\Delta G = -RT\ln(K_{A'})$

![Diagram of M, ML, and ML2 binding sites with $K_{ON}$ and $K_{OFF}$ arrows showing equilibrium between states.]

**Macroscopic** $K_A$:
- This is the observed $K_A$ based on the experimental measurement of the concentrations of the various species. $K_{A1} = [ML]/[M][L]$ and $K_{A2} = [ML2]/[ML][L]$

![Diagram of M, ML, and ML2 binding sites with $K_{ON}$ and $K_{OFF}$ arrows and $2K_{OFF}$ and $2K_{ON}$ showing equilibrium between states.]

For the first binding event, $K_{A1} = K_{ON\ Total}/K_{OFF\ Total} = 2K_{ON}/K_{OFF}$ (There are two ways to form the ML species)

For the second binding event: $K_{Aw} = K_{ON\ Total}/K_{OFF\ Total} = K_{ON}$ (Second ligand can bind only one way, but there are two ways to leave).

**Important parameters and how to obtain them:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>One-site or n- non-cooperative sites</th>
<th>Cooperative</th>
</tr>
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<tbody>
<tr>
<td>$N_m$ (Hill-coefficient)</td>
<td>=1</td>
<td>Slope of the Hill plot at log $(Y/1-Y)=0$</td>
</tr>
<tr>
<td>$\Delta G^\circ$</td>
<td>$\Delta G^\circ = -RT\ln K_A = -RT\ln(1/K_D)$</td>
<td>$\Delta G^\circ \neq -RT\ln K_{A(ave)}$</td>
</tr>
</tbody>
</table>
Binding affinity

$K_A = \frac{K_{on}}{K_{off}}$.

binding affinity for each subunit.

$K_A^m$ - microscopic binding constant.

$\text{Macroscopic/Observed binding constant}$

$[ML]$ - formed in two ways $K_{A_1} = \frac{2 \cdot K_{on}}{K_{off}}$.

$K_{A_2} = \frac{K_{on}}{2 \cdot K_{off}}$. 2 different ways to loose the 2nd ligand or have one form $[ML]$.

$K_A = 2^m (K_{A_2})$.

$K_{A_2} = \frac{1}{2} \cdot K_A$.

$\Delta G = -RT \ln [K_{eq}]$. 
Microscopic and Macroscopic Binding Constants:

Microscopic $K_A (K_A^\mu)$: This is the association constant for a single site, and is just the ratio of the on- and off-rates: $K_A^\mu = k_{on}/k_{off}$. It reflects the intrinsic affinity between the protein and the ligand.

$\Delta G^\ddagger = -RT \ln K_A^\mu$.

This is what would be measured for a single distinct binding site.

If $K_A^\mu$ changes from one binding step to another, the system is cooperative:

- **Non-cooperative**: $K_A^\mu$ is the same for each step.
- **Positive cooperativity**: $K_A^\mu$ increases ($K_0^\mu$ decreases)
- **Negative cooperativity**: $K_A^\mu$ decreases ($K_0^\mu$ increases)

Macroscopic $K_A$: This is the observed $K_A$ based on the experimental measurement of the concentrations of the various species, i.e. $K_A = [ML]/[M][L]$.

The macroscopic $K_A$ values are related to the microscopic ones.

**A) Statistical factors from concentrations.**

For a dimeric system, there are two possible intermediates where one ligand is bound. They are labeled $ML'$ and $ML''$ in the diagram on the right. These are indistinguishable from each other by experimental measurement and equal in concentration. The microscopic and macroscopic binding constants for both steps are:

$$K_{1A}^\mu = \frac{[ML']}{[M][L]} = \frac{[ML'']}{[M][L]}$$

$$K_{1A} = \frac{[ML]}{[M][L]} = \left(\frac{[ML'] + [ML'']}{[M][L]}\right) = \frac{[ML']}{[M][L]} + \frac{[ML'']}{[M][L]} = K_{1A}^\mu + K_{1A} = 2 \times K_{1A}^\mu$$

$$K_{2A}^\mu = \frac{[ML'_2]}{[ML'][L]} = \frac{[ML'_{2A}]}{[ML'][L]}$$

$$K_{2A} = \frac{[ML_2]}{[ML'][L]} = \left(\frac{[ML'] + [ML'']}{[M][L]}\right) = \frac{[ML_2]}{2[ML'][L]} = \frac{1}{2} K_{2A}^\mu$$

**B) Statistical factors from kinetic-rates.**

For any reaction, the equilibrium constant is: $K_A = \frac{k_{on}^{Total}}{k_{off}^{Total}}$

The microscopic binding constants are just: $K_{A1}^\mu = k_{on-1}/k_{off-1}$.

For the first binding event: $K_{A1} = \frac{k_{on}^{Total}}{k_{off}^{Total}} = \frac{2k_{on}}{k_{off}} = 2 \times K_A^\mu$

(There are two ways to form the $[ML]$ species, but only one way for the ligand to leave.)

For the second binding event: $K_{A2} = \frac{k_{on}^{Total}}{k_{off}^{Total}} = \frac{k_{on}}{2k_{off}} = \frac{1}{2} K_A^\mu$

(There is only one way for the second ligand to bind, but there are two ways for the ligand to leave.)

**Example – Trimeric System:**
Biochemistry I

Summary of Ligand Binding:

- Y = Fractional saturation. Varies from 0 to 1.
- Y = [ML]/(ML)+[M]) (one site).
- n = Number of binding sites.
- $K_A$ = Association (binding) equilibrium constant, $K_A$=[ML]/[M][L].
- $K_D$ = Dissociation constant, $K_D$=1/$K_A$. Y = 0.5 when [L]=$K_D$.
- $K_{D,OBs}$ = Observed $K_D$ for coop binding, [L] to give Y=0.5
- $n_h$ = Hill coefficient, measure of cooperativity, maximum value is n (inf pos coop).

How to Measure Y:

i) Equilibrium dialysis.
- Protein (M$_1$) inside dialysis bag, cannot leave (semi-permeable).
- Add ligand to outside, after equilibrium is reached, L$_{IN}$ = [ML] + L$_{OUT}$.
- Y = (ML)/(MT) = (LN-L$_{OUT}$)/MT, at a ligand concentration of L$_{OUT}$.

ii) Spectrophotometric.
- Measure absorption [L]=0, this gives $A_M$
- Measure absorption with saturating concentrations of [L], this gives $A_{ML}$.
- Vary [L], measure A
- Y = ($A_A$-$A_M$)/($A_{ML}$ - $A_M$)

Data Analysis—How to obtain $K_D$ and Hill coef.

i) Binding Curve: Plot Y versus [L].
- $K_D$ is [L] to give Y=0.5. This is true $K_D$ for non-cooperative binding, $K_{D,OBs}$ for cooperative binding.

ii) Hill Plot: Plot log(Y/(1-Y)) versus log[L]
- $K_D$ - Ligand concentration when curve crosses x-axis (Y=0.5). This is true $K_D$ for non-cooperative binding, $K_{D,OBs}$ for cooperative binding.
- $n_h$: Slope, $\Delta$(log(Y/(1-Y))/log([L])), when curve crosses x-axis.

Type & degree of cooperativity:

- $n_h$=1 for non-cooperative binding. No interaction between binding sites.
- $n_h$>1 for positive cooperativity: Binding of the 1st ligand enhances the binding of additional ones.
- $n_h$<1 for negative cooperativity: Binding of the 1st ligand impairs the binding of additional ones.
- The closer $n_h$ is to n, the stronger the cooperativity, maximum value is n, # of sites.

Microscopic $K_A$($K_A^\mu$): This is the association constant for binding to a single site. It is the ratio of the on- and off-rates: $K_A^\mu = k_{on}/k_{off}$. It reflects the intrinsic affinity between the protein and the ligand: $\Delta G^0 = -RT \ln K_A^\mu$. This is what would be measured for a monomeric protein or an isolated site. Compare microscopic binding constants when assessing cooperativity. These will all be the same for a non-cooperative system.

Macroscopic $K_A$: This is the observed $K_A$ based on the experimental measurement of the concentrations of the various species, i.e $K_A$=[ML]/[M][L]. The macroscopic binding constants are related to the microscopic, e.g. for a protein that binds two ligands: $K_A^\mu = 2K_A^\mu$, $K_A^\mu = 1/2 K_A^\mu$

Important parameters and how to obtain them:

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<th>Cooperative</th>
</tr>
</thead>
</table>
| $K_D$ (This is always [L] that gives Y=0.5) | 1. Binding Curve, Y=0.5, [L] = $K_D$
2. Hill Plot, x-intercept= log $K_D$ | 1. $Y = 0.5$ on binding curve, [L]=$K_{D,OBs}$
2. x-intercept of Hill Plot = log $K_{D,OBs}$ |
| $n_h$ (Hill coefficient) | =1 | Slope of Hill plot when Y=0.5 (log[Y/(1-Y)] = 0) |
| $\Delta G^0$ | $\Delta G^0 = -RT \ln K_A = -RT \ln (1/K_D)$ | $\Delta G^0 = -RT \ln (1/K_{D,OBs})$ |