Lecture 14: Analysis of Cooperative Binding

Goals: Obtain $K_0$ and $n_h$ from Hill plot, correctly interpret $n_h$, in terms of type of cooperativity.

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

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 - 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.

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:

$$[M] + [L] \xrightarrow{K_{D1}} [ML]$$

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

i) $n_h = 1$. Non-cooperative. The binding constant remains the same for both binding events. $K_{D1} = K_{D2}$

ii) $n_h < 1$. Negative cooperativity: $K_{D2} > K_{D1}$ i.e. the second binding is lower in affinity.

iii) $n_h > 1$. Positive cooperativity: $K_{D2} < K_{D1}$ i.e. the second binding is higher in affinity.

iv) $n_h = n$. Infinitely strong positive cooperativity, $K_{D2} \ll K_{D1}$.

<table>
<thead>
<tr>
<th>Hill Coefficient ($n_h$)</th>
<th>Interpretation</th>
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<tbody>
<tr>
<td>$= 1$</td>
<td>Non-cooperative</td>
</tr>
<tr>
<td>$&lt; 1$</td>
<td>Negative cooperativity.</td>
</tr>
<tr>
<td>$&gt; 1$</td>
<td>Positive cooperativity.</td>
</tr>
<tr>
<td>$= n$, # of binding sites</td>
<td>Infinitely strong pos. cooperativity.</td>
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</table>
Hill Equation and Plot: The Hill coefficient, and the observed $K_D$ can be obtained from a Hill Plot. The Hill plot is based on the following transformation of the above binding equation:
- Plot of $\log \left[ \frac{Y}{1-Y} \right]$ versus $\log [L]$
- The Hill coefficient, $n_h$, is the slope as the line crosses the $x$-axis.
- The $\log K_{D-obs}$ is the intersection of the Hill curve with the $x$-axis. This is the $[L]$ to give $Y=0.5$.

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

$$Y = \frac{[L]}{K_D + [L]} \rightarrow \log \frac{Y}{1-Y} = -\log K_D + \log [L]$$

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

$$0 = \log \frac{1}{K_D} + \log [L]$$

$$-\log \frac{1}{K_D} = \log [L]$$

$$\log K_D = \log [L]$$

$$K_D = [L]$$

Cooperative Systems. 

Intermediate Ligand Concentration ($@Y=0.5$):
- Slope: Hill coefficient ($0 \leftrightarrow 1 \leftrightarrow n$)
- Intercept: Ligand concentration to give $Y=0.5 = K_D^{Ave}$.

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 $\log K_{D1}$.

High ligand: At very high ligand concentration, the binding also appears non-cooperative because most of the macromolecule is in the [MLn] form. Therefore the Hill plot is again linear, with a slope $= 1$, intersecting the $x$-axis at $\log K_{Dn}$.

$K_{D-obs}$: For a macromolecule that binds n-ligands with infinite cooperativity:

$$Y = \frac{[L]^n}{K_D^n_{obs} + [L]^n}$$

When $Y = 0.5$ what is $[L]$? $[L]_{Y=0.5} = K_{D-obs} = \sqrt[n]{K_{D1}K_{D2}...K_{Dn}}$

For less cooperative systems, the fractional saturation can be approximated by the Hill equation:

$$Y = \frac{[L]^n}{K_D^n_{obs} + [L]^n}$$

When $Y=0.5$, $[L] = K_{D-obs}$ $K_{D-obs} = f(K_{D1}, K_{D2}, ... K_{Dn})$. Although the observed $K_D$ can be easily obtained, it cannot be easily related to the individual $K_D$ values unless $n_h=N$.

Exception: for two binding sites $K_{D-obs} \approx \sqrt[K_D]{K_{D1}K_{D2}}$, regardless of $n_h$. 

ML2 $\rightarrow$ ML $\rightarrow$ M. $\frac{K_{D2}}{K_D}$
Lecture 14b: K_{D,\text{OBS}}: Microscopic & Macroscopic Binding Constants.

K_{D,\text{OBS}} = \text{Ligand concentration to give } Y=1/2.

<table>
<thead>
<tr>
<th>Non-cooperative Binding</th>
<th>Infinitely Positive Cooperative (n-sites)</th>
<th>Cooperative systems (Pos &amp; Neg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y = \frac{[L]^n}{K_D^n + [L]^n}$</td>
<td>$Y = \frac{[L]^n}{K_{D,\text{OBS}}^n + [L]^n}$</td>
<td>$Y \approx \frac{[L]^n}{K_{D,\text{OBS}}^n + [L]^n}$</td>
</tr>
</tbody>
</table>

For systems that bind two ligands, $K_{D,\text{OBS}} = \sqrt{K_{D,1}\times K_{D,2}}$, regardless of the level of cooperativity. Note: In OLI $K_{D,\text{OBS}} = K_{D,\text{Ave}}$.

Microscopic and Macroscopic Binding Constants:

A. Microscopic $K_A (K_A^n)$: This is the association constant for a single site, and is just the ratio of the on- and off-rates: $K_A^n = k_{on}/k_{off}$. It reflects the intrinsic affinity between the protein and the ligand. The standard energy of binding can be obtained as usual: $\Delta G^o = -RT \ln K_A^n$.

This is what would be measured for a single distinct binding site. If $K_A^n$ changes from one binding step to another, the system is cooperative:
- Non-cooperative: $K_A^n$ is the same for each step.
- Positive cooperativity: $K_A^n$ increases ($K_0^n$ decreases)
- Negative cooperativity: $K_A^n$ decreases ($K_0^n$ increases)

In the diagram on the right, two of the four microscopic equilibria are indicated by gray boxes.

B. Macroscopic $K_A$: This is the experimentally observed $K_A$ based on the measurement of the concentrations of the various species, i.e. $K_A = [ML][M][L]$. In the diagram on the right, one of these is indicated with the green shading.

The macroscopic $K_A$ values are related to the microscopic ones by statistical factors, e.g. for a system that binds two ligands:

- $K_{1,\text{A}} = 2K_A^n$
- $K_{2,\text{A}} = \frac{1}{2}K_A^n$
- $K_{1,\text{A}}'' = \frac{[ML][M][L]}{[ML][M][L]} = \frac{[ML][M][L]}{[ML][M][L]} = 2K_{1,\text{A}}''$
- $K_{2,\text{A}}'' = \frac{[ML][M][L]}{[ML][M][L]} = \frac{[ML][M][L]}{[ML][M][L]} = \frac{[ML][M][L]}{[ML][M][L]} = 2K_{2,\text{A}}''$

In 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:

Using Rate Constants to determine Statistical Factors: $K_A = k_{\text{forward}}/k_{\text{reverse}}$

- $K_{1,\text{A}}$: There are two ways to make (ML), the ligand can bind to either free site, so the forward rate would be twice as fast. There is only one way for (ML) to return to (M): $K_{1,\text{A}} = 2k_{10}/k_{20} = 2K_{1,\text{A}}''$
- $K_{2,\text{A}}$: There is only one way to make (ML2), the ligand can only bind to the remaining free site, so the forward rate would be just $k_{20}$. There is two ways for (ML2) to return to (ML) since the ligand can exit from either site: $K_{2,\text{A}} = k_{20}/2k_{20} = (1/2)K_{2,\text{A}}''$

Unless told otherwise, you should always assume you are given microscopic $K_A$ values.
Summary of Ligand Binding:
- \( Y = \) Fractional saturation. Varies from 0 to 1.
- \( Y = \frac{[ML]}{([ML]+[M])} \) (one site).
- \( n = \) Number of binding sites.
- \( K_A = \) Association (binding) equilibrium constant, \( K_A = \frac{[ML]}{[M][L]} \).
- \( K_D = \) Dissociation constant, \( K_D = \frac{1}{K_A} \). \( Y = 0.5 \) 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 (\( M_s \)) inside dialysis bag, cannot leave (semi-permeable).
  - Add ligand to outside, after equilibrium is reached, \( L_{in} = [ML] + L_{out} \).
  - \( Y = \frac{[ML]}{[M]_T} = \frac{L_{in} - L_{out}}{M_T} \), at a ligand concentration of \( L_{out} \).

  ii) Spectrophotometric.
  - Measure absorbance \( [L]=0 \), this gives \( A_M \).
  - Measure absorbance with saturating concentrations of \([L]\), this gives \( A_{ML} \).
  - Vary \([L]\), measure \( A \).
  - \( Y = \frac{(A-M)}{(A_{ML} - M)} \)

Data Analysis—How to obtain \( K_D \) and Hill coeff.
  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, \( \Delta(\log(Y/(1-Y)))/\Delta\log([L]) \), 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 \( K_A(K_A^n) \): This is the association constant for binding to a single site. It is the ratio of the on- and off-rates: \( K_A^n = K_{on}/K_{off} \). It reflects the intrinsic affinity between the protein and the ligand: \( \Delta G^p = -RT \ln K_A^n \). 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: \( K_A = 2K_A^2, \quad K_A = \frac{1}{2}K_A^2 \).

Important parameters and how to obtain them:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>One-site or ( n )-sites non-cooperative</th>
<th>Cooperative</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_D ) (This is always ([L]) that gives ( Y=0.5 ))</td>
<td>1. Binding Curve, ( Y=0.5 ), ([L]=K_D)</td>
<td>1. ( Y = 0.5 ) on binding curve, ([L]=K_D_{OBS})</td>
</tr>
<tr>
<td>( n_h ) (Hill coefficient)</td>
<td>2. Hill Plot, x-intercept= ( \log(K_D) )</td>
<td>2. x-intercept of Hill Plot = ( \log(K_D_{OBS}) )</td>
</tr>
<tr>
<td>( \Delta G^p )</td>
<td>( \Delta G^p = -RT \ln K_A = -RT \ln (1/K_D) )</td>
<td>( \Delta G^p = -RT \ln (1/K_D_{OBS}) )</td>
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</tbody>
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