Ligand Binding

A. Binding to a Single Site:

The equilibrium constant (also known as association constant or affinity constant) for the binding of a ligand to a protein is described by the following equation (note: $K_{eq} = K_A$):

$$K_{eq} = \frac{[ML]}{[M][L]} \qquad (1)$$

where K_{eq} is the equilibrium constant for the reaction, [ML] is the concentration of the protein-ligand complex, [M] is the concentration of the protein, and [L] is the concentration of the free ligand (not the total ligand present in solution). Note that the dissociation constant, K_D, is just the inverse of K_{eq}:

$$K_D = \frac{[M][L]}{[ML]} \qquad (2)$$

A ligand is usually considered to be a small molecule, however, anything that binds with specificity can be considered a ligand.

A1. Importance of the Association constant:

Provides a qualitative measure of the binding affinity - useful for comparisons. Provides information on the energetics of binding by the relationship: $\Delta G = -RT\ln K_{eq}$. The enthalpy of binding can be obtained from the temperature dependence of K_{eq}.

A2. Measurement of the Association Constant:

In order to obtain an experimental measurement of K_{eq} it is necessary to measure the concentration of [ML] as a function of [L]. This is usually done in one of two ways:

Monitor a change in the spectroscopic properties of the protein (or ligand). For example, the binding of the hapten dinitrophenyl to Fab fragments would likely alter the extinction coefficient of the two Trp residues that interact with the hapten.

Utilize equilibrium dialysis. The protein (in solution) is placed inside a sealed bag composed of dialysis membrane. The properties of this membrane are such that the small ligand can freely diffuse across the membrane but the protein cannot. The ligand is added to the outside of the bag and after equilibrium is reached ($[L]_{IN} = [L]_{OUT}$) it is possible to measure the total concentration of the ligand inside the bag. This is equal to [ML]+[L]. The concentration of free ligand, [L], can be obtained by sampling outside of the bag, thus giving [ML].

A3. Single Site Binding & Fractional Saturation:

The fractional saturation, Y, is defined as the fraction of protein molecules that are saturated with ligand. Y varies from 0 to 1.0. In the case of a protein that binds only one ligand it is the same as the ratio of the moles of ligand bound/mole of protein. In the form of an equation:

$$Y = \frac{[ML]}{[M]+[ML]}$$
$$= \frac{[M][L]K_{eq}}{[M]+[M][L]K_{eq}}$$
$$= \frac{K_{eq}[L]}{1+K_{eq}[L]} \qquad (3)$$
$$= \frac{[L]}{K_D+[L]} \qquad (4)$$

The above manipulations utilized the following equation: [ML] = [M][L]$K_{eq.}$

This equation gives a hyperbolic curve (example below). When the protein is half saturated with the ligand the ligand concentration is equal to K_D. This can be easily seen:

$$\frac{1}{2} = \frac{[L]_{1/2}}{K_D+[L]_{1/2}}$$
$$(K_D+[L]_{1/2}) = 2[L]_{1/2}$$
$$K_D = [L]_{1/2} \qquad (5)$$

B. Effect of Inhibitor Binding:

A protein can often bind more than one ligand at the same site. If the second ligand binds, but its binding is not biologically productive, then it is termed an inhibitor. For example, an enzyme will bind a substrate and transform it into a product. The same enzyme can bind a structurally related compound and not be able to transform it into a product. The presence of the latter compound reduces the ability of the enzyme to bind to substrate and thus *inhibits* the reaction.

The effect of an inhibitor on the affinity of the ligand is to reduce the association constant by an amount that depends on the inhibitor concentration, [I], and the association constant of the inhibitor for the protein, K_I.

$$K_{eq}^{'} = K_{eq}\frac{1}{(1+K_I[I])} \qquad (6)$$

The origin of this equation can be seen by substituting $K_{eq}^{'}$ into the above equation for fractional saturation:

$$Y = \frac{K_{eq}^{'}[L]}{1+K_{eq}^{'}[L]}$$
$$= \frac{\frac{K_{eq}}{(1+K_I[I])}[L]}{1+\frac{K_{eq}}{(1+K_I[I])}[L]}$$
$$= \frac{K_{eq}[L]}{1+K_{eq}[L]+K_I[I]}$$

The latter equation is the correct equation to describe the fractional saturation of the protein in the presence of ligand and inhibitor (in that case Y=([ML]/([M]+[ML]+[MI])).

Note that the binding curve has *exactly* the same shape in the presence of the inhibitor, but will show a reduced association constant.

C. Scatchard Plot:

The hyperbolic binding curve can be put in a linear form by plotting Y/[L] versus Y. The equation of this line is:

$$\frac{Y}{[L]} = K_{eq} - K_{eq}Y \qquad (7)$$

$$\frac{Y}{[L]} = \frac{1}{K_D} - \frac{Y}{K_D} \qquad (8)$$

The slope of this plot is $-K_{eq}$ (or $1/K_D$); the y-intercept is $1/K_D$.

This equation that describes the Scatchard plot is obtained in the following fashion:

$$\frac{Y}{(1-Y)} = (1+K_{eq}[L])\frac{K_{eq}[L]}{(1+K_{eq}[L])}$$

$$Y = (1-Y)K_{eq}[L]$$

$$Y = K_{eq} - YK_{eq}[L]$$

$$\frac{Y}{[L]} = -K_{eq}Y + K_{eq}$$

D. Multiple Independent Binding Sites:

Proteins often have more than one binding site for the same ligand (i.e. the intact immunoglobulin molecule). If the binding events are independent it is quite easy to write the equation for the fractional saturation for a protein that contains *n* sites. In this case we define a new variable, ν:

$$\nu = n\sum_{i=1}^{n} Y_i \qquad (9)$$

$$= \frac{nK_{eq}[L]}{1+K_{eq}[L]} \qquad (10)$$

Note that Y has been replaced by ν. This is defined as the moles of bound ligand over the total protein concentration. If only one ligand can bound to the protein then the two are equal, otherwise $\nu = nY$. Not that ν varies from 0 to n (instead of 0 to 1 for Y)

This also gives a hyperbolic binding curve, but the maximum value of ν is now *n,* the number of ligand binding sites. The Scatchard plot in this case is just:

$$\frac{\nu}{[L]} = nK_{eq} - K_{eq}\nu$$

$$= \frac{n}{K_D} - \frac{\nu}{K_D}$$

Note that the slope is the same (the association constant), but the x-intercept now gives, n, the number of binding sites. The y-intercept is n/K_D.

E: Independent sites - microscopic and macroscopic binding affinities.

Consider the binding of two ligands to an immunoglobulin to proceed in the following fashion:

$$M + L \xrightarrow{K_1} (ML) \xrightarrow{K_2} (ML_2)$$

The equilibrium association constants are defined as follows:

$$K_1 = \frac{[ML]}{[M][L]}$$

$$K_2 = \frac{[ML_2]}{[ML][L]}$$

Note that these equilibrium constants refer to successive ligand binding steps and are termed *macroscopic binding* constants because they only report on the total number of half-liganded states. For example, there are actually two (indistinguishable) forms of [ML] in solution. Starting from the unliganded sample there are two ways for forming [ML] by binding to either of the two empty ligand binding sites.

The *microscopic association* constant reflects the equilibrium that would be measured if only one ligand can bind (as is the case for an isolated Fab fragment). The relationship between the microscopic binding constant K_{micro}, and the two macroscopic binding constants can be obtained by considering the reaction rates. The microscopic binding constant is defined below. The kinetic rate constant for ligand binding is k_{on} and that for release of the ligand is k_{off}.

$$K_{micro} = \frac{k_{on}}{k_{off}}$$

In the two-step binding curve the first binding constant is equal to: $K_1 = \frac{2k_{on}}{k_{off}}$

The factor of two comes from the fact that there are two ways to make [ML].

Similarly, the second macroscopic binding constant is: $K_2 = \frac{k_{on}}{2k_{off}}$

The factor of two in this case occurs because there are two ways of forming [ML] starting from $[ML_2]$.

Thus, in the case of independent binding events, even though there is no difference in the molecular events of binding, the observed affinity constants, K_1 and K_2 are not equal due to statistical factors.

F. Cooperative Binding

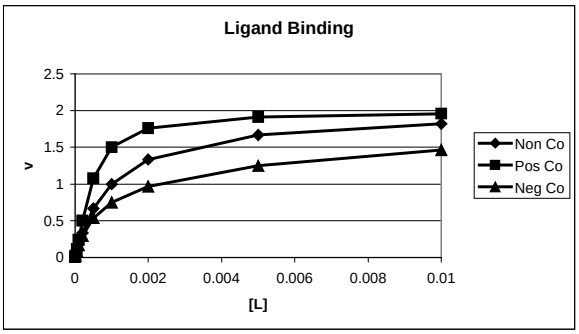
In many proteins (such as hemoglobin) the binding of the first ligand to the protein can change the affinity for the second ligand beyond that which would be observed for the above statistical factors. This can lead to cooperative binding, an important regulatory mechanism in biochemistry.

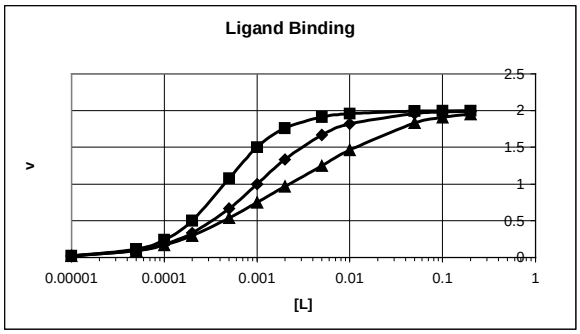
- Positive cooperativity is observed when K_2 is larger than $k_{on}/2k_{off}$.
- Negative cooperativity is observed when K_2 is less than $k_{on}/2k_{off}$

Whether binding is cooperative or not can be determined from the shape of the binding curve. It is difficult to distinguish non-cooperative from cooperative binding from a plot of ν (or Y) versus [L]. Differences in cooperative behavior are easier to recognize when ν is plotted versus log[L].

The following graphs show non-cooperative, positive cooperative, and negative cooperative binding. The left panel shows a plot of ν versus [L], with both plotted on a linear scale. The right panel shows a plot of ν versus [L] plotted on a semi-log scale with [L] on the log scale.

Each plot contains three curves, one for non-cooperative binding, one for positive cooperative binding and one for negative cooperative binding.





- In the case of positive cooperativity (Pos Co) a small change in ligand concentration gives rise to a large change in the concentration of the liganded protein.
- In the case of negative cooperativity (Neg Co) a large change in ligand concentration is required to obtain an equivalent change in the concentration of the liganded protein.
- The semi-log can be used to distinguish the type of cooperativity in the following way. For non-cooperative binding find the location where the protein is half-saturated with ligand, at 0.001M in this case. Now, check the fractional saturation at 10 times more ligand and 10 times less ligand. For non-cooperative binding these values will be 91% and 9% saturated, respectively (ν=1.82 and 0.18 in this example).

G. Hill Plot

The degree of cooperativity can be characterized by the Hill coefficient (n) which is the slope of the Hill plot at log(θ)=0. The Hill plot is a plot of : $\log\theta$ versus log[L]. Where θ is defined by the following equation:

$$\theta = \frac{Y}{1-Y} \qquad (13)$$

Y is the fractional saturation of the protein: the number of bound ligands/total number of binding sites (Y varies from 0 to 1).

The Hill coefficient is less than or equal to the number of binding sites(N).

- For non-cooperative binding, the slope (n)=1.
- If the slope (n) >1 the binding shows positive cooperativity
- For highly cooperative binding, n is close to N.
- If the slope(n)<1 the binding shows negative cooperativity.

G1. Single Site: (i.e. binding of antigen to Fab)

$\theta = K_{eq}[L]$. Thus, the Hill plot is of the following equation:

$$\log\theta = \log K_{eq} + \log[L]$$

A plot of $\log\theta$ *versus log([L])* will be a straight line with a slope of 1. When θ=1 ($\log\theta$ =0) then $\log K_{eq}$ = -log[L], or [L] = K_D. The intersection of the Hill plot when log(θ) = 0 gives K_D.

G2. Independent Two Site binding (i.e. binding of antigen to intact immunoglobulin)

$$\nu = \frac{[ML]+2[ML_2]}{[M]+[ML]+[ML_2]}$$

$$= \frac{K_1[L]+2K_1K_2[L]^2}{1+K_1[L]+K_1K_2[L]^2}$$

$$\theta = \frac{\nu/2}{(1-\frac{\nu}{2})} = \frac{K_1[L]+2K_1K_2[L]^2}{2+K_1[L]} \qquad (Y = \nu/2)$$

The behavior of this function can be understood by looking at the limits of low and high ligand concentration:

$$\theta_{[L]\to 0} = \frac{1}{2}K_1[L]$$

$$\theta_{[L]\to\infty} = 2K_2[L]$$

The Hill plot at these two extremes will have a slope of 1 and intercepts of $1/2K_1$ and $2K_2$, respectively. Remember that: $K_1 = \frac{2k_{on}}{k_{off}}$ and $K_2 = \frac{k_{on}}{2k_{off}}$.

gives the following two functions at low and high ligand concentration:

$$\log\theta_{[L]\to 0} = \log(\frac{1}{2}K_1) + \log[L]$$

$$\log\theta_{[L]\to\infty} = \log(2\frac{K_1}{42}) + \log[L]$$

$$= \log(\frac{K_1}{2}) + \log[L]$$

Both of these curves have the same intercept ($K_1/2$), equal to the microscopic binding constant, thus the slope of a Hill plot for two independent binding sites is the same as that found for one site (unity) and the intersect at log(θ)=0 gives the binding constant.
In summary, for non-cooperative binding, regardless of the number of ligands, the Hill plot is always linear with a slope of 1.

G3. Binding in a Highly Cooperative Manner:
If the affinity constant for the second step (K_2) is higher than that for the first step then the system will show cooperative binding. That is, once the first ligand binds, the second one binds more readily. If K_2 is very large compared to K_1 then the major species present in solution are either [M] or [ML_2] and the ligand binding equation can be *approximated* as:

$$Y = \frac{K[L]^2}{1 + K[L]^2}$$

this equation is obtained from the equilibrium constant for both binding steps occurring at the same time (e.g. [MX] is assumed to be zero)

$$K = \frac{[ML_2]}{[M][L]^2}$$

The fraction saturated is:

$$\theta = \frac{Y}{1-Y} = K[L]^2$$

the Hill plot is this case is:

$$\log\theta = \log K + 2\log[L]$$

Note that this has a slope of 2.

In general, for *n* ligands binding in a highly cooperative manner the degree of ligation is described by the general Hill equation:

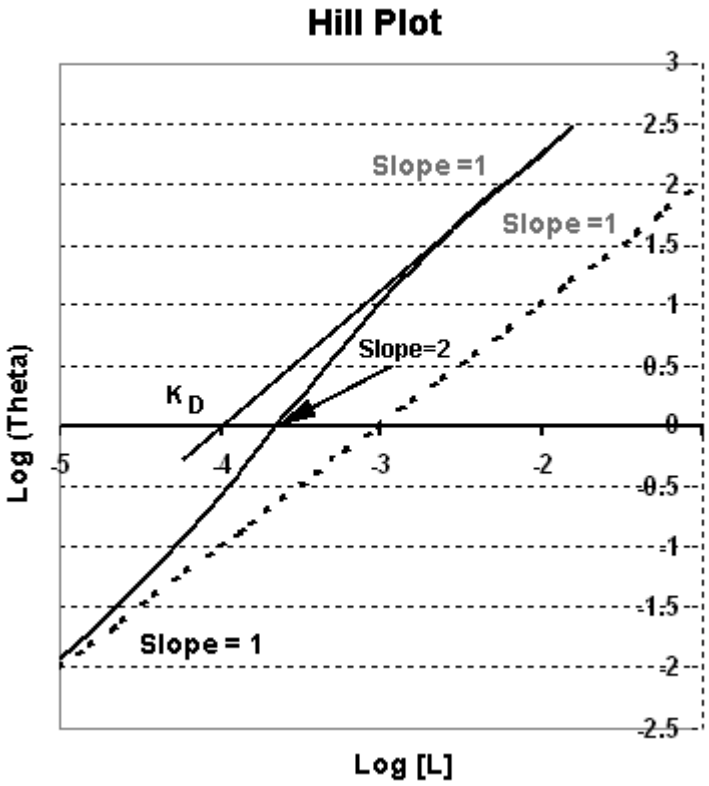
$$Y = \frac{K[L]^n}{1 + K[L]^n} \qquad (14)$$

This gives a Hill plot of the following form:

$$\log\theta = \log(K) + n\log([L]) \qquad (15)$$

For "absolute cooperativity", the slope of the Hill plot = n, the number of ligands bound. However, the binding properties of a highly cooperative system are only high cooperative for values of [L] near the middle to the binding curve. The slope of the Hill plot at low and high values of the ligand have a slope of one because under these conditions only one ligand is binding (to either the empty molecule or the molecule with one more space).

Hill plots for the binding of two ligands to a protein is shown below. This example shows non-cooperative binding and highly cooperative binding.



1. For non-cooperative binding (dashed line) a straight line is observed. Note that the intercept on the log(θ)=0 axis gives the value of K_D (10^{-3} in this case).
2. The curve for highly cooperative binding is shown (solid line). In this case the affinity constant for the second binding event was dramatically increased. Note the following points:
   - The slope at low and high ligand concentration is unity
   - The slope at log(θ)=0 is equal to 2, the number of binding sites
   - Extrapolation of the line from low ligand concentration gives K_D for the 1st binding event (10^{-3} in this case).
   - Extrapolation of the line from high ligand concentration gives K_D for the *last* binding event (10^{-4} in this case). If four ligands bound, this would be the K_D for the fourth binding step.

G4. Binding with Moderate Cooperativity

Biological systems usually display moderate cooperativity.

- A slope>1 in the middle (log(θ)=0) of the plot indicates some degree of positive cooperativity.
- The closer this slope is to the number of bound ligands, the higher the degree of cooperativity.
- A slope<1 in the middle indicates some degree of negative cooperativity.