Lecture 11: Ligand Binding Measurements

Recommended reading: Ligand Binding Handout by Dr. Rule

Key Terms:

- **Association constant**
- **Dissociation constant**
- **Equilibrium dialysis**
- **Scatchard equation & Scatchard plot**

Measurements of the binding affinity and binding stoichiometry between molecules and macromolecules provide essential information on topics that range from mechanism to specificity. In the case of enzymes, binding of the substrate necessarily preceeds the catalytic event.

Energetics: The formation of a ligand-protein complex can involve any and all of the fundamental forces in biochemistry:

Energetic Term	Native Protein Stability	Contribution to Ligand Binding
Conformational entropy	-------	
Hydrophobic effect	++++	
Van der Waals interactions	+++	
Hydrogen Bonds	++	
Electrostatic interactions	+	

11.1 Fundamentals of Ligand Binding:

- Ligands collide with their targets at a rate constant of k_{on}. Usually this is diffusion limited and occurs at about 10^8 $sec^{-1}M^{-1}$.

- The ligand leaves its binding site with a rate constant that depends on the strength of the interaction between the ligand and the binding site. Rate constants for dissociation (k_{off}) can range from $10^6 sec^{-1}$ (weak binding) to 10^{-2} sec^{-1} (strong binding).

- The equilibrium constant for binding is given by:

$$K_{eq} = \frac{[ML]}{[M][L]} = \frac{k_{on}}{k_{off}} = K_A$$

where [ML] is the macromolecule-ligand complex, [M] is the concentration of free macromolecule, and [L] is the concentration of free ligand. For example, M could be an Fv fragment and L could be DNP.

K_A is the term for the *equilibrium constant for association*. Note that Keq is no longer dimensionless as it was for protein folding, so remember to work in units of $molarity^{-1}$.

It is very useful to define *a dissociation constant*, K_D:

$$K_D = \frac{[M][L]}{[ML]} = \frac{\mathbf{1}}{K_A}$$

K_D has units of molarity and is the ligand concentration where [M] = [ML]. The K_D serves a similar role as the acidity constant, marking the point of half-saturation.

- Ligands with low K_D values bind tightly.
- Ligands with high K_D values bind weakly.

The fractional saturation, Y, is defined as:

$$Y = \frac{[ML]}{[M]+[ML]} = \frac{[ML]}{[M_T]}$$

Y is just the amount of macromolecule with bound ligand [ML], divided by the total concentration of macromolecule [M_T].

Using the expression for K_{eq} and K_D:

$$Y = \frac{K_A[L]}{\mathbf{1}+K_A[L]} = \frac{[L]}{K_D+[L]}$$

Y varies from 0 to 1. A plot of Y versus [L] is referred to as a **saturation binding curve**.

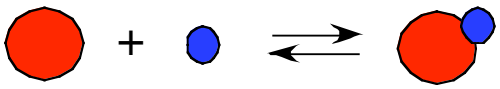
When [L] = K_D, Y = 0.5.

Measurement of K_D: The dissociation constant, K_D, is obtained by measuring Y as a function of free ligand concentration [L]. Once the K_D has been determined for a particular macromolecule-ligand combination (e.g. antibody and DNP) then it is possible to predict the fractional saturation at any ligand concentration.

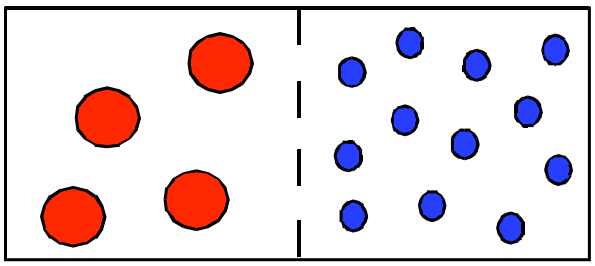
11.2 Equilibrium Dialysis: It is common to use equilibrium dialysis for this measurement. A dialysis membrane will allow small ligands to pass through, but will retain macromolecules such as proteins (M) as well as protein-ligand complexes (ML).

The following figures show how the equilibrium dialysis experiment can be used to determine the concentrations of M, L, and ML at binding equilibrium.

Model reaction: M + L $\rightleftharpoons$ ML, or schematically:



At the start of the measurement, the protein (M) is present only in the left cell of the dialysis chamber. The small molecule (L) is present only in the right cell. The left and right cells are separated by a semi-permeable membrane, through which only the ligand can pass. For illustration we will use concentration units of “balls/box” instead of mol/liter.

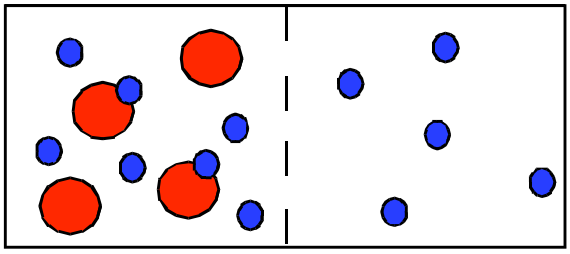


Starting concentrations: (b/B):

Left cell: [ML]=0; [L]=0; [M]=4.

Right cell: [ML]=0; [L]=12; [M]=0.

When equilibrium is reached, the concentration of *free ligand* [L] will be the same in both cells. However, because the protein can bind the ligand, the concentration of *total ligand* will be higher in the left cell.



Equilibrium concentrations (b/B):

Left cell: [ML]=2; [L]=5; [M]=2. **Observable [L_T]=7.**

Right cell: [ML]=0; [L]=5; [M]=0. **Observable [L_T]=5.**

Experimentally, the ligand concentration inside $[L]_{in}$ and outside $[L]_{out}$ the dialysis bag is measured.

The total amount of macromolecule, [M_T], is also known.
To calculate the fractional saturation, Y, it is necessary to determine the following:

[L] =

$[ML] = [L]_{in} - [L]_{out} =$

$[M] + [ML] = [M_T] =$

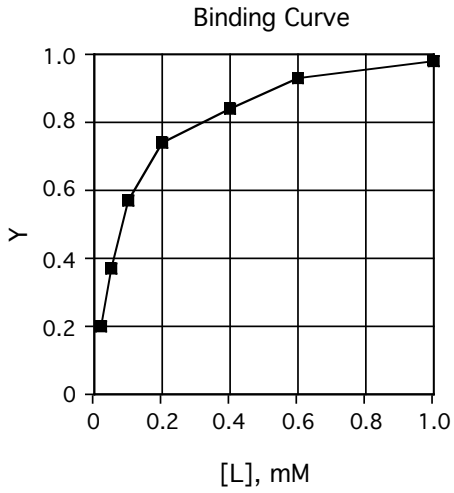
$$Y = \frac{[ML]}{[M] + [ML]} =$$

A complete binding curve is generated by measuring Y at different ligand concentrations.

11.3 Example of Data Analysis:
1. Acquire binding data as a function of ligand concentration. To do so you need a method of measuring [ML]. This could be by radioactivity (i.e. radioactive ligand) or by measuring changes in absorption of the protein due to binding of the ligand.

The following are some example data of Y as a function of [L]:

[L], mM	Y
0.02	0.20
0.05	0.37
0.1	0.57
0.2	0.74
0.4	0.84
0.6	0.93
1.0	0.98



2. Estimate KD from the binding data.

3. Plot the data on a **Saturation Binding Curve**:

Equation plotted: $$Y = \frac{[L]}{K_D + [L]}$$

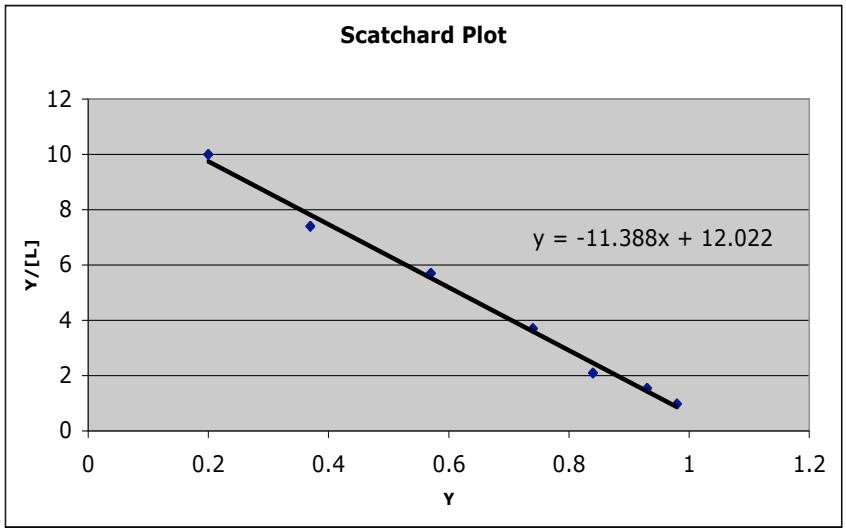
K_D is just the concentration of [L] that gives Y = 0.5 (half fractional saturation).

4. Plot the data on a **Scatchard Plot**:

From the saturation equation, the following rearrangement can be made to give a useful analytical tool, the Scatchard equation:

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

A plot of Y/[L] versus Y will give a straight line with a slope of –1/K_D. This is a useful transformation of the original hyperbolic binding curve to a simple line, from which the dissociation constant can be readily obtained.



This transformation essentially facilitates:

- Interpolation of the data in the region of [L]=K_D
- Extrapolation to infinite ligand concentration

Slope of line = -1/K_D = -K_A