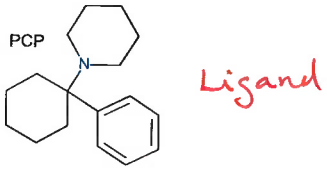
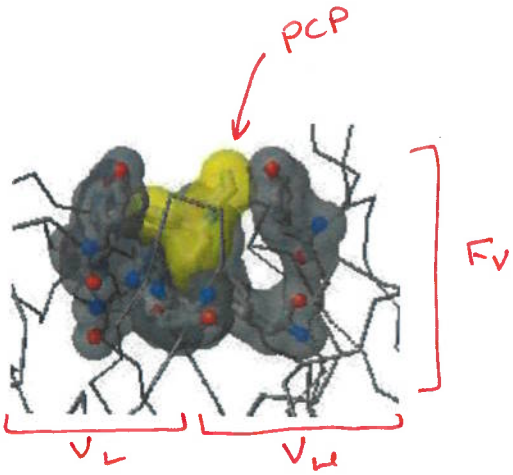
Lecture 11: Introduction to Ligand Binding

- Identify protein-ligand interactions
- Relate binding kinetics to interactions
- Use K_D to characterize strength of binding
- Experimental determination of binding
  - Equilibrium Dialysis
  - Spectrophotometric
- Obtain K_D from:
  - Binding curve
  - Hill plot



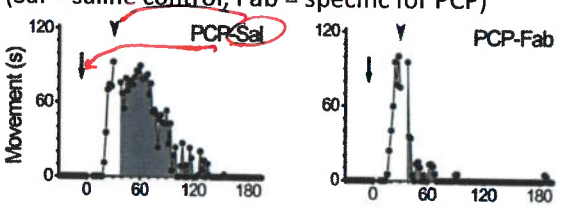


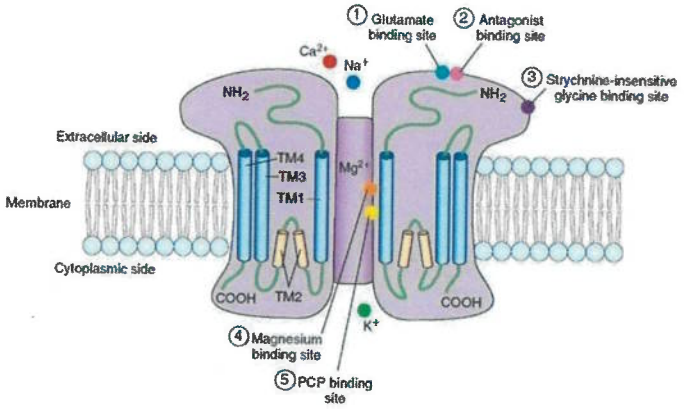
A. Stabilization of Antibody-Antigen Complexes:

Any (and all) of the following energetic terms may be important for binding of ligands to proteins. Which of the following stabilize the bound PCP?

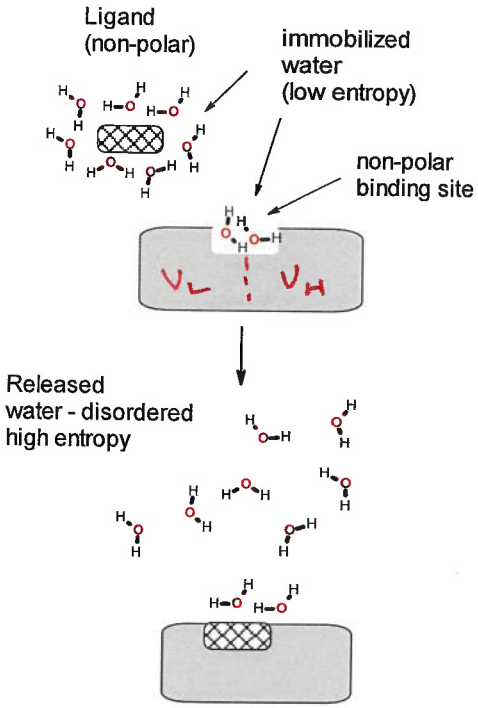
	Anti-PCP
Hydrophobic effect	Yes!
hydrogen bonding	Minor
van der Waals	Yes!
Electrostatic Interactions	No!

Anti-Phencyclidine (PCP, angel dust) antibody – drug detoxification
(Sal = saline control, Fab = specific for PCP)





A.



Fundamentals of Ligand Binding:

M = macromolecule **L = ligand** **ML = complex**

- Ligands collide with their targets, at a rate of k_{on}. Usually this is diffusion limited and occurs at a rate of about 10^8 $sec^{-1}M^{-1}$ (10^8 collisions/sec at a concentration of 1M).
- The ligand leaves its binding site at a rate that depends on the strength of interaction between the ligand and the binding site. Off-rates (k_{off}) can range from 10^6 sec^{-1} (weak binding) to 10^{-4} sec^{-1} (strong binding).
- Binding of multiple ligands can be cooperative, the binding of one affects the subsequent binding. If only one ligand binds, it must bind in a non-cooperative manner.

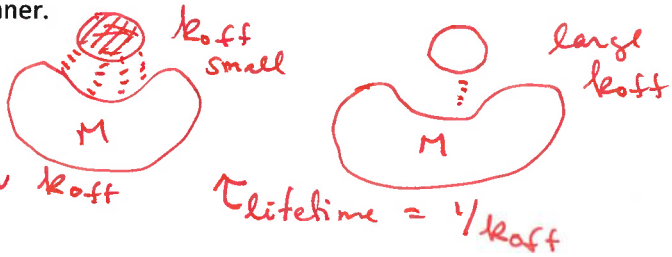
$k_{ON} \approx 10^8\ sec^{-1}\ M^{-1}$ (same for all)

$(M) + (L) \rightleftharpoons (ML)$

F_V PCP k_{off} Varies $(F_V \cdot PCP)$

k_{off} depends on # of interactions

more interactions, smaller k_{off}



The equilibrium constant for binding is called K_A, K-"association": $\frac{[ML]}{[M][L]} = K_{eq} = K_A = \frac{k_{on}}{k_{off}}$

The representation of K_A in term of kinetic rates (k_{on}, k_{off}) comes from expressing the change in [ML] as a function of time, and then setting this change to zero at equilibrium.

$$M + L \underset{k_{OFF}}{\overset{k_{ON}}{\rightleftharpoons}} ML$$

- The overall forward rate (formation of (ML) depends on k_{on} times the concentration of both (M) and (L). k_{on} is the rate of complex formation when (M) and (L) are at 1M.
- The reverse rate depends on how much (ML) is present and the rate (k_{off}) that the ligand falls off.
- At equilibrium, there is no next change in (ML) (but binding and dissociation still occur, but with equal rates).

$$\frac{d[ML]}{dt} = +k_{on}[M][L] - k_{off}[ML]$$

$$0 = +k_{on}[M][L] - k_{off}[ML]$$

$$k_{on}[M][L] = k_{off}[ML]$$

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

Don't get K_A confused with the acidity constant used to describe acid-base reactions.

It is very useful to define a **dissociation constant, K_D** (equilibrium constant for reverse reaction, or dissociation).

$$K_D = \frac{[M][L]}{[ML]} = \frac{1}{K_A} = \frac{k_{off}}{k_{on}}$$

$$ML \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} M + L$$

$K_D = \frac{k_{off}}{k_{on}}$

- The K_D has units of molarity.
- What is the value of [L] when [M]=[ML]?

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

if $[L] = K_D$

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

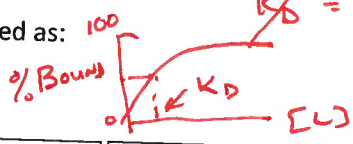
Ligands that bind tightly have low k_{off} and therefore small K_D values.

Ligands that bind weakly have high k_{off} and therefore higher K_D values.

Experimental Measurement of Ligand Binding - Fractional Saturation.

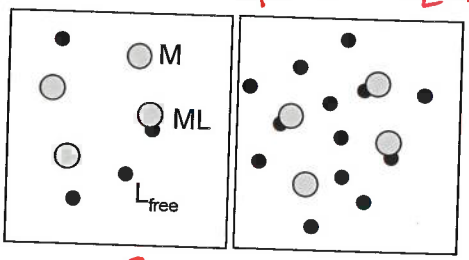
The **fractional saturation**, Y (θ is also used in some textbooks), is defined as:

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



Y is just the amount of protein with bound ligand, divided by the *total* concentration of protein, $[M_T]$. Y various from 0 to 1.

Note: $[M_T]$ is usually constant in a given experiment, the ratio of [M] to [ML] changes as the ligand concentration changes.



$M_{TOT} = 4$

$[L_{free}]$? 3 10

Y? $\frac{1}{4} = .25$ $\frac{3}{4} = .75$

Y can be related to [L] and K_A or K_D:

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

$$Y = \frac{K_A[L]}{1+K_A[L]} = \frac{1/K_D[L]}{1+(1/K_D)[L]} = \frac{[L]}{K_D+[L]}$$

Note: [L] is the **free** ligand concentration, not the total, typically the approximation $[L]_{TOTAL}=[L]_{FREE}$ is used.

$Y_{obs} = \frac{[L]}{K_D+[L]}$

$K_A = \frac{[ML]}{[M][L]}$ $M + L \rightleftharpoons (ML)$

$K_A[M][L] = [ML]$

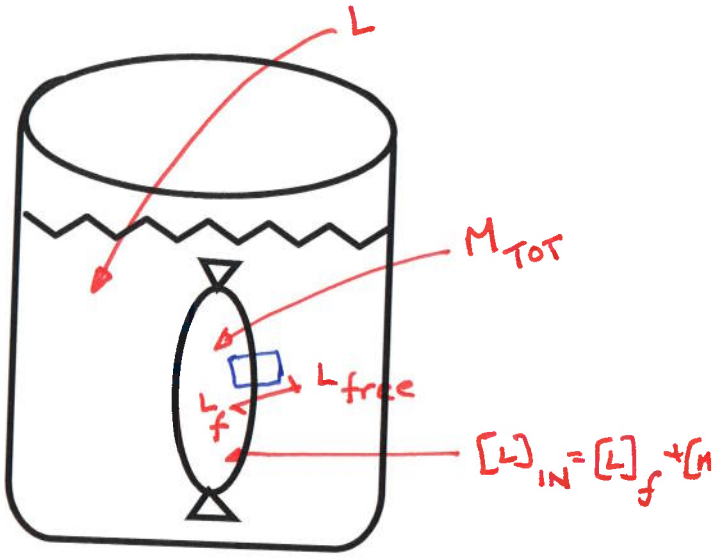
If [L]≈[M] and the binding is high affinity such that a significant amount of the ligand is bound the assumption that $[L]_{Free}=[L]_{Total}$ is a poor one. In which case the equation that describes the binding is:

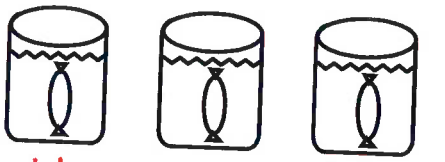
$$[ML] = \frac{(M_{TOT} + L_{TOT} + K_D) - \sqrt[2]{(M_{TOT} + L_{TOT} + K_D)^2 - 4(M_{TOT}L_{TOT})}}{2}$$

K_D is obtained by fitting the data using non-linear fitting programs, such as Solver in Excel.

A. Measurement of Y using Equilibrium Dialysis:

- A dialysis membrane is a *closed* bag that will allow small ligands to pass through, but will retain proteins (M) as well as protein-ligand complexes (ML).
- A series of different experiments are performed, with the ligand concentration varied. The fractional saturation is obtained for each ligand concentration.
- Experimentally, the ligand concentration inside ($[L]_{IN}$) and outside ($[L]_{OUT}$) the dialysis membrane is measured. Given that the total amount of macromolecule, $[M_T]$, is also known, it is possible to calculate Y from these two measurements.

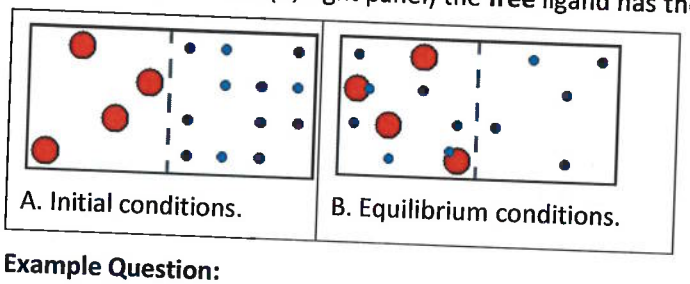




Model reaction: [M] + [L] ↔ [ML]

$$Y = \frac{[ML]}{M_{TOT}} = \frac{[L]_{IN} - [L]_{free}}{M_T} = \frac{[ML]}{M_T}$$

Initially (A, left panel), 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 semi-permeable membrane only allows ligand to pass; M (and ML) is too large. At equilibrium (B, right panel) the **free** ligand has the same concentration on both sides of the membrane.



A. Initial conditions. B. Equilibrium conditions.

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

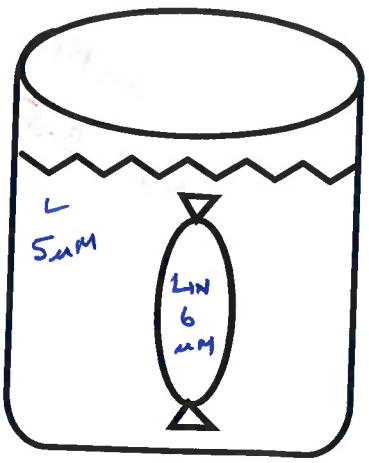
$[L]_{free} = 5$ & $[ML] = 7 - 5 = 2$

$$Y = \frac{2}{4} = 0.5$$

Example Question:

- Protein is added to a dialysis bag to give a concentration of 8 uM.
- Ligand is added and after equilibrium, its concentration outside the bag is 5 uM.
- The amount of ligand inside the bag was found to be 6 uM.

1. What is the free ligand concentration?
2. What is the total ligand concentration inside the bag?
3. What is the bound ligand concentration?
4. What is the fractional saturation?



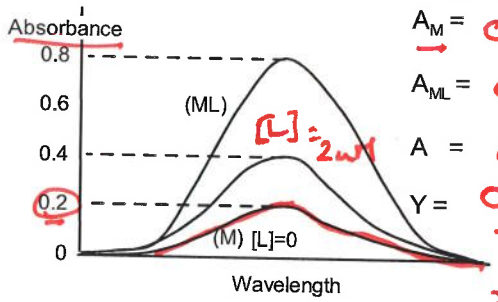
$$Y_{obs} \text{ at } [L] = 5uM = \frac{[ML]}{[M_{TOT}]} = \frac{1uM}{8uM} = .125$$

B. Measuring fractional Saturation by Spectrophotometry

If the absorption (or NMR, or fluorescence) spectra of the free macromolecule (M) is different from the macromolecule-ligand complex (ML), then the absorbance of the solution can be used to measure the fractional saturation.

A_M = absorbance of free macromolecule.
A_{ML} = absorbance of macromolecule with ligand bound, (ML) complex.
A = absorbance with an arbitrary mixture of M and (ML), i.e. at non-saturating ligand concentrations.

- The largest change in absorption: $A_{ML} - A_M$ corresponds to a change in fractional saturation of 1.0.
- The fractional saturation is just the fractional absorbance change: $Y = \frac{A - A_M}{A_{ML} - A_M}$



$[L]_{free} = 2\,\mu M$
$A_M = 0.2$
$A_{ML} = 0.8$
$A = 0.4$
$Y = \frac{0.4 - 0.2}{0.8 - 0.2} = 0.2/0.6 = .33$

Amount of change / Total pos change

Derivation:
The absorption if none of the macromolecules had ligand bound:
$A_M = \varepsilon_M \times l \times [M_T]$
The absorption if all of the macromolecules had ligand bound:
$A_{ML} = \varepsilon_{ML} \times l \times [M_T]$
The absorption if we have a mixture of bound and unbound (Y=fraction bound, (1-Y)=fraction unbound):
$A = \varepsilon_{ML} \times l \times (Y \times [M_T]) + \varepsilon_M \times l \times ((1-Y) \times [M_T])$
$A = Y \times A_{ML} + (1-Y) \times A_M$
$A = Y \times A_{ML} + A_M - Y \times A_M$
$A = Y(A_{ML} - A_M) + A_M$
$Y = \frac{A - A_M}{A_{ML} - A_M}$

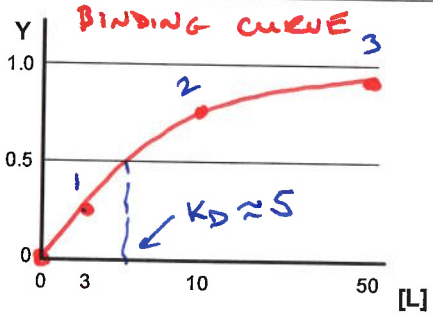
Data Analysis Methods – Obtaining K_D from Y.

The fractional saturation as a function of free ligand concentration is measured→

$[L]_{free}$	Y
0	0.00
3 uM	0.25
10 uM	0.75
50 uM	0.90

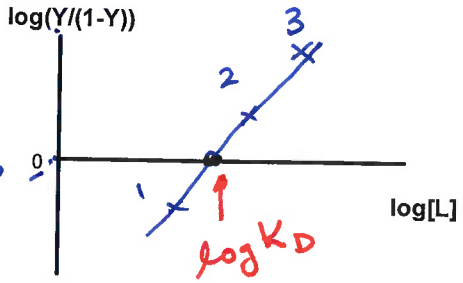
The K_D can be determined by one of the following three methods:

1. Directly from the **binding curve** (or raw data) by estimating the [L] which gives Y = 0.5. A drawback is that this approach typically does not utilize all of the collected data, but only data near Y=0.5.



2. Direct fitting of the data to the binding equation using Solver in Excel.
$$Y = [L]/(K_D + [L])$$

3. By plotting a **Hill plot**. *log*(Y/(1-Y)) *versus* *log*[L]
x-intercept is *log*K_D.
When Y=0.5, [L]=K_D: *log* (0.5/0.5)=*log*(1)=0, therefore x-int. = *log*K_D.
Slope is one for non-cooperative binding (single site, or no interaction between ligands for multiple sites).



$\log \frac{0.5}{(1-0.5)} = \log \frac{0.5}{0.5} = \log 1 = 0$ $\quad Y = \frac{1}{2}$

Binding Energetics: This equilibrium constant is related to the free energy of binding in the usual way. Notice that K_A is no longer dimensionless, as it was for protein folding, so lnK_A is undefined. To fix this problem, everyone agrees to work in molar units when calculating energies. Usually the reaction direction is M+L→(ML), i.e. the ΔG° represents the energy *released* when binding occurs. The enthalpy of binding is obtained from the temperature dependence of the equilibrium constant.

$$K_A = [ML]/[M][L]$$
$$\Delta G^o = -RT \ln K_A$$
$$= \Delta H^o - T\Delta S^o$$
$$\frac{d \ln(K_A)}{d(1/T)} = -\Delta H^0 / R$$

Protein folding
$N \rightleftharpoons U$
$\Delta G^\circ = -RT \ln \frac{[U]}{[N]}$

$\Delta G^\circ = -RT \ln \frac{[ML]}{[M][L]}$ ← use moles/L.