Medchem 528
Biophysical Enzymology and Biopharmaceuticals
W, F 2:30 – 4:00 pm

Bill Atkins
winky@uw.edu
685 0379

Course website:
http://courses.washington.edu/medch528

***Tentative* Lecture Schedule**

Lecture	Instructor	Date	Lecture Topic
Part I: Protein-Ligand Interactions			
1	Atkins	January 6	Ligand Binding at Equilibrium
2	Atkins	January 8	Ligand Binding at Equilibrium/Regulation
3	Atkins	January 13	Ligand Binding Dynamics/Allostery
4	Atkins	January 15	Ligand Binding Dynamics/Antibody Basics
Part II: Methods			
5	Nath	January 20	UV-Vis, CD, Fluorescence, Light Scattering
6	Nath	January 22	Optical Methods, Single Molecule
7	Schuman/GE Healthcare	January 27	Surface Plasmon Resonance
8	Sumida	January 29	Calorimetry (ITC/Thermodynamics and Drug Design)
9	Sumida	February 3	Calorimetry (DSC, protein stability)
10	Sumida	February 5	Analytical Ultracentrifugation
11	Lee/Guttman	February 10	H/DX MS Protein Dynamics
12	Lee	February 12	Structural Analysis: SAXS, EM
Part III: Applications in Biopharmaceuticals			
13	Pearson	February 17	Antibody Clearance and Disposition
14	Lyon	February 19	Antibody Drug Conjugates
15	Atkins	February 24	Therapeutic Protein Scaffolds/dabs/FC-fusions
16	Hill	February 26	Examples from Protein Therapeutic Development
17	Hill	March 2	Examples from Protein Therapeutic Development
18	-	March 4	*Student presentations*
19	-	March 9	*Student presentations*
20	-	March 11	*Student presentations*

Protein-Ligand Interactions:
Thermodynamics, Kinetics, and Allostery

References:

Cantor & Schimmel. Biophysical Chemistry vol. III, Chapter 15. ✔ web site

Wyman & Gill. Binding and Linkage: Functional Chemistry of Biological Macromolecules (1990, University Science Books) Chapters 1, 2.

G. Weber. Protein Interactions. (1992, Chapman & Hall).

Advances in Protein Chemistry, vol. 51 (1998, ed. E. Di Cera, Academic Press).

Outline:

- **I. Definition of Terms**
- **II. Equilibrium Methods – experimental and analytical**
  - **single binding**
  - **multiple binding - entropy**
- **III. Thermodynamics, Coupling, Specificity**
  - **Multiple binding sites: independent vs. interacting; Avidity**
- **IV. Allostery – generic principles and a case study**
- **V. Forces**
- **VI. Kinetics**
  - **Methods**
  - **Examples**

I. Ligand Binding: Definition of Terms

The term 'ligand' is a problem because of the range of things that must be considered in a unifying theory. 'Ligands' include:

electrons

metal ions, other ions

small polar molecules (sugars, nucleotides)

small nonpolar molecules (lipids, steroids)

macromolecules (DNA, proteins, RNA)

'transition states'

water, a 'special ligand'

Can there be a general theory for such a broad range of ligands? Something that works for 'P' and 'L'?

I. Definitions (con't)

$$aA + bB \Leftrightarrow aAbB$$

$$K_a = \frac{[aAbB]}{[A]^a[B]^b} \qquad K_d = \frac{1}{K_a}$$

units of K_a = liters/mole; units of K_d = moles/liter

for the special case of 1:1 stoichiometry:

$$P + L \Leftrightarrow [P \bullet L]$$

$$K_d = \frac{[P][L]}{[P \bullet L]}$$

where [L] is free L concentration
Fractional occupancy, $\overline{X}$ is the fraction of total sites occupied by L, and varies with L via a hyperbolic relationship:

$$\overline{X} = \frac{[P \bullet L]}{[P] + [P \bullet L]} \quad \text{and} \quad \overline{X} = \frac{K_a[L]}{1 + K_a[L]}$$

$\overline{X}$ varies 0 → 1 for any stoichiometry
vs. 'number moles L bound/mole protein' which can be > 1 if multiple binding is present.

I. Definitions: thermodynamic terms

For $K_a = \dfrac{[aAbB]}{[A]^a[B]^b}$

$$\Delta G_{bind} = \underbrace{\Delta G^\circ}_{} + -RT\ln K_a = \Delta G^\circ + RT\ln K_d$$

Free energy change for moving reagents from their standard state to the state of comparison; for biochemists, usually pH 7.0, 37 C, but usually ignored.

$R = 1.985\ \text{calK}^{-1}\text{mol}^{-1} = 0.001985\ \text{kcalK}^{-1}\ \text{mol}^{-1}$

T= temperature in K

e.g.

for K_d = 1 micromolar at 37 C, $\Delta G = (0.001985)(310)\ln[1X10^{-6}] = -8.49$ kcal/mol.

for K_d = 1 nanomolar at 37 C, $\Delta G = -12.5$ kcal/mol.

Conversely, 2-fold change in K_d at 37 C is only 0.4 kcal/mol $\Delta\Delta G$.

BIG change in K_d doesn't require much change in energy.

II. Methods- experimental

The useful parameters that describe the equilibrium are K_d, $\overline{X}$, and ΔG.
Methods for measuring K_d and $\overline{X}$ include, but are not limited to:

<u>Partition techniques, in which [L] or [PL] is directly measured (calculate $\overline{X}$)</u>:

equilibrium dialysis
filter binding assays (radiometric)
gel filtration

<u>Perturbation Methods, in which a fractional response is measured (Calculate [L])</u>:

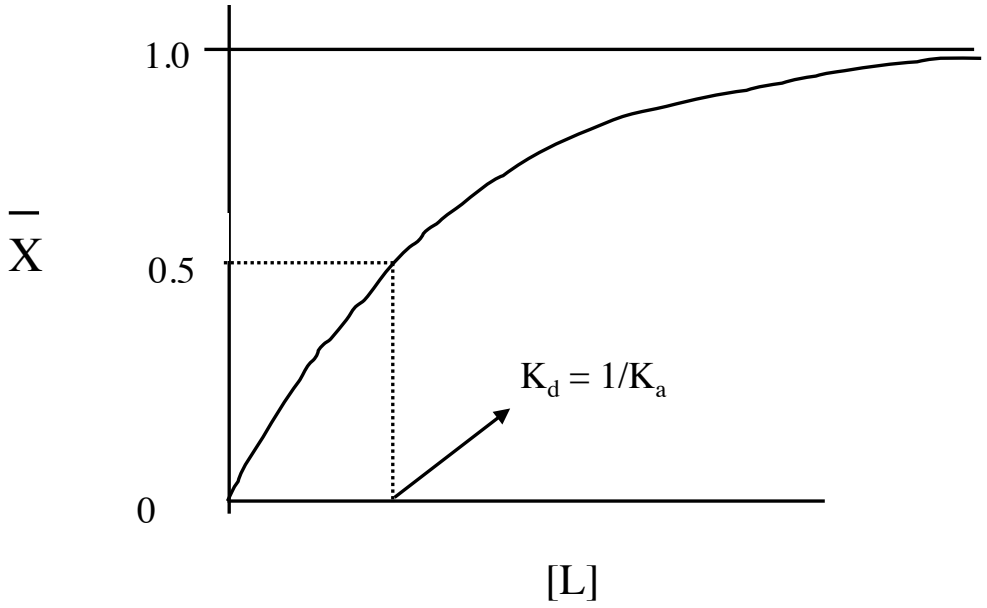
absorption, UV-visible spectroscopy
Surface Plasmon Resonance
fluorescence, CD
NMR
titration calorimetry
analytical ultracentrifugation

<u>Analytical methods include, but are not limited to,</u> fitting of the data to functions that express $\overline{X}$ in relation to [L].

hypberbolic plots, Scatchard plots, 'binding isotherms', Hill Plots

II. Methods - Analytical Approaches for simple 1:1 binding:

Hyperbolic Plot

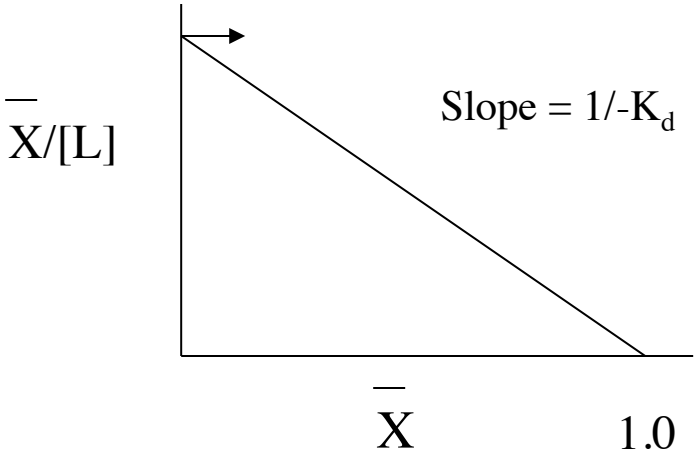


$$\overline{X} = \frac{K_a[L]}{1 + K_a[L]}$$

$$\overline{X} = \frac{[P \bullet L]}{[P] + [P \bullet L]}$$

Scatchard Plots

$$\frac{\overline{X}}{[L]} = K_a - K_a\overline{X}$$



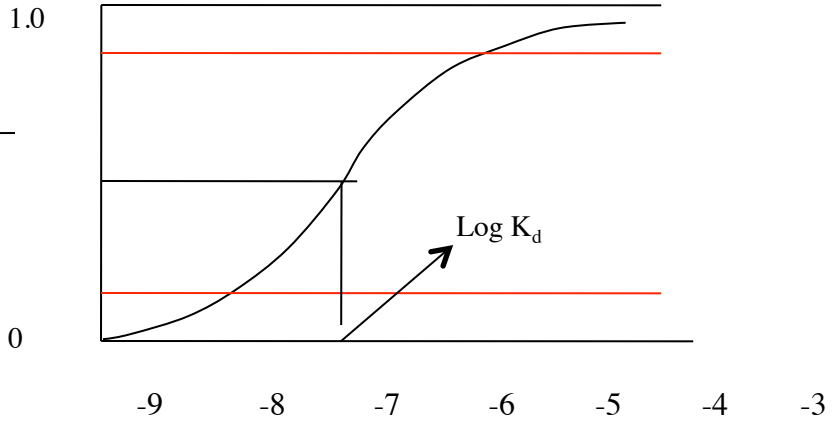
these plots tend to distort the data and artificially weight data near the intercepts. Useful qualitatively to seek deviation from linearity, easier than deviation from hyperbola.

II. Methods Analytics

"Binding Isotherms"

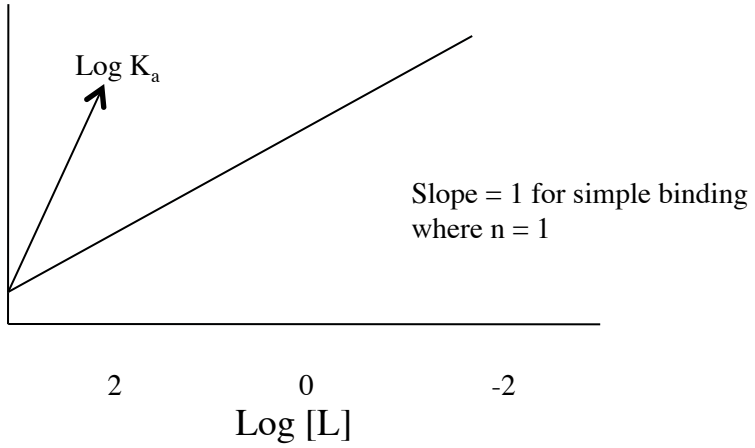
For simple binding, no cooperativity, $\overline{X}$ = 0.1 to 0.9 spans 1.8 log units.

Preferred method: ΔG is directly proportional to Log [L]. Free energy of the reaction is least 'sensitive' to [L] near the K_d. Fractional occupancy is most sensitive in this region,



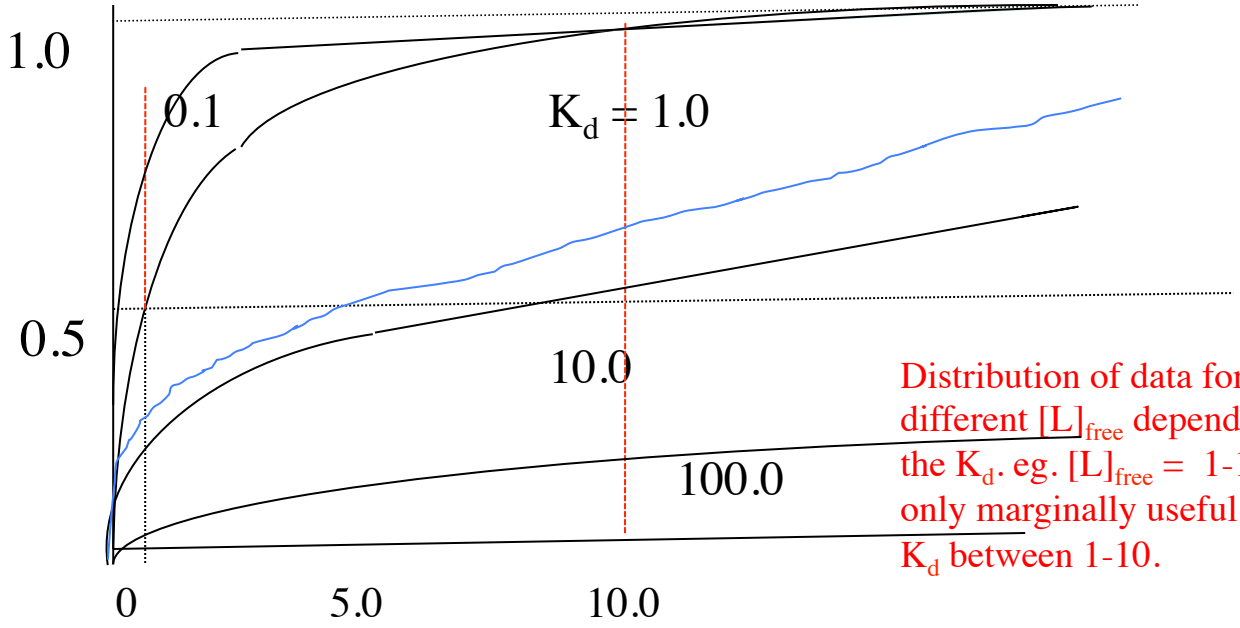
Hill Plots

Log $[\overline{X}/(1-\overline{X})]$



Log $[\overline{X}/(1-\overline{X})]$ = nLog [L] + Log Ka

II. Methods Analytics: How does $\overline{X}$ vary with K_d?



Distribution of data for different $[L]_{free}$ depends on the K_d. eg. $[L]_{free}$ = 1-10 is only marginally useful for K_d between 1-10.

For $K_d = 100$ P_0

$$\overline{X} = \frac{K_a[L]}{1 + K_a[L]}$$

$$\overline{X} = \frac{[L]/K_d}{1 + [L]/K_d}$$

Typically have known total $[P]_0$ and known total $[L]_0$, we measure $\overline{X}$ and calculate $[L]_{free}$ from $[L]_0 - \overline{X}[P]_0$. Error in $[L]_{free}$ is wholly dependent on error in $\overline{X}$. Two co-dependent variables used to get K_d. Need to plot $[L]_{free}$ is a limitation.

Often assume $[L]_{free} = [L]_0$, ok if $[L]_0 > [P]_0$ and $[P]_0 < K_d$

II. **Analytics and Experimental Design: What determines the experimental range of $[L]_{free}$ or $[L]_0$?**

Solubility: Many ligands/proteins are insoluble even in the micromolar range. Limits work at higher concentrations.

Sensitivity: Many techniques are insufficiently sensitive to detect low concentrations. Limits work at low concentrations.

These experimental considerations often oppose each other.

II. Methods/ Analytics: Practical Examples Concerning the Ratio of L_0/P_0 at Constant K_d – How much protein should I use?

Consider a series of experiments with a constant K_d = 10 μM, moderately weak binding, with varying $[P_0]$. **If $P_0 << K_d$, then $[L_0] \approx [L]$ and**

$$\overline{X} = [L_0]/K_d/[1+ [L_0]/K_d]]$$

– easy to calculate the total $[L_0]$ and $[L_0]/[P_0]$ needed to achieve $\overline{X}$ = 0.95 (95%) saturation of binding sites, which is a good target for accurately defining 100%.

At variable $[P_0]$ and constant K_d, the ratio of $[L_0]/[P_0]$ must change, even though the total $[L_0]$ needed for 95% saturation is constant (note X is independent of $[P_0]$ and this has practical consequences):
For $[P_0]$ = 1 μM; K_d = 10 μM ➔ $[L_0]$ = 190 μM, and $[L_0]/[P_0]$ = 190. For most ligands 190 μM is not limited by solubility, a signal from 1 μM $[P_0]$ is usually sensitive enough.

Now decrease $[P_0]$ to $[P_0]$ = 0.1 μM (save P, L?), ➔ $[L_0]$ = 190 μM, still, and $[L_0]/[P_0]$ = 1900. Ratio goes way up due to mass action effect, lower $[P_0]$ requires more ligand/ protein to saturate. Because $[L_0]$ is constant no new solubility 'advantage' but possibly problems with sensitivity due to lower $[P_0]$. And no savings in L, but savings in P_0.

At $[P_0]$ = 10 μM, $[L_0]/[P_0]$ = 19 μM, need much lower ratio, still $[L_0]$ = 190 μM. BUT, deviation from 'hyperbolic' 'equilibrium' conditions – $[P_0] \approx K_d$ - introduces error.

II. Methods/ Analytics: Practical Examples Concerning the Ratio of L_0/P_0 at Constant $[P_0]$

Similar considerations are relevant for a series of experiments in which the protein concentration is held constant and the K_d changes. There are trade offs in accuracy, sensitivity, and solubility restrictions.

See homework #1.

II. Methods Analytics: How does $\overline{X}$ vary with K_d?

Key points:

Experimentally it is critical to use a range of ligand concentrations such that $[L]_{free}$ spans at least two orders of magnitude including data on both sides of K_d, and at least some data need to be at $[L]_{free}$ way above K_d, within ~ 95% of $\overline{X} = 1.0$.

Error in $\overline{X}$ leads to error in both axes of a binding curve – calculation of $[L]_{free}$ from L_0 and [PL] is required for the most commonly used analytical expressions but not the most general solution. Error in $\overline{X}$ can arise if 'saturation' is not clearly established – all values on the y-axis are determined by $\overline{X} = 1.0$ so if $\overline{X}= 1.0$ has error, all y-values have error.

Although $[L]_{free}$ is 'formally' required to calculate K_d, at $[P_0] << K_d$, $[L] \rightarrow [L_0]$. Total [L] can be a surrogate for decent approximation.

II. Methods:Analytics- Stoichiometric vs. Equilibrium Binding

Consider K_d in terms of what is more easily measurable or known:

$$K_d = \frac{(P_0 - [PL])(L_0 - [PL])}{[PL]}$$

P_0, L_0 = total protein and total ligand

$$0 = [PL]^2 - (P_0 + L_0 + K_d)[PL] + P_0 L_0$$

Note symmetrical with respect to L_0 and P_0, so L and P are 'interchangeable'

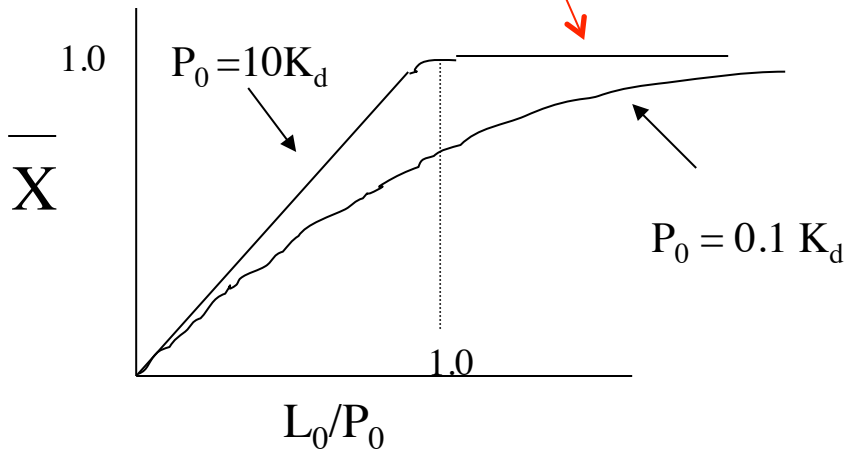
Don't need L_{free}, it's just 'mathematically' convenient

When $P_0 >> K_d$, With algebraic tricks, factoring, substitution, 2 solutions apparent:

$$(\frac{L_0}{P_0} - \overline{X})(1 - \overline{X}) = 0$$

$$\frac{L_0}{P_0} = \overline{X}$$

That is, the fractional saturation is identical to the molar ratio of L_0, P_0. The added L binds completely, no $[L]_{free}$. Plot of $\overline{X}$ vs. L_0/P_0 is linear with slope = 1.



For n = 1, L_0/P_0 is a max at 1.0. For n > 1, the fraction saturation occurs at L_0/P_0 = n, the 'equivalence point'.

Equivalence point yields stoichiometry (n) of binding, but K_d can not be determined, because there is no free L. No titration technique can yield both K_d and n from a single experiment. Conditions that yield high precision in K_d have great error in n and vice versa.

II. Methods - analytics for 'stoichiometric' binding conditions.

When $K_d < P_0$ the formalism dependent on free [L] breaks down. The full 'quadratic equation' resulting from the use of $[P]_0$ - [PL] and $[L]_0$ - [PL] yields the expressions above which when factored yields the new expression for [PL]

$$K_d = \frac{(P_0 - [PL])(L_0 - [PL])}{[PL]}$$

$$0 = [PL]^2 - (P_0 + L_0 + K_d)[PL] + P_0 L_0$$

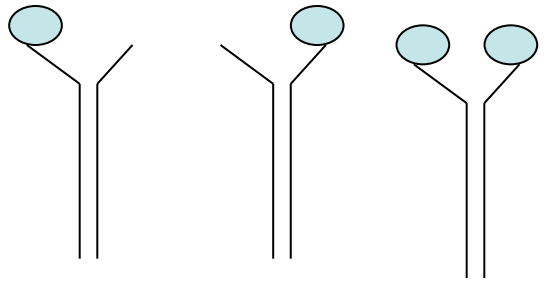
$$[P \bullet L] = \frac{([P]_0 + [L]_0 + K_d) - \sqrt{([P]_0 + [L]_0 + K_d)^2 - 4[P]_0[L]_0}}{2}$$

This 'general' equation should be used to calculate K_d if $[P]_0 > K_d$ or if there is no way to accurately get $[L]_{free.}$ NOTE: THE QUADRATIC EQUATION Still requires data at low and high $[L]_0$, on both side of the equivalence point.

II. Methods Analytics: Multiple Binding with Independent Sites

Consider a protein with *n* noninteracting sites, e.g. the IgG or other immunoglobulins. The 'fractional' saturation of protein can now be >1. The fractional saturation of protein must be distinguished from fractional saturation of 'sites.' More clearly, the number of moles ligand/protein, $\bar{\upsilon}$, can be > 1, but the average number of moles ligand/site can still only vary 1 to 0. This has implications for the various analytical solutions, and the information that can be extracted.

Average number of ligands bound/protein $\bar{\upsilon} = n\sum_{i=1}^{n} \bar{X}_i$



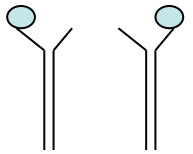
$$\bar{\upsilon} = n\bar{X} = \frac{nK_a[L]}{1 + K_a[L]}$$

But still a hyperbolic equation! Each of plots above will have the same 'shape' - can't detect multiple binding with experiments that measure fractional response. Plots of 'fractional saturation' of sites can not yield n directly, n is 'hidden.' If you have independent measure of *n*, can be included to get the real K_a.

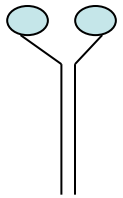
II. Methods Analytics - Multiple noninteracting sites

Lets take a closer look at what contributes to $\bar{\nu}$

$$P + L \Leftrightarrow PL_1 \qquad K_1 = \frac{[PL_1]}{[P][l]}$$



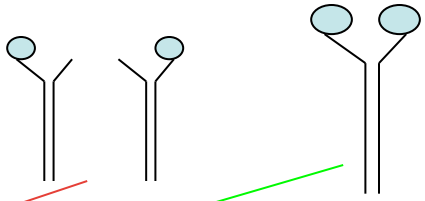
$$PL_1 + L \Leftrightarrow PL_2 \qquad K_2 = \frac{[PL_2]}{[PL_1][L]}$$



$$PL_{n-1} + L \Leftrightarrow PL_n \qquad K_n = \frac{[PL_n]}{[PL_{n-1}][L]}$$

When n sites/protein, **moles L bound/ moles P = $\bar{\nu}$ =**

$$= \frac{\sum_{i=1} i[PL_i]}{\sum_{i=0}^{n}[PL_i]}$$

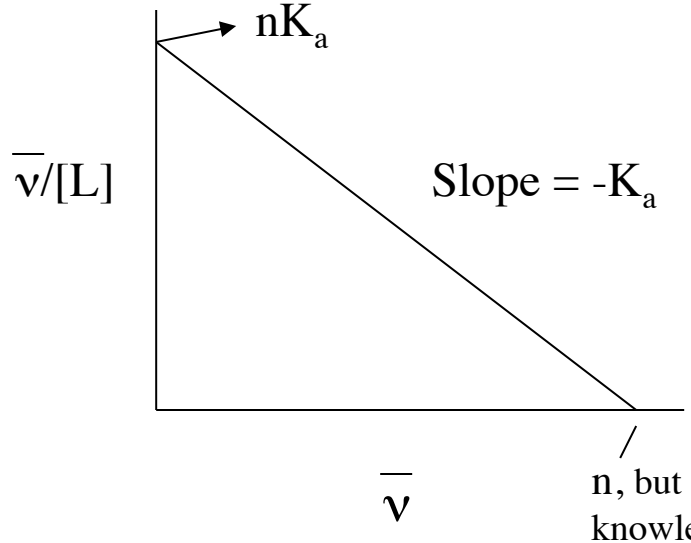


$$= \frac{[PL_1] + 2[PL_2] + 3[PL_3] + \bullet\bullet\bullet n[PL_n]}{[P] + [PL_1] + [Pl_2] + [PL_3] + \bullet\bullet\bullet[PL_n]} = \frac{n\mathrm{K_a[L]}}{1+\mathrm{K_a[L]}}$$

II. Analytics: Consider Multiple Binding and the Scatchard or Hill Plots

Scatchard

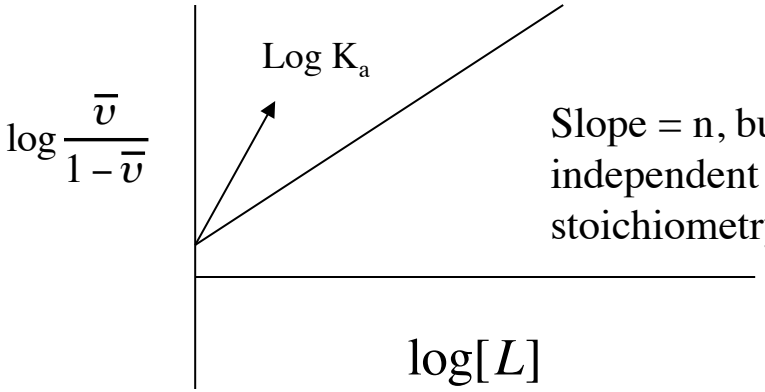
$$\frac{\bar{\upsilon}}{[L]} = nK_a - K_a\bar{\upsilon}$$



n, but 1.0 if no independent knowledge that more than 1 ligand can bind.

Hill

$$\log\frac{\bar{\upsilon}}{1-\bar{\upsilon}} = \log K_a - n\log[L]$$



II. Methods- Analytics:Ligand Distribution

When proteins have multiple binding sites, 'affinity' or $\bar{\upsilon}$ are not the only determinants of biological responses to ligands. At subsaturating concentrations of L, their distribution matters. From above we can see that there are multiple ways to distribute, for example, 2 ligands:

Two possibilities that contribute equally to $\bar{\nu}$

[PL]+[LP] vs. [LPL]

There are multiple, energetically degenerate, ways to distribute a fixed number of ligands. But proteins control this distribution - this is a distinguishing feature of biological systems.

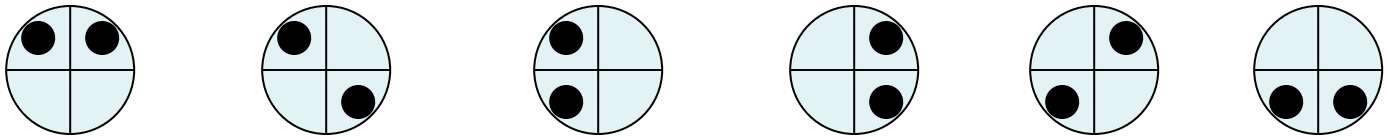
To fully understand ligand distribution we must consider statistical effects, and we must distinguish between macroscopic and microscopic equilibrium constants.

III. Thermodynamics and Statistical Mechanics of Ligand Distribution

Macroscopic vs. Microscopic Equilibrium Constants

How many ways can we arrange ligands among available sites? Consider a protein with 4 identical sites,  with 2 ligands to 'distribute.':

For n = 4, i = 2, there are 6 species that contribute to $[PL_2]$

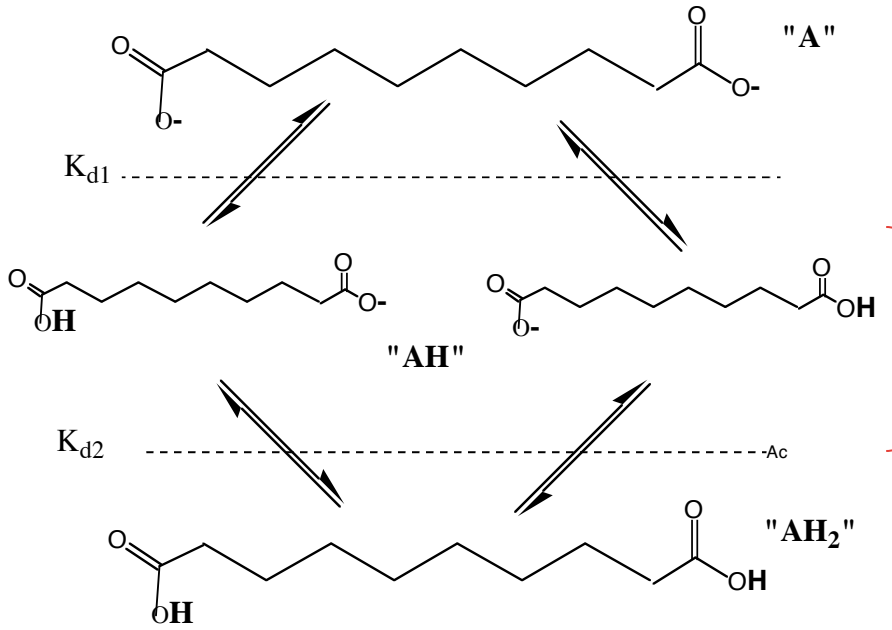


From the definition of a binomial distribution in statistics, the number of ways to partition i outcomes with equal probability into n total possible events is:

$$\Omega_{n,i} = \frac{n(n-1)(n-2)\bullet\bullet\bullet(n-i+1)}{i!} = \frac{n!}{(n-i)!\,i!}$$

III. Thermodynamics of Ligand Binding: Microscopic vs. Macroscopic Binding Equilibria

Consider a diacid, with ionizable groups at either end of an 'insulating linker' - so the ends do not 'sense' each other:



Macroscopic $K_{d1} = [A]/[AH]$

Macroscopic $K_{d2} = [AH]/[AH_2]$

Two ways to form AH from A, two ways to lose AH to form AH_2

Consider $K_d = k_{off}/k_{on}$: then

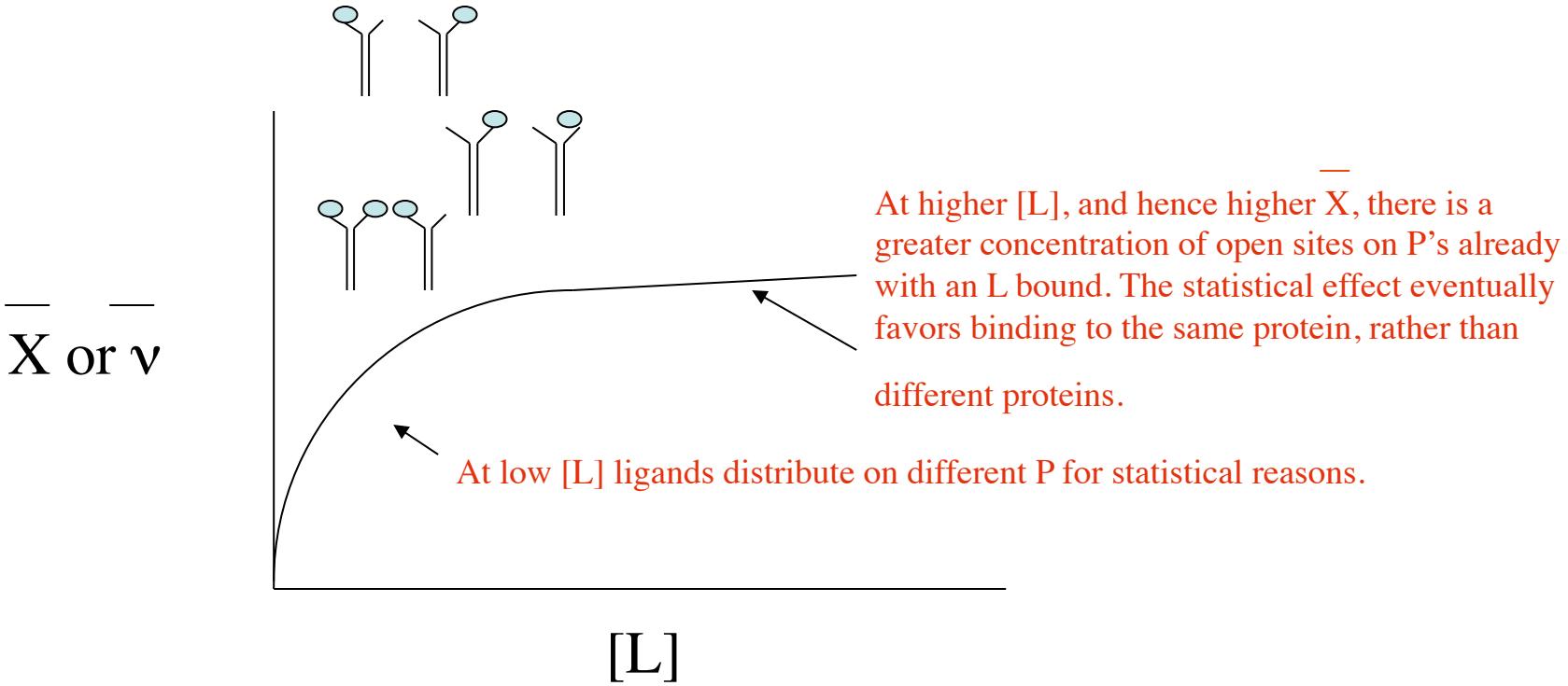
$K_{d1} = k_{off}/2k_{on}$ and $K_{d2} = 2k_{off}/k_{on}$

$4K_{d1} = K_{d2}$

Statistical effects make Kd1 appear higher affinity even though sites are chemically identical!! Apparent negative cooperativity with respect to proton binding!!

III. Thermodynamics, Multiple Binding

But . . . In an ensemble of proteins with multiple binding sites where we can measure $\bar{X}$ or $\bar{\nu}$ we can't see this apparent negative cooperativity. The macroscopic affinity looks uniform, determined by the intrinsic K_{d1}, K_{d2}. Plots are hyperbolic.



III. Thermodynamics - Multiple Binding and entropy as an introduction to the thermodynamics of ligand binding

This statistical effect is an entropic one. The general relationship for n sites is:

$$K_i = \frac{\Omega_{n,i-1}}{\Omega_{n,i}} k$$

Here K is the macroscopic dissociation constant for site *i*, and k is the intrinsic constant.

HOMEWORK #2: Calculate the K_i for the 2nd and 3rd ligands binding to a protein with 3 equivalent sites; and for the 2nd, 3rd, and 4th ligands for a protein with 4 equivalent sites.

$$S \propto \ln \Omega$$

S = entropy here (not substrate)

Remember that entropy is related to 'probability.' Higher entropy of a system at subsaturating ligand is with ligands distributed over many proteins. Entropically unfavorable to 'park' many ligands on one protein and none on other proteins.

Entropy works against proteins, by counteracting their ability to control ligand distribution.

III. Thermodynamics and Ligand Distribution

Consider a 'ligase' that joins two substrates, S, into a single larger product. At subsaturating concentrations of S the entropy that favors binding to different proteins results in wasted binding energy. The enzyme population can't catalyze any reaction if individual enzyme molecules are singly-ligated!

The enzyme wants to do this:

$$E + S + S \longleftrightarrow [E \bullet S \bullet S] \longleftrightarrow E + S{-}S$$

But [E•S] + [E•S] can't do this, only [E • S • S] can.
Much of the binding isotherm below saturation would include enzymatically unfunctional complexes. Proteins would be victims of chance (statistics) if they couldn't control ligand distribution.

Summary of key points so far:

- Parameters needed to describe ligand binding at equilibrium are K_d, $\overline{X}$, ΔG.

- For simple 1:1 binding or for multiple binding at noninteracting sites, K_d should be measured at $[P]_0 << K_d$, using $[L]_{free}$. Stoichiometry can be measured at $[P]_0 >> K_d$.

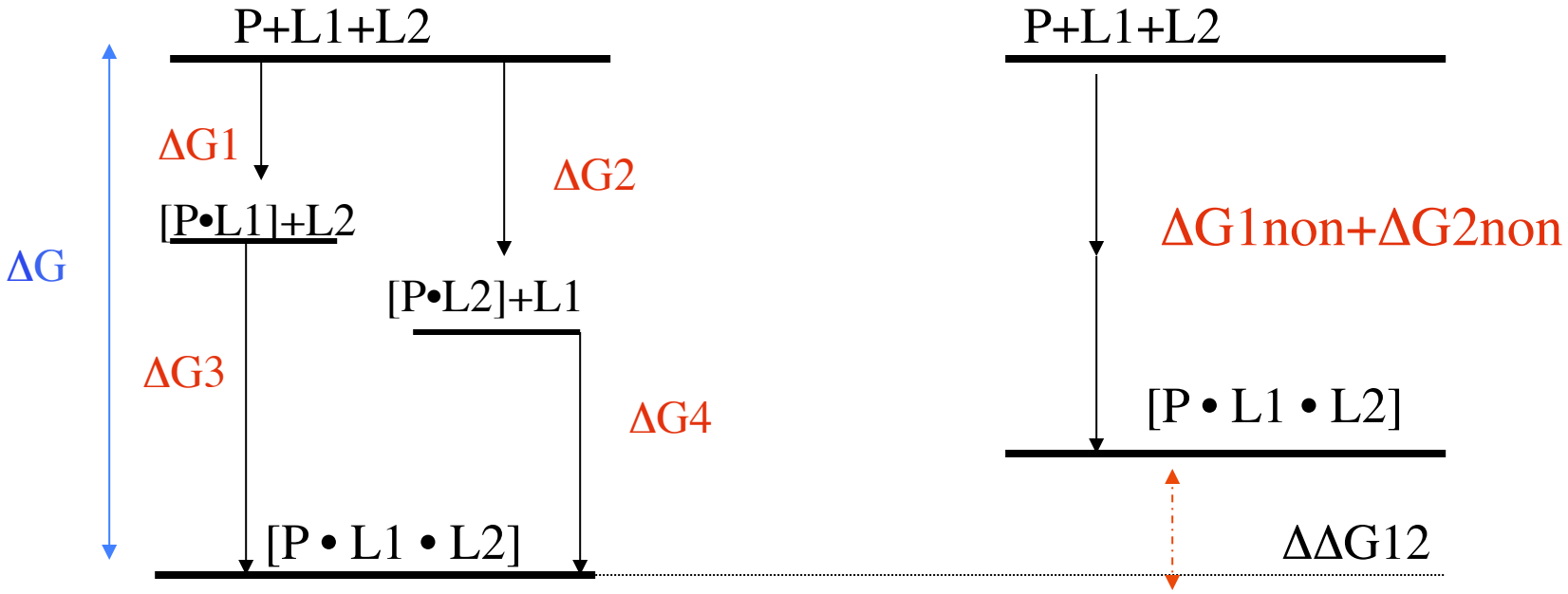
- When $[P]_0 >> K_d$, the K_d must be determined from the 'quadratic equation' because there is no $[L]_{free}$.

- For multiple noninteracting sites, there is a statistical bias against binding multiple ligands to the same protein molecule, at low [L]. This results from a higher entropic cost.

- Without a mechanism to control the statistical bias, proteins would be victims of chance at low [L].

III. Thermodynamics, Coupling Free Energy

Consider two distinct ligands L1, L2 with free energy of binding to P, ΔG1 and ΔG2. P can form a ternary complex, and ΔG3 is the free energy of binding L2 to the complex [PL•1] and ΔG4 is the free energy for L1 binding to the complex [PL•2]. We can express this pictorially on a free energy diagram.

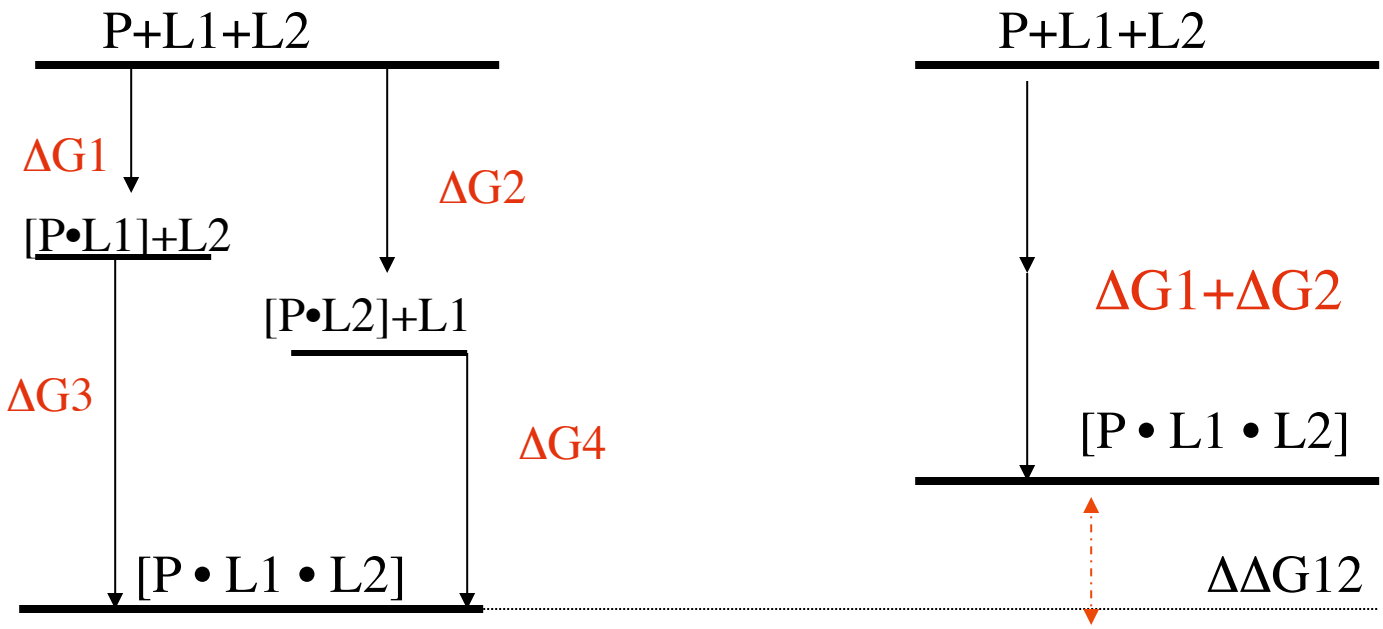


ΔG1+ΔG3 = ΔG2+ΔG4 but no requirement that ΔG1 = ΔG2 or ΔG3 = ΔG4

Required: ΔG4 - ΔG1 = ΔG3 - ΔG2 = ΔΔG12, the coupling free energy

Coupling free energy is the effect that the binding of L1 has on the binding of L2 which must equal the effect that the binding of L2 has on the binding of L1.

III. Thermodynamics, coupling free energy



If **ΔΔG12 = 0**, ligands have no effect on each other, **no cooperativity**

If **ΔΔG12 < 0** , then ΔG of binding two ligands simultaneously is more negative, more favorable, than individual ligands, **positive cooperativity.**

If **ΔΔG12 > 0**, then ΔG of binding individual ligands is more positive, less favorable, than binding two ligands simultaneously, **negative cooperativity.**

III. Thermodynamics, Coupling

The coupling free energy is critically important in biology - it determines ligand distribution and biological response. It can be considered in another thermodynamic context. The $\Delta\Delta G12$ is equal to the ΔG for the disproportionation reaction:

$[P \bullet L1] + [P \bullet L2] \longleftrightarrow P + [L1 \bullet P \bullet L2]$ where $K_{eq} = \dfrac{[P \bullet L_1 \bullet L_2][P]}{[L_1 \bullet P][P \bullet L_2]}$

Consider the case when [L1] and [L2] are adjusted to $\overline{X}_1 = \overline{X}_2 = 1/2$

If $\overline{X}_1 = \dfrac{[P \bullet L_1] + [P \bullet L_1 \bullet L_2]}{P_0}$ $\overline{X}_2 = \dfrac{[P \bullet L_2] + [L_1 \bullet P \bullet L_2]}{P_0}$ $\overline{X}_{1,2} = \dfrac{[P \bullet L_1 \bullet L_2]}{P_0}$

Then at $\overline{X}_1 = \overline{X}_2 = 1/2$ $K_{eq} = \dfrac{[\overline{X}_{1,2}]^2}{(1/2 - [\overline{X}_{1,2}])^2}$

and $\overline{X}_{1,2} = \dfrac{1/2\sqrt{K_{eq}}}{(1 + \sqrt{K_{eq}})}$

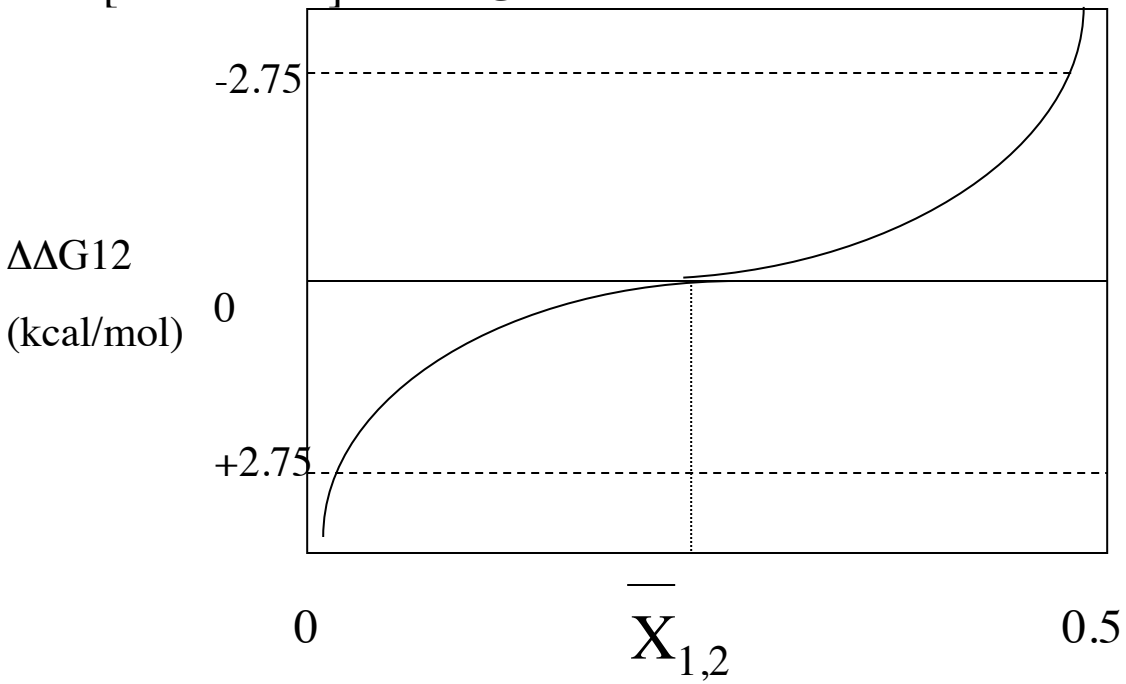
Knowing that $\Delta\Delta G12 = -RT\ln K_{eq}$ we can solve for $\Delta\Delta G12$ in terms of $\overline{X}_{12}$

$$\Delta\Delta G12 = -RT \ln \frac{2\overline{X}_{1,2}}{[1 - 2\overline{X}_{1,2}]}$$

III. Thermodynamics, coupling

$$\Delta\Delta G12 = -RT\ln\frac{2\bar{X}_{1,2}}{[1-2\bar{X}_{1,2}]}$$ for $[P \bullet L1] + [P \bullet L2] \longleftrightarrow P + [L1 \bullet P \bullet L2]$

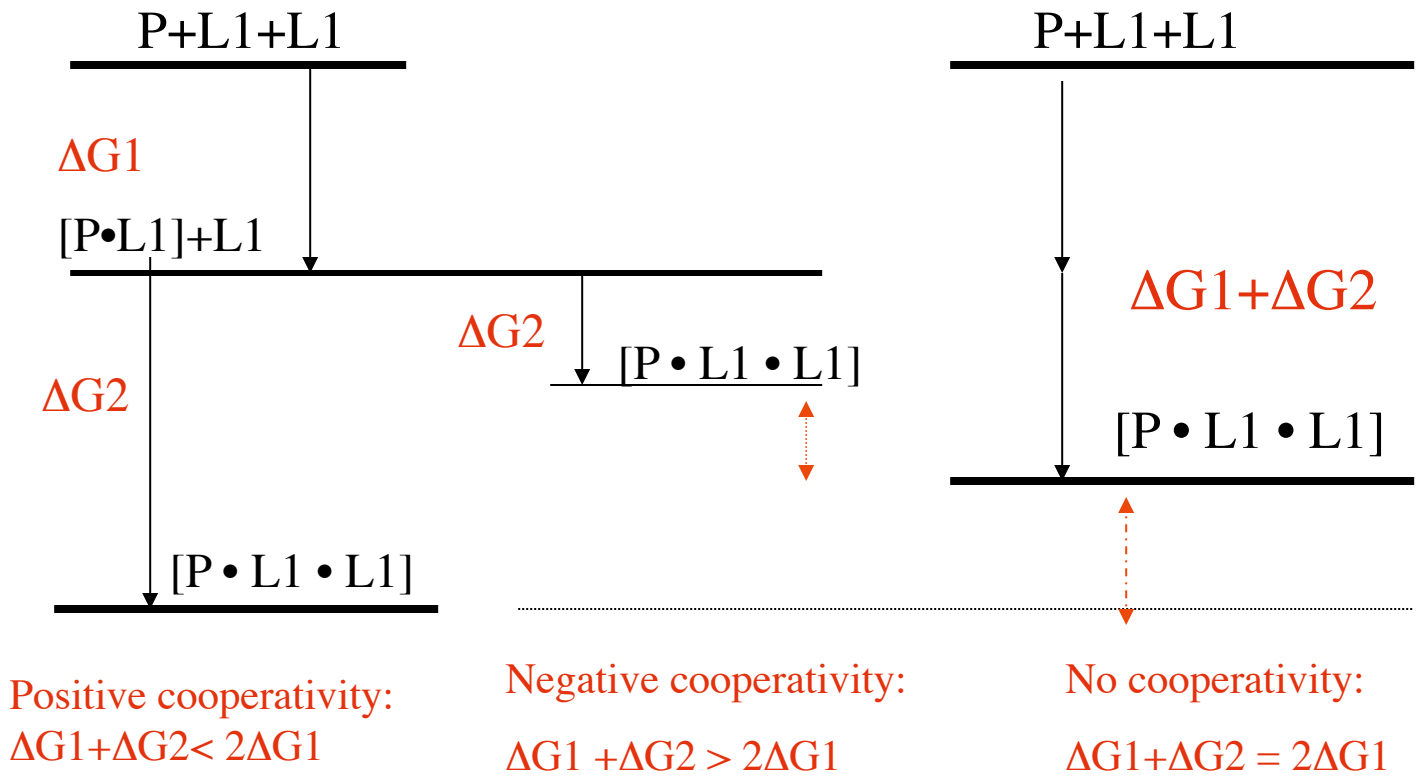
A few special features: when ΔΔG12 = 0, no coupling, half the ligands L1 are in [L1P] or [L1PL2] and half the L2 ligands are in [PL2] or [L1PL2], so $\bar{X}1,2 = 0.25$ at zero coupling. Note, as coupling is favorable (more negative ΔΔG12) there is an increase in $\bar{X}1,2$, the fraction of protein with two ligands bound, and vice versa. Also, using known values of RT, ~90% of bound P is [L1PL2] at -2.75 kcal/mol and 90% is [L1P + PL2] for ΔΔG12 +2.75 kcal/mol.



This plot tells us that very good coupling (90%) can be obtained for small ΔΔG12, on the order of 3 kcal/mol or - 3 kcal/mol. For the price of a hydrogen bond proteins can get very good control of ligand distribution. On the other hand, due to the asymptotic nature of the plot, with respect to ΔΔG12 = 0 and ΔΔG1,2 = 0.5, it is very expensive to get perfect control.

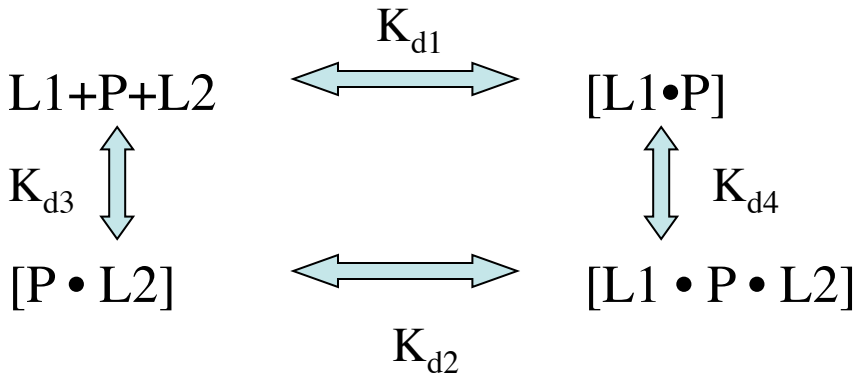
III. Thermodynamics, coupling

Of course there can be homotropic cooperativity as well, directly analogous to the heterotropic case above.



III. Thermodynamics, cooperativity and linkage

The previous free energy diagrams emphasize the path-independent nature of the state function ΔG, and hence of binding affinity. They can be recast in the framework of a thermodynamic box.



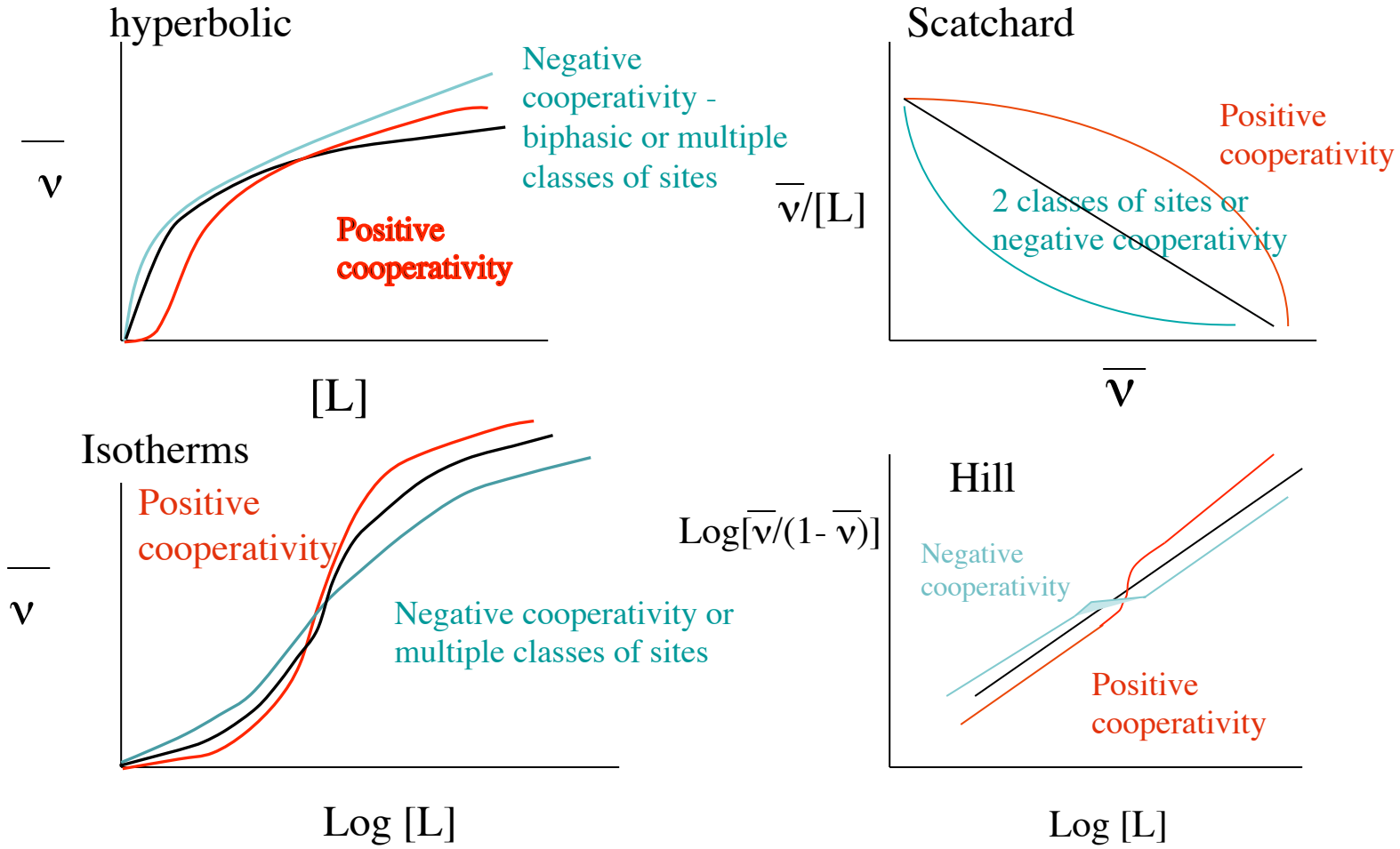
$K_{d1}K_{d4} = K_{d2}K_{d3}$

Because of the path independence, and in relation to the coupling free energy, it is obvious that any effect that L2 has on the binding of L1 must be equal to the effect that L1 has on the binding of L2. This reciprocity was first discussed in the context of hemoglobin, O2, and CO by Hendersen, and Wyman elaborated a theory of thermodynamic 'linkage' based on chemical potential (rather than ΔG).

$\delta(\ln\bar{X}2)/\ \delta\bar{X}1 = \ \delta(\ln\bar{X}1)/\ \delta\bar{X}2$

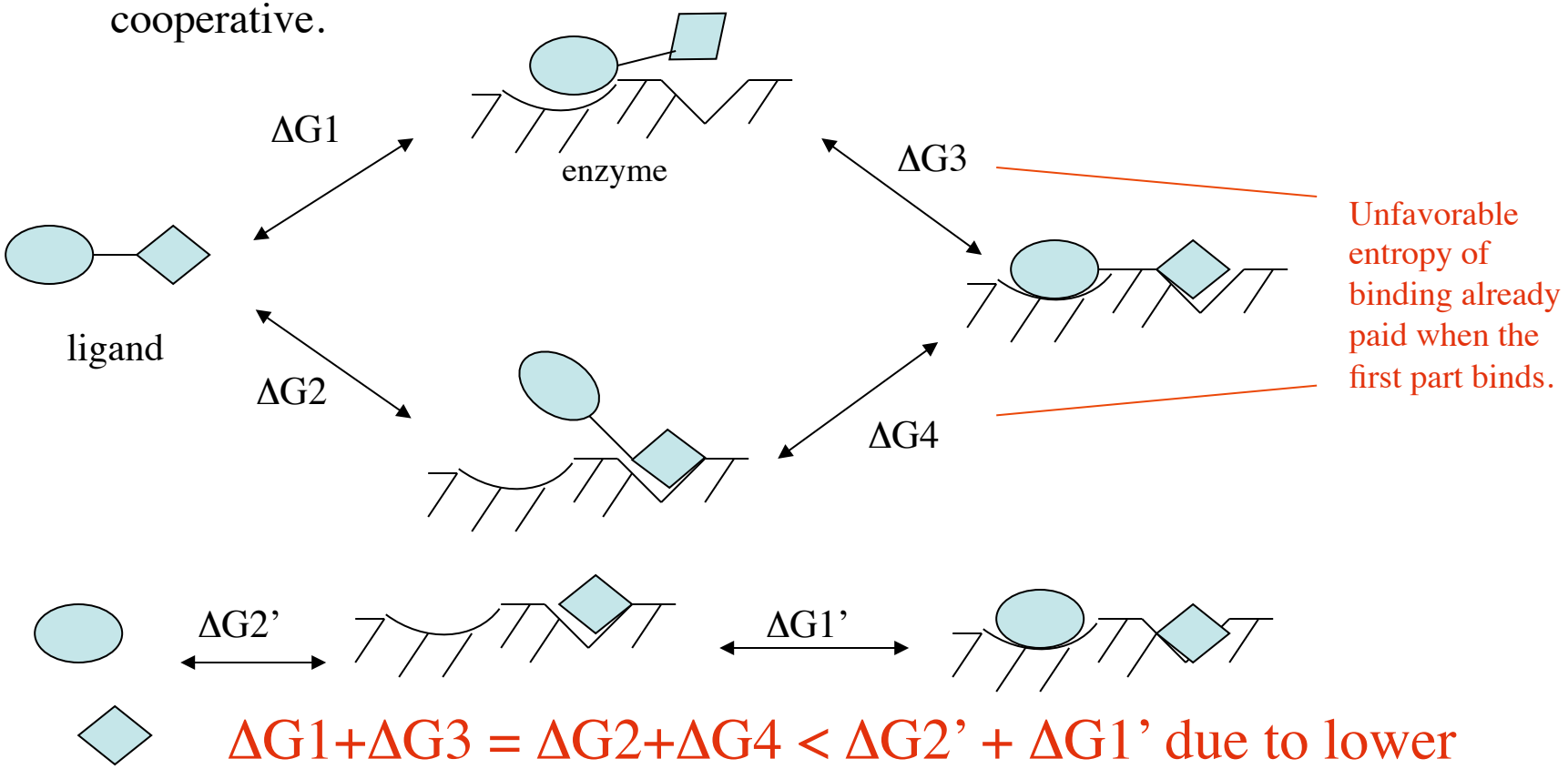
III. Thermodynamics - Cooperativity, Multiple Binding and Binding Curves- Homotropic Effects

When ligand interactions take place, they result in changes in the appearance of the analytical plots discussed above.



III. Thermodynamics and intra-ligand cooperativity: the basis for specificity.

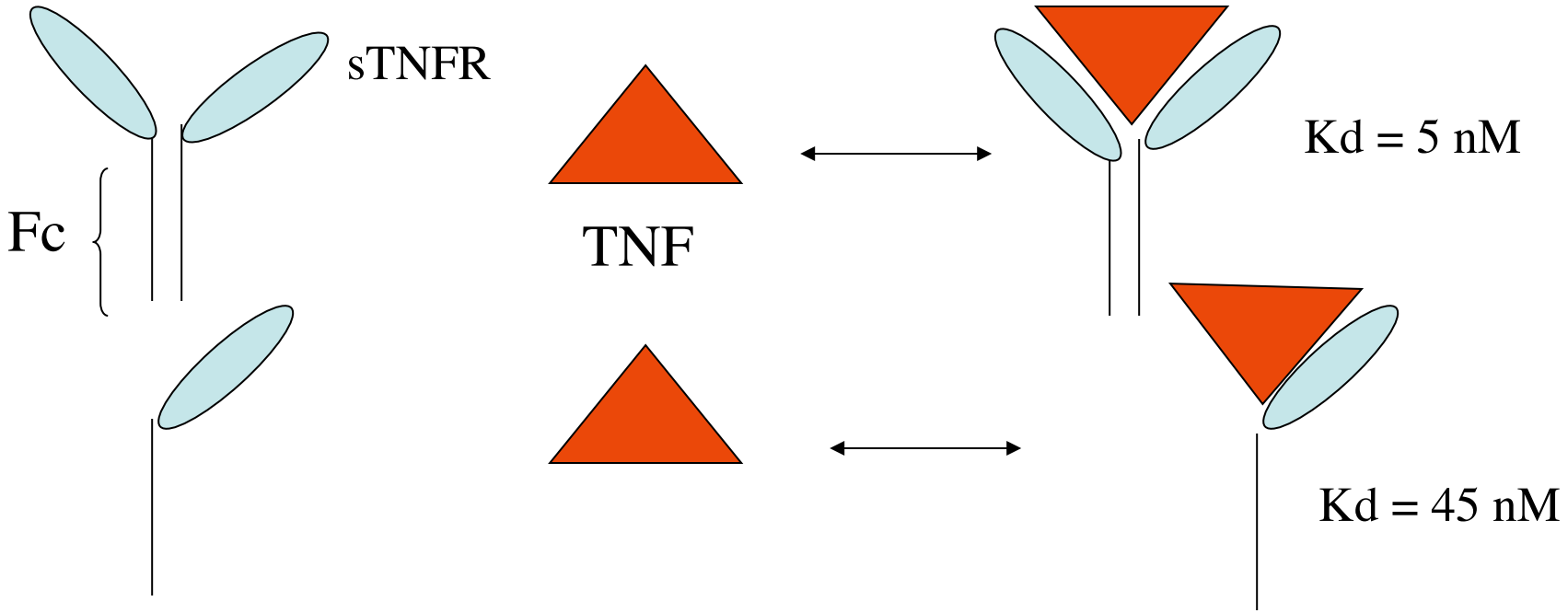
Consider two parts of a single ligand, rather than two separate ligands. Binding of each part is 'coupled' to the other parts, and hence binding of the parts is cooperative.



$\Delta G1+\Delta G3 = \Delta G2+\Delta G4 < \Delta G2' + \Delta G1'$ due to lower entropy cost, once part of a molecule is bound.

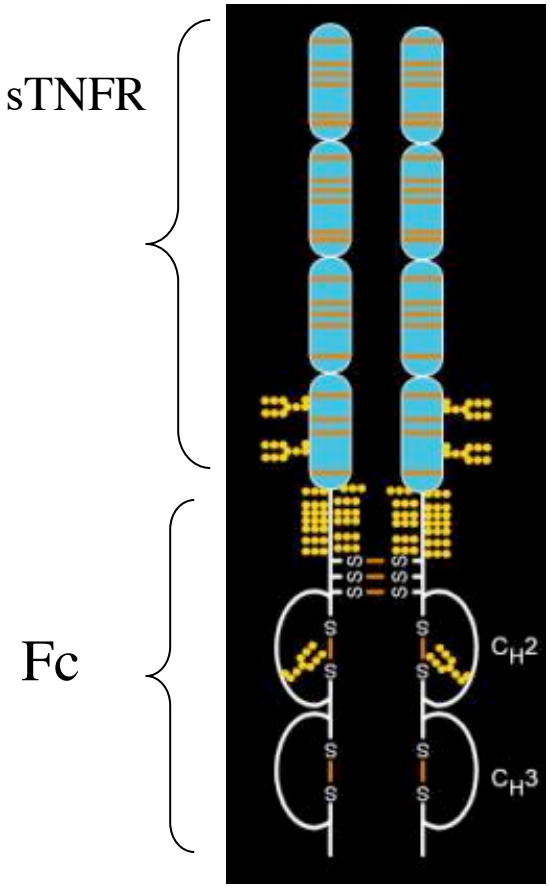
III. Thermodynamics, coupling: An example of a therapeutic protein

Enbrel marketed first by Immunex (Seattle) in 1998 is an example of the use of coupling free energy, via bivalency. TNF mediates rheumatoid arthritis. A strategy to reduce systemic TNF was to 'sponge' it up with a soluble TNF receptor construct. TNF is a trimer of identical subunits and the TNF receptor (TNFR) was known to bind at a subunit-subunit interface of the trimer. Immunex fused a soluble fragment of TNFR to the Fc region of an IgG, thus resulting in a 'bivalent' TNFR. A blockbuster drug!

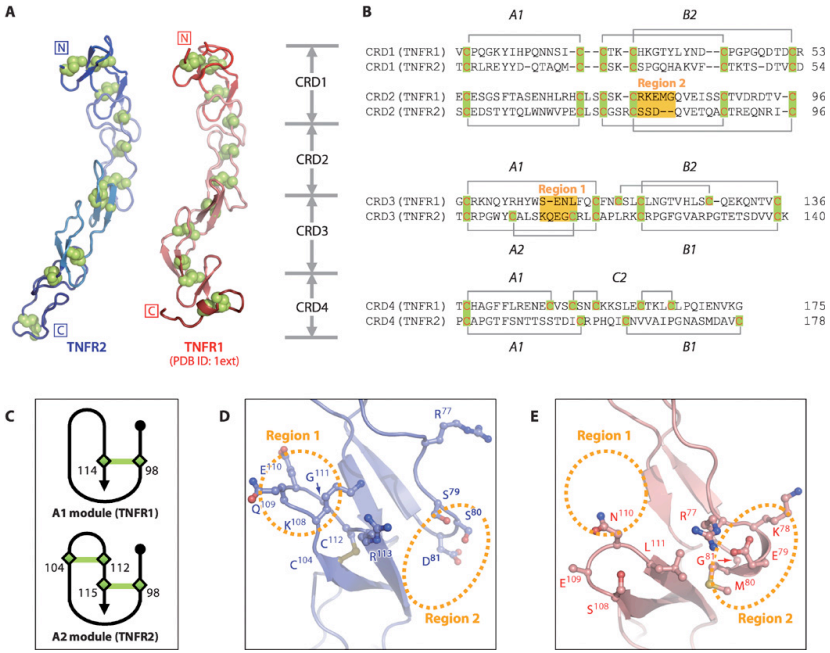


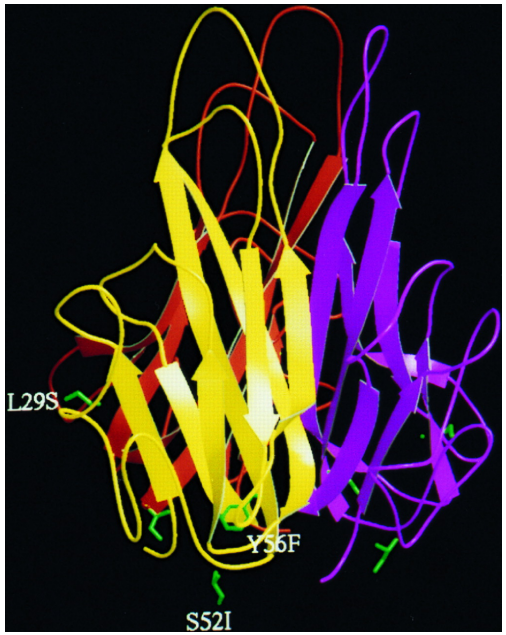
TNF and Enbrel, structures

sTNFR



enbrel

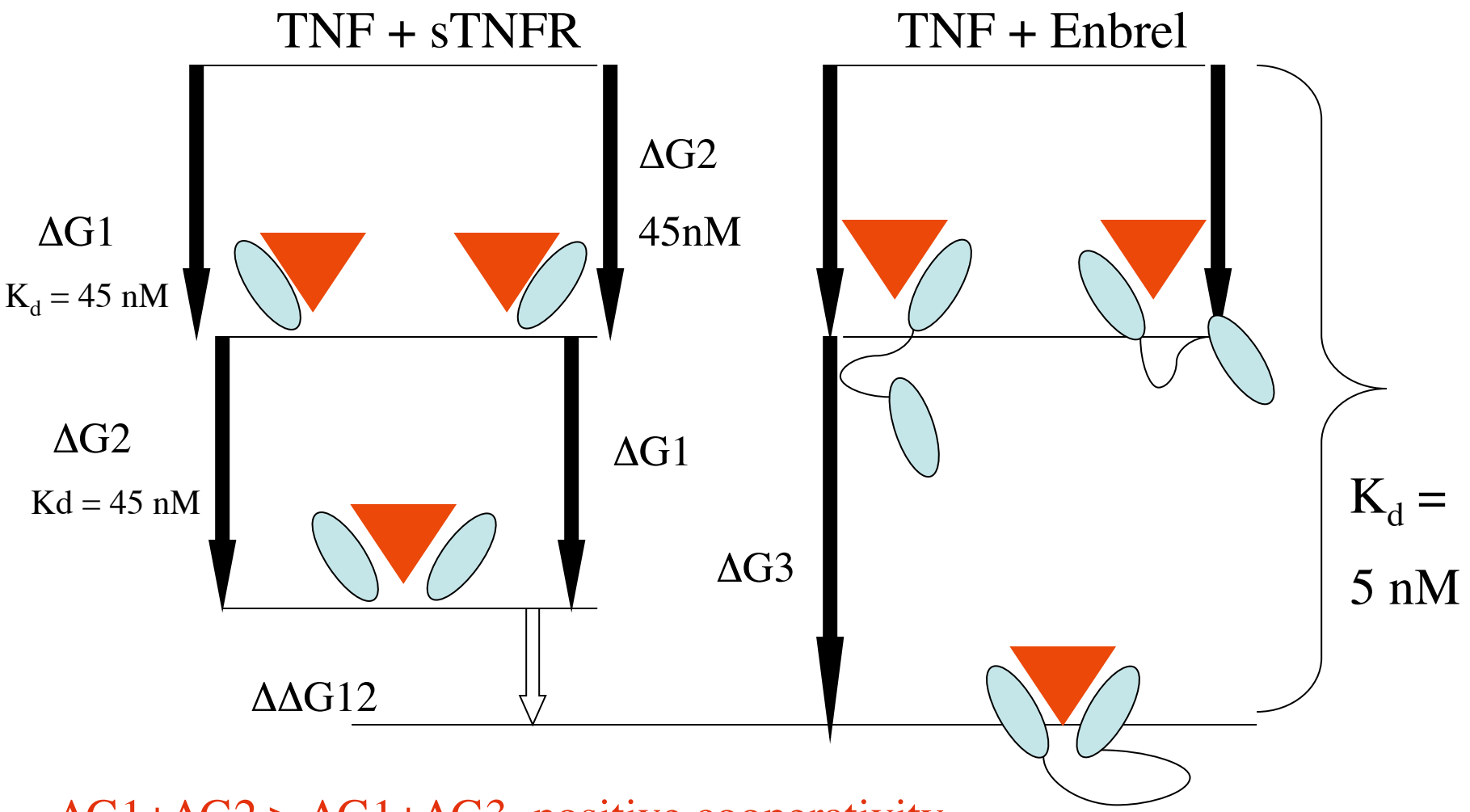




Trimeric TNF

Mukai et al,
Science Signaling
vol 3, 143, 2010

III. Thermodynamics, coupling: Enbrel as an example of bivalency.



ΔG1+ΔG2 > ΔG1+ΔG3, positive cooperativity between 2 sTNFR

K_d = 45 nM: ΔG = -10.4 kcal/mol K_d = 5 nM;

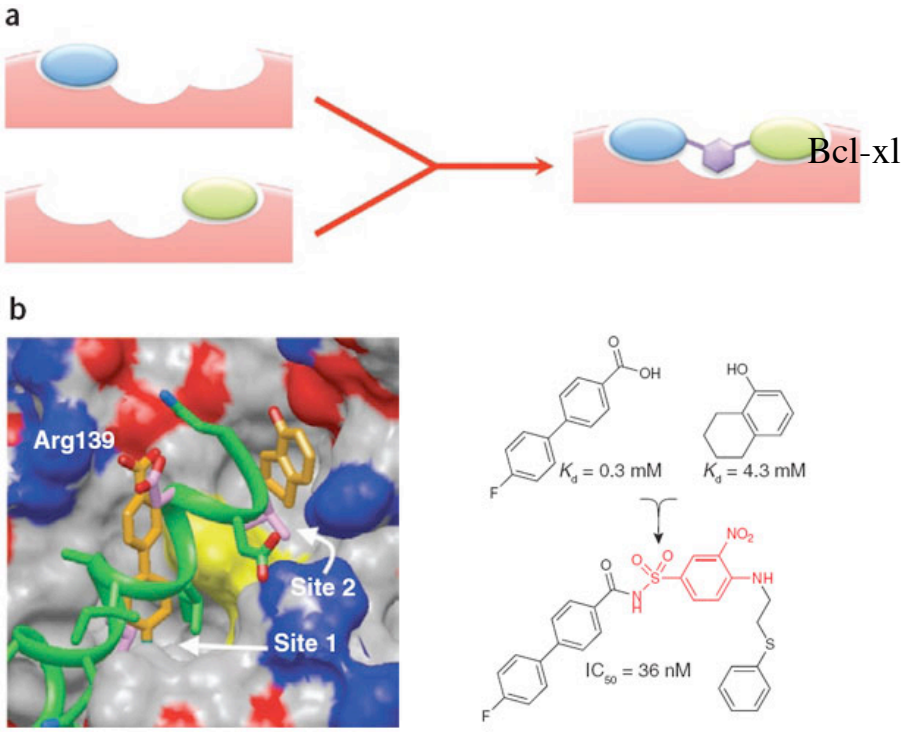
ΔG = -11.7 kcal/mol

ΔΔG = - 1.3 kcal/mol

III. Thermodynamics, coupling - bivalent inhibitors

While the concept of ‘multivalency’ has been appreciated in drug design circles for a long time, recent advances in high throughput NMR and computational methods have brought into focus as ‘fragment-based drug discovery.’

FBDD uses NMR/computation to screen libraries of ‘small fragments’ that bind simultaneously to a target. Lead Fragments with the best ‘ligand efficiency’ are linked together to create a ‘multivalent’ lead compound. This is exploitation of coupling free energy.



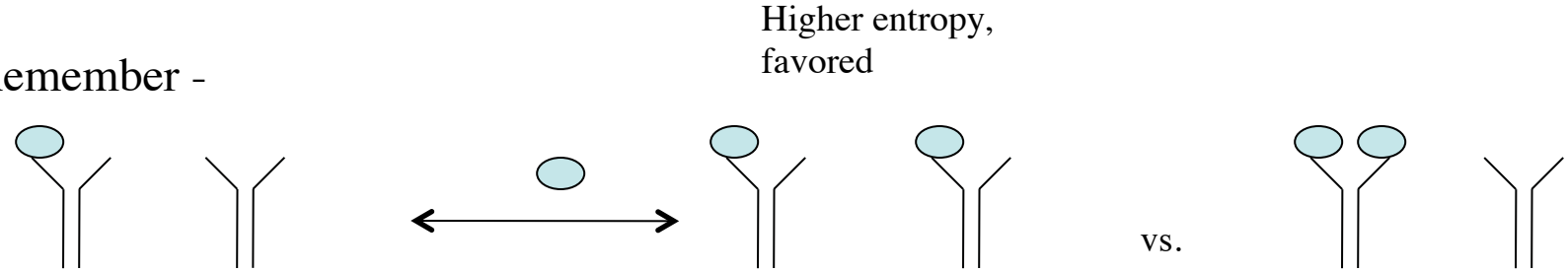
III. Thermodynamics, bivalent inhibitors, linker considerations

The ‘entropic advantage’ of multivalency is only realized if the linker is entropically neutral. Both the binding elements (fragments) and linker forfeit conformational, rotational, translational, vibrational degrees of freedom when they bind. If the linker is long and flexible, the entropic cost of binding the linker offsets any advantage from the multivalency. Best linkers are short. For long flexible linkers, their conformational distribution becomes an important design element, and they also contribute directly to enthalpic interactions with the protein.

III. Thermodynamics, Multiple Binding and Avidity.

For multivalent interactions with antibodies, the term 'avidity' is commonly used, maybe paradoxical. There is not a clear consensus on the definition of 'avidity' but people use it to acknowledge that antibodies are bivalent and therefore there is intrinsic cooperativity between binding sites, when the ligand is multivalent in solution or when monovalent ligands are 'clustered' on surfaces. The avidity seems to acknowledge effects on 'ligand distribution' in regard to antibody-receptor interactions on surfaces or with multivalent ligands in solution. Here the receptor density has an effect on the 'apparent' affinity for antibody due to avidity effects. Avidity is a special type of coupling, between antigens at high density or covalently connected. The resulting proximity of antigens leads to coupling.

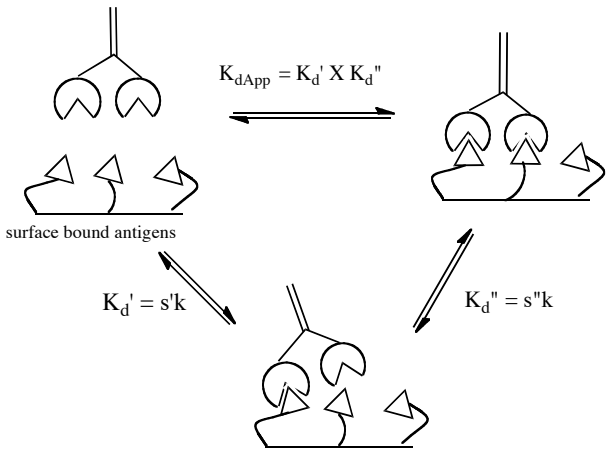
Remember -



Statistical effects favor low occupancy with monovalent antigens

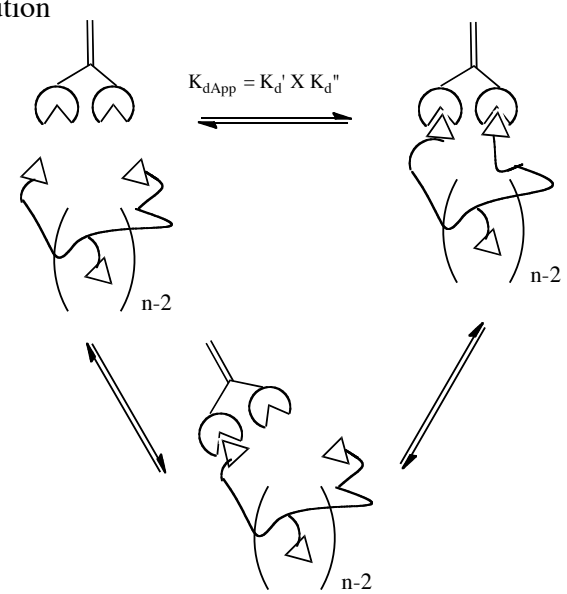
III. Thermodynamics of 'Avidity'

On Surfaces



if antigen density is appropriate $K_{dApp} < K_d'$ or K_d''

In Solution



if antigen antigen density is appropriate $K_{dApp} < K_d'$ or K_d''

With antigens immobilized on surfaces, such as on cells (receptors) or on SPR chips, the antigens now 'appear' multivalent. In effect the cell surface or the SPR chip surface is the 'linker.' The properties of the surface and the density of antigens can result in apparent increases in affinity (avidity). Thus the measurement of antibody affinity on cells and in SPR experiments can be tricky.

Each part of the step-wise binding process has statistical effects ('s' terms) that vary with antigen density. Unlike statistical effects in multivalent proteins with monovalent ligands, the statistical effects can now cause 'apparent positive cooperativity' which is conceptually analogous to the multivalent examples above (FBDD).

Similar considerations in solution if antigen is multivalent, as with Enbrel above.

Read: Mack et al. (2012) Thermodynamic Analysis to Assist in the Design of Recombinant Antibodies. Crit Rev Immun 32: 503-527.

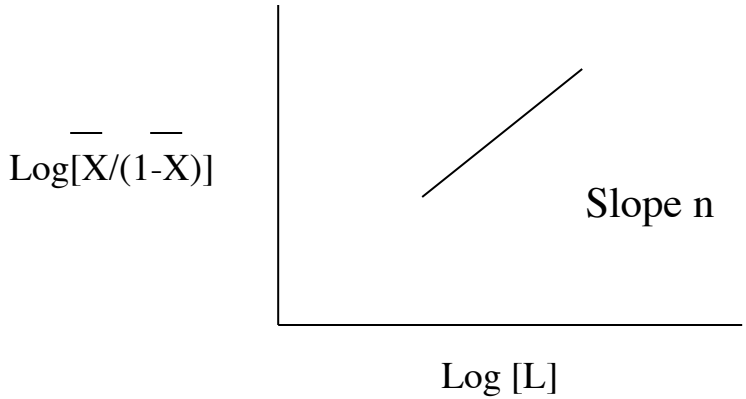
IV. Allostery - 'other site'

Allostery is the useful exploitation of control of ligand distribution for some biological advantage. Although we are now comfortable with the concept of allostery, it was controversial when first proposed in the early 1960's. A 'traditional' analytical approach is the application of the Hill plot, as described above. Whereas we already considered the ''stepwise' nature of multiple ligand binding, Hill considered multiple ligand binding as a two state process, in distinct contrast to all the examples above which explicitly recognize intermediate states of ligation.

$$P + nL \leftrightarrow P \bullet nL \qquad K_a = \frac{[P \bullet nL]}{[P][L]^n}$$

$$\overline{X} = \frac{[P \bullet nL]}{[P]_{total}} = \frac{[L]^n}{[L]^n + K_d}$$

$$\log\left(\frac{\overline{X}}{(1-\overline{X})}\right) = \log K_d + n \log[L]$$



Hill considered the slope, n, as the cooperativity index - a perfectly cooperative system with stoichiometry n would yield a line of slope n. As a result, n, has often mistakenly been used as a measure of stoichiometry. The Hill model is physically unreasonable and no 'physical' meaning can be applied to the Hill coefficient. It is however a useful comparator of the degree of cooperativity for a system with known or fixed 'n'.