Chem 728 Lec Notes – Part 4 – Ligand Binding & Kinetics

The following are lecture notes for Chem 728 (by C. Martin, with minor modifications by L.!Thompson). Much of the material is taken directly from the indicated references (old vH, some refs updated to new). This is not intended to replace the original references, but is made available solely for the convenience of students in the class.

VH = "Principles of Physical Biochemistry," Kensal E. van Holde, W. Curtis Johnson, & P. Shing Ho, Prentice Hall, NJ, 1998 (ISBN 0-13-720459-0)

CS = "Biophysical Chemistry, Volumes I-III" Charles R. Cantor & Paul R. Schimmel, W. H. Freeman, NY, 1980 (ISBN 0-7167-1188-5, 0-7167-1190-7, 0-7167-1192-3)

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Ligand Binding and Related Equilibria

The simplest ligand binding reaction can be written: P + L ↔ PL with $K = \frac{[PL]}{[P][L]}$

$$\bar{v} = \frac{\#\ \text{moles ligand bound}}{\#\ \text{moles protein}} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]}{[P]+[PL]}$$

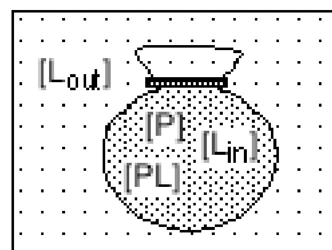
For the simple case described above, it will range from 0 to 1.

Methods of measurement

Equilibrium Dialysis**:**

For the binding of a small molecule (ligand, L) to a large macromolecule (protein, P), we can often measure binding via a technique called equilibrium dialysis. The requirements are:

1) a semiporous dialysis membrane to which L is freely permeable and P is not
2) a method for detecting [L_{out}] and/or [L_{in}] - a radioactive tag on L is most commonly used.



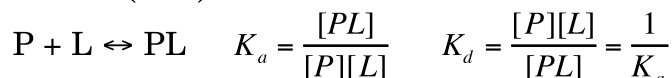
Perturbation of Ligand or Protein

Alternatively, if we can measure a perturbation which occurs on binding, we can readily measure the *fraction saturation* $\theta = \frac{\bar{v}}{n} = \frac{\Delta Obs}{\Delta Obs_T}$

This measurement *assumes* $\bar{v}$ is linear in *Obs* and is the same for different binding sites. Note that for n=1, $\bar{v}$=θ.

Theoretical Treatment

Single Site per Macromolecule **(n=1)**



$$\text{P + L} \leftrightarrow \text{PL} \qquad K_a = \frac{[PL]}{[P][L]} \qquad K_d = \frac{[P][L]}{[PL]} = \frac{1}{K_a}$$

The equilibrium can be expressed either as an association (K_a) or a dissociation (K_d).

$$\bar{v} = \frac{[PL]}{[P]+[PL]} = \frac{1}{\left([P]/[PL]\right)+1} = \frac{1}{\left([P]/K_a[P][L]\right)+1}$$

$$\bar{v} = \frac{K_a[L]}{1+K_a[L]} = \frac{[L]}{1/K_a + [L]} = \frac{[L]}{K_d + [L]}$$

Always!check the behavior of your equation at simple, extreme limits:

Various results: as [L]→∞, $\bar{v}$ →1 (makes sense)
also when $\bar{v}$ =0.5, [L]=1/K_a=K_d.

Rearrange to linear function (but watch for distortion of error)

$$\bar{v} = \frac{K_a[L]}{1+K_a[L]} = \frac{[L]}{\left(1/K_a\right)+[L]} \quad \text{then} \quad \left(1/K_a\right) + [L] = \frac{[L]}{\bar{v}}$$

$$\left(1/[L]K_a\right) = \frac{1}{\bar{v}} - 1 = \frac{1-\bar{v}}{\bar{v}} \quad \text{and} \quad \frac{\bar{v}}{[L]} = K_a(1-\bar{v})$$

The last form of the equation is known as a **Scatchard Plot**.

Alternatively, $\left(\frac{1}{[L]K_a} \right) = \frac{1-\bar{v}}{\bar{v}}$ leads to $\frac{\bar{v}}{1-\bar{v}} = [L]K_a$

Multiple Binding Sites

If we assume, more than one ligand binding site per protein, we have the more general form:

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

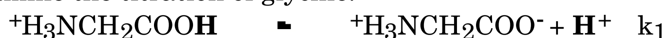
It should be obvious that as we approach saturation in this case, all protein is pushed to the $[PL_n]$ limit, and this ratio should approach n, the total number of binding sites per protein.

In equilibrium dialysis, if we know the concentration of protein and can measure the total concentration of ligand inside the bag (radioactive labeling is frequently employed), then we can measure this parameter.

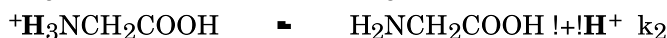
$P + nL \leftrightarrow PL_n$ multiple sites, could be same or different, independent or cooperative.

Digression - Microscopic vs. Macroscopic

Examine the titration of glycine:



$^{+}H_3NCH_2COO\mathbf{H} \rightleftharpoons {}^{+}H_3NCH_2COO^{-} + \mathbf{H}^{+}$ k_1



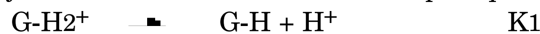
$^{+}\mathbf{H}_3NCH_2COOH \rightleftharpoons H_2NCH_2COOH + \mathbf{H}^{+}$ k_2

$^{+}\mathbf{H}_3NCH_2COO^{-} \rightleftharpoons H_2NCH_2COO^{-} + \mathbf{H}^{+}$ k_3

$H_2NCH_2COO\mathbf{H} \rightleftharpoons H_2NCH_2COO^{-} + \mathbf{H}^{+}$ k_4

The reactions above represent *microscopic* equilibria (hence the lower case k).

More commonly we would measure the macroscopic equilibria:



$G\text{-}H_2^{+} \rightleftharpoons G\text{-}H + H^{+}$ K1

$G\text{-}H \rightleftharpoons G^{-} + H^{+}$ K2

where

$G\text{-}H_2^{+} = {}^{+}H_3NCH_2COOH$

$GH = {}^{+}H_3NCH_2COO^{-} + H_2NCH_2COOH$

$G^{-} = H_2NCH_2COO^{-}$

then

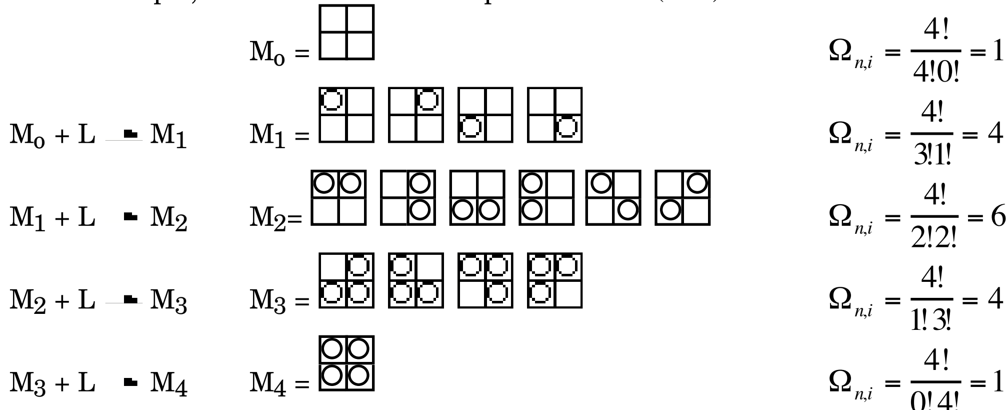
$$K_1 = k_1 + k_2 \quad \text{and} \quad K_2 = \frac{1}{\left(\frac{1}{k_3} + \frac{1}{k_4} \right)} \quad \text{(try it!)}$$

Note also that all four microscopic constants k_1, k_2, k_3, and k_4 are *not* independent.

Ligand Binding - Identical Independent Sites

In general, one can arrange i ligands on n identical sites $\Omega_{n,i} = \frac{n!}{(n-i)!i!}$ ways

As an example, let's consider four independent sites (n=4)



$M_0 =$ $\qquad \Omega_{n,i} = \frac{4!}{4!0!} = 1$

$M_0 + L \rightleftharpoons M_1 \qquad M_1 =$ $\qquad \Omega_{n,i} = \frac{4!}{3!1!} = 4$

$M_1 + L \rightleftharpoons M_2 \qquad M_2 =$ $\qquad \Omega_{n,i} = \frac{4!}{2!2!} = 6$

$M_2 + L \rightleftharpoons M_3 \qquad M_3 =$ $\qquad \Omega_{n,i} = \frac{4!}{1!3!} = 4$

$M_3 + L \rightleftharpoons M_4 \qquad M_4 =$ $\qquad \Omega_{n,i} = \frac{4!}{0!4!} = 1$

As before, we want to calculate the macroscopic parameter

$$\bar{v} = \frac{[L_B]}{[P]+[PL]} = \frac{[L_B]}{[P_T]} \qquad = \frac{[PL]+2[PL_2]+\ldots+n[PL_n]}{[P]+[PL]+[PL_2]+\ldots+[PL_n]}$$

more generally

$$\bar{v} = \frac{\sum_{i=0}^{n} iM_i}{\sum_{i=0}^{n} M_i} \qquad \left(= \frac{\sum_{i=0}^{n} i[PL_i]}{\sum_{i=0}^{n} [PL_i]} \right)$$

We have the *macroscopic* equilibrium constants: $K_i = \frac{[M_i]}{[M_{i-1}][L]}$

or $[M_i] = K_i[L][M_{i-1}]$

Since these are identical sites, $k_i = k_j$, however, $K_i \neq K_j$

You can show that $[M_i] = [L]^i[M_o]\prod_{j=1}^{i} K_j$ (try it, solve for M_1, then for M_2, etc.)

It can be shown that $K_i = \frac{\Omega_{n,i}}{\Omega_{n,i-1}}k$ (in above example, $\Omega_{n,i}$ is the number of microstates)

as an aside, in example above K_1=4k, K_2=(6/4)K, K_3=(4/6)k, K_4=(1/4)k
This says that equilibrium favors adding a ligand to a fully unbound form is more favorable than adding ligand to a partially bound form. Does this make qualitative sense from your understanding so far?

then $K_i = \frac{\Omega_{n,i}}{\Omega_{n,i-1}}k = \frac{(n-i+1)!(i-1)!}{n!}\frac{n!}{(n-i)!i!}k = \frac{(n-i+1)}{i}k$

So that $[M_i] = [L]^i[M_o]\prod_{j=1}^{i}\left(\frac{(n-j+1)}{j}\right)k$

and finally $[M_i] = \left[\prod_{j=1}^{i}\left(\frac{(n-j+1)}{j}\right)\right](k[L])^i[M_o]$

which simplifies somewhat to $[M_i] = \left[\frac{n!}{(n-i)!i!}\right](k[L])^i[M_o]$

Returning to the macroscopic observable:

$$\bar{v} = \frac{\sum_{i=0}^{n} iM_i}{\sum_{i=0}^{n} M_i} = \frac{\sum_{i=0}^{n}\left\{i\left[\frac{n!}{(n-i)!i!}\right](k[L])^i[M_o]\right\}}{\sum_{i=0}^{n}\left\{\left[\frac{n!}{(n-i)!i!}\right](k[L])^i[M_o]\right\}} = \frac{[M_o]\sum_{i=0}^{n}\left\{i\left[\frac{n!}{(n-i)!i!}\right](k[L])^i\right\}}{[M_o]\sum_{i=0}^{n}\left\{\left[\frac{n!}{(n-i)!i!}\right](k[L])^i\right\}}$$

$$= \frac{0 + \sum_{i=1}^{n}\left\{i\left[\frac{n!}{(n-i)!i!}\right](k[L])^i\right\}}{1 + \sum_{i=1}^{n}\left\{\left[\frac{n!}{(n-i)!i!}\right](k[L])^i\right\}}$$

However, the denominator is a binomial expansion:

$$1 + \sum_{i=1}^{n}\left\{\left[\frac{n!}{(n-i)!i!}\right](k[L])^i\right\} = (1+k[L])^n$$

eg. for n=4 above, $1 + \sum_{i=1}^{4}\left\{\left[\frac{4!}{(4-i)!i!}\right](k[L])^i\right\} = 1 + 4kL + 6k^2L^2 + 4k^3L^3 + k^4L^4 = (1+k[L])^4$

save this, we'll need it. But in the meantime, take it and

differentiate both sides with $\frac{\partial}{\partial(k[L])}$ $$\sum_{i=1}^{n}\left\{i\left[\frac{n!}{(n-i)!i!}\right](k[L])^{i-1}\right\} = n(1+k[L])^{n-1}$$

then multiply both sides by k[L] $$\sum_{i=1}^{n}\left\{i\left[\frac{n!}{(n-i)!i!}\right](k[L])^{i}\right\} = nk[L](1+k[L])^{n-1}$$

Combining the above two results,

$$\bar{v} = \frac{nk[L](1+k[L])^{n-1}}{(1+k[L])^{n}} = \frac{nk[L]}{1+k[L]}$$

Compare:

Before *for a single site*: $\bar{v} = \frac{K_a[L]}{1+K_a[L]}$, which led to $\frac{\bar{v}}{[L]} = K_a(1-\bar{v})$ and $\frac{\bar{v}}{1-\bar{v}} = [L]K_a$

Before, as [L]→∞, $\bar{v}$ →1 (single site)

Now, as [L]→∞, $\bar{v}$ →n (n multiple sites)

Both of these results should be completely and simply obvious. At complete binding, the number of ligands bound per protein is simply the number of ligand binding sites per protein.

Now, similarly for multiple, independent sites we have $\frac{\bar{v}}{[L]} = k(n-\bar{v})$

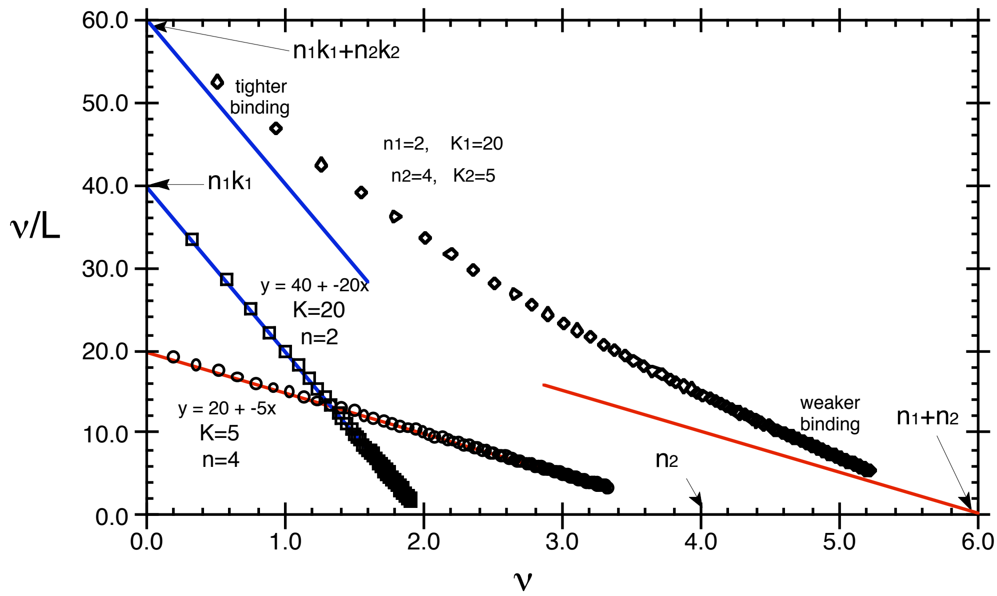
Ligand Binding - Multiple (Different) Classes of Independent Sites

If a protein has multiple class of sites in which sites within a given class are identical and independent, but there is more than one such class of sites, then for m different classes of sites, in which each class is composed of n_i identical sites with association constant k_i:

$$\bar{v} = \sum_{i=1}^{m}\frac{n_i k_i[L]}{1+k_i[L]} \quad \text{or} \quad \frac{\bar{v}}{[L]} = \sum_{i=1}^{m}\frac{n_i k_i}{1+k_i[L]} \quad \text{(Scatchard)}$$

Notice that if we plot $\frac{\bar{v}}{[L]}$ vs. $\bar{v}$, then the intercept ($\bar{v}$=0 at [L]=0) corresponds to $\frac{\bar{v}}{[L]} = \sum_{i=1}^{n} n_i k_i$

Similarly, the x-intercept ($\frac{\bar{v}}{[L]} = 0$) corresponds to $\bar{v} = n_1 + n_2$ (extra credit for the first to derive it!).



Ligand Binding - Multiple Identical but Interdependent Sites

If we assume a protein (or other macromolecule) with multiple ligand binding sites, for which binding of each ligand alters the energetics of binding of the subsequent ligand.

If the inherent (independent) binding constant is k_o, and we can express the change in free energy associated with increasing fractional binding as RTφ(ν),

then $\Delta G^o = \Delta G^o_o + RT\phi_\nu$ where $\Delta G^o_o = -RT\ln k_o$

and then $k(\nu) = e^{-\Delta G^o / RT} = e^{-(\Delta G_o^o + RT\phi_\nu)/RT} = e^{-\Delta G_o^o / RT} e^{-\phi_\nu} = k_o e^{-\phi_\nu}$

Stop and analyze:

As ligand binds, if:

$\Delta\phi_\nu<0$ then $k>k_o$ tighter binding cooperative

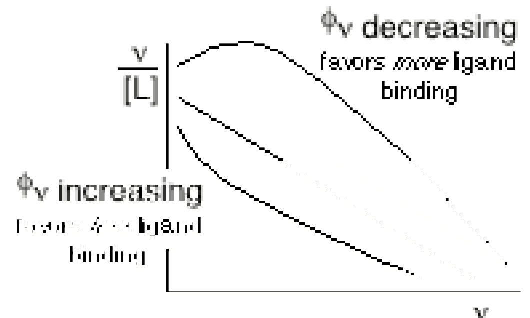
$\Delta\phi_\nu>0$ then $k<k_o$ weaker bindinganticooperative

One can then replace k in the multiple independent sites model:

$$\bar{\nu} = \frac{nk[L]}{1+k[L]} = \frac{n[L]k_o e^{-\phi_\nu}}{1+[L]k_o e^{-\phi_\nu}} = \frac{n[L]k_o}{e^{\phi_\nu} + [L]k_o}$$

$$\frac{\bar{\nu}}{[L]} = k(n-\bar{\nu}) = (n-\bar{\nu})k_o e^{-\phi_\nu}$$

The plot at right shows behavior characterized by these kinds of interactions. (Note that our convention for k is the inverse of that used by Cantor & Schimmel, such that a decreasing ϕ_ν corresponds to a decreasing energy cost of *association*, and consequently more ligand is bound than in the independent site model. Conversely, an increasing ϕ_ν indicates a larger barrier to binding of second, third, and fourth ligands, so that less is bound at any point in the titration.



N.B. - the behavior predicted for ϕ_ν increasing (anticooperative binding) is similar in general shape to that predicted for multiple classes of independent sites. In practice, it may be hard to distinguish the two. C & S suggests that multiple classes of independent sites is more simple. ***One should always choose the most simple model which satisfactorily explains the data.***

In contrast, the cooperative behavior predicted for ϕ_ν decreasing cannot be explained by a simpler model.

Finally, one can (with great caution in real experimental situations) apply the same type of reasoning to a system with multiple class of interacting sites, each class having a different interaction function ϕ_ν.

N.B. - It is less important that you memorize these different equations, and more important that you understand them and can derive them. It may well be that a system you encounter in the future requires a slight twist from these stock solutions - you want to be able to come up with a correct, custom solution.

Ligand Binding - Infinite Cooperativity

If binding to multiple sites is infinitely cooperative, then binding is all or none and can be written quite simply as:

$$M_o + nL \longleftrightarrow M_n \quad \text{then} \quad K_a^n = \frac{[M_n]}{[M_o][L]^n}$$

we can derive ν as before, $\nu = \frac{n[M_n]}{[M_o]+[M_n]} = \frac{nK_a^n[M_o][L]^n}{[M_o]+K_a^n[M_o][L]^n} = \frac{nK_a^n[L]^n}{1+K_a^n[L]^n}$

$$\nu = \frac{n[L]^n}{\left(\frac{1}{K_a^n}\right)+[L]^n} \quad \text{and} \quad \nu = \frac{nK_a^n[L]^n}{1+K_a^n[L]^n} \quad \text{or trivially} \quad \frac{\nu}{[L]} = \frac{nK_a^n[L]^{n-1}}{1+K_a^n[L]^n}$$

*** MISSING EQUATION – FIX!!! ***

bringing $\left(\frac{1}{K_a^n[L]^n}\right)+1 = \frac{n}{\nu}$

from here we can go to $\frac{\nu}{n} = \theta = \frac{K_a^n[L]^n}{K_a^n[L]^n+1}$ ← Another useful form.

Note that $\dfrac{\theta}{1-\theta} = \left(\dfrac{K_a^n[L]^n}{K_a^n[L]^n+1}\right)\left(\dfrac{1}{1-\dfrac{K_a^n[L]^n}{K_a^n[L]^n+1}}\right) = \dfrac{K_a^n[L]^n}{K_a^n[L]^n+1-K_a^n[L]^n} = K_a^n[L]^n$

So that $\ln\left(\dfrac{\theta}{1-\theta}\right) = \ln\left(K_a^n[L]^n\right) = n\ln\left(K_a[L]\right) = n\ln K_a + n\ln[L]$

and plotting $\ln\left(\dfrac{\theta}{1-\theta}\right)$ vs. $\ln[L]$ is predicted to yield a straight line with slope of n and an intercept of n ln K.

You will also see this in other forms. Note that $\dfrac{\theta}{1-\theta} = \dfrac{\nu}{n-\nu}$ (easy, try it!)

Ligand Binding - Intermediate Cooperativity

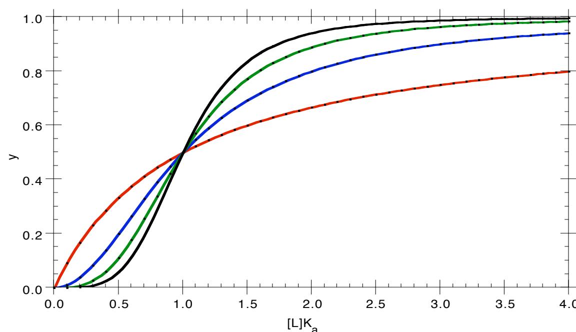
A *semiemperical* derivative of this which is often used is $\dfrac{\nu}{[L]} = \dfrac{nK_a^{\alpha_H}[L]^{\alpha_H-1}}{1+K_a^{\alpha_H}[L]^{\alpha_H}}$

In this case α_H is called the Hill constant and indicates the degree of cooperativity. As α_H approaches n, then the binding is infinitely cooperative.

Other manipulations of these equations are:

$$\theta = \frac{\nu}{n} = \frac{K_a^n[L]^n}{1+K_a^n[L]^n} \longrightarrow \frac{K_a^{\alpha_H}[L]^{\alpha_H}}{1+K_a^{\alpha_H}[L]^{\alpha_H}} = \frac{\left(K_a[L]\right)^{\alpha_H}}{1+\left(K_a[L]\right)^{\alpha_H}}$$

The figure below plots θ vs. K[L] from the above equation and demonstrates it's behavior for infinite cooperativity for α_H!=!n!=!1,2,3,4.



Note that n =!1 is a single site (of course, uncooperative) and shows a typical titration profile. In the other cases, ligand binds slowly at first, but as a few bind, more bind with little (no) energetic cost.

NOTE - α_H is not a constant in this treatment - see below.

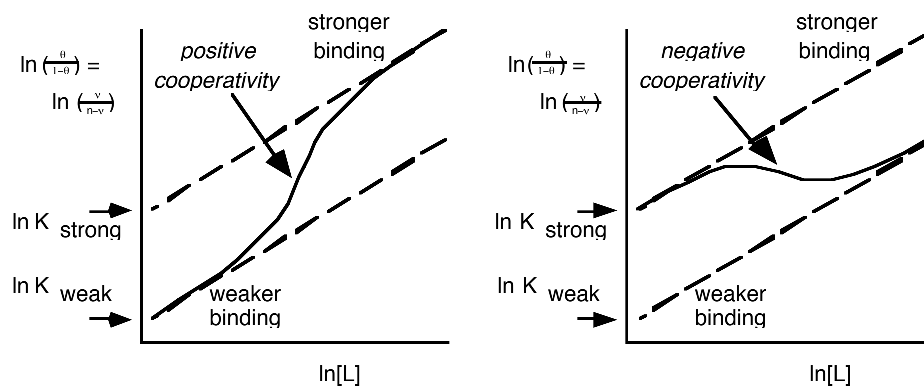
Hill Plots

Also, as before we have $\ln\left(\dfrac{\theta}{1-\theta}\right) = \alpha_H \ln K_a + \alpha_H \ln[L]$

A plot of $\ln\left(\dfrac{\theta}{1-\theta}\right)$ vs. ln[L] yields a (varying) slope of αH (these are usually called Hill Plots)

For the plot above (infinite cooperativity), this manipulation would yield a straight line with slope!=!α_H!=!n. Again, for **no** cooperativity, the slope would be 1.0.

Now, what happens when we have intermediate cooperativity?



For positive cooperativity, binding is “weak” early in the titration when no sites are occupied, and so the sites titrate as if there is no cooperativity (in this region α_H=1). Very late in the titration, as you are filling the last remaining sites (now with a strong binding constant), you again see little evidence of cooperativity (in this region, again α_H=1).. In the middle, you see the largest evidence of cooperativity (α_H>1).

For negative cooperativity, binding starts off at the “strong” limit, and then gets weaker as more sites are filled.

The plots above are usually called “Hill Plots.” Note that you will see different nomenclature for the same plot. As before, the limiting conditions have α_H=1, while in the middle of the titration α_H<1. The Hill constant is usually defined as the slope of the curve at the midpoint of the titration. In other words, where cooperativity (or anticooperativity) is *greatest*.

Review - Ligand Binding

NOTE: van Holde uses K_a as we do, however, Cantor & Schimmel use K_d (= $1/K_a$). Be warned. Similarly, van Holde uses n_α instead of α_H (as we and Cantor & Schimmel do...).

Terms:

$$\bar{v} = \frac{[L_B]}{[P_T]} = \frac{\text{conc of ligand bound}}{\text{total concentration of protein}}$$ can range from 0 to n (number of binding sites/protein)

fraction saturation $\theta = \frac{\bar{v}}{n} = \frac{\Delta Obs}{\Delta Obs_T}$ can range from 0 to 1

Single Site

$$\frac{\bar{v}}{[L]} = \frac{K_a}{1 + K_a[L]} = -K_a\bar{v} + K_a$$

Plot $\frac{\bar{v}}{[L]}$ vs. $\bar{v}$, slope = $-K$, y-intercept = K

Plot $\frac{\bar{v}}{[L]}$ vs. $[L]$, y-intercept = K

Multiple Identical, Independent Sites

$$\frac{\bar{v}}{[L]} = \frac{nK_a}{1 + K_a[L]} = -K_a\bar{v} + nK_a$$

Plot $\frac{\bar{v}}{[L]}$ vs. $\bar{v}$, slope = $-K$, y-intercept = nK

Plot $\frac{\bar{v}}{[L]}$ vs. $[L]$, y-intercept = K

Multiple Independent *Classes* of Multiple Identical, Independent Sites

$$\frac{\bar{v}}{[L]} = \sum_{i=1}^{n_{classes}} \frac{n_i K_{a_i}}{1 + K_{a_i}[L]}$$

Plot $\frac{\bar{v}}{[L]}$ vs. $[L]$, y-intercept = $\sum_{i=1}^{n_{classes}} n_i K_{a_i}$

Multiple Identical, Infinitely Cooperative Sites

$$\frac{\bar{v}}{[L]} = \frac{nK_a^n[L]^{n-1}}{1 + K_a^n[L]^n} \qquad \theta = \frac{\bar{v}}{n} = \frac{K_a^n[L]^n}{1 + K_a^n[L]^n}$$

then

$$\ln\left(\frac{\theta}{1-\theta}\right) = n\ln K_a + n\ln[L]$$

Plot $\ln\left(\frac{\theta}{1-\theta}\right)$ vs. $\ln[L]$, slope = n, y-intercept = $n\ln K_a$

Multiple Identical Sites, Intermediate Cooperativity

$$\frac{\bar{v}}{[L]} = \frac{nK_a^{\alpha_H}[L]^{\alpha_H - 1}}{1 + K_a^{\alpha_H}[L]^{\alpha_H}} \qquad \theta = \frac{\bar{v}}{n} = \frac{K_a^{\alpha_H}[L]^{\alpha_H}}{1 + K_a^{\alpha_H}[L]^{\alpha_H}}$$

then

$$\ln\left(\frac{\theta}{1-\theta}\right) = \alpha_H \ln K_a + \alpha_H \ln[L]$$

Plot $\ln\left(\frac{\theta}{1-\theta}\right)$ vs. $\ln[L]$, slope = α_H y-intercept = $\alpha_H \ln K_a$

For positive cooperativity, $1 \le \alpha_H \le n$ (Fully Independent ≤!≤!Infinitely Cooperative)

Models for Cooperativity

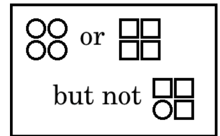
MWC - Monod, Wyman, Changeux

Assume that each subunit of a multisubunit protein can exist in two states:

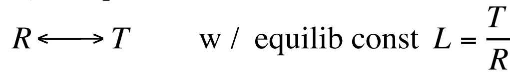
○ R (stronger ligand binding) and

□ T (weaker ligand binding).

Assume that all subunits are symmetric and that they can be either all R or all T, but not intermediate (you can argue that this *should* follow from the symmetry of the system).



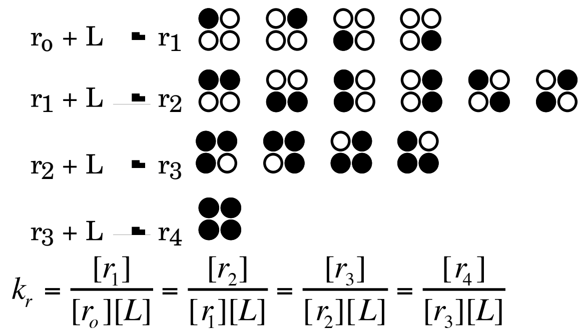
In the absence of ligand, an equilibrium exists:

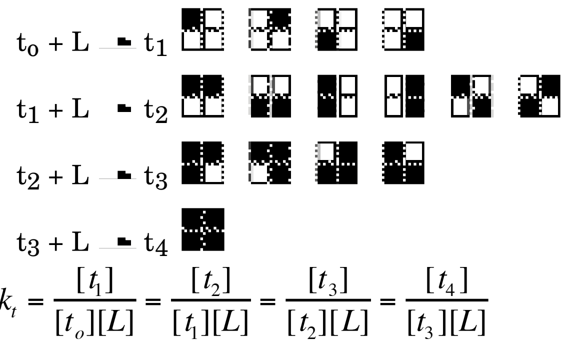


Le Chatelier tells us that addition of ligand will shift the equilibrium to the left (L will decrease).

Backing up to examine the R and T states separately, each can be considered a system with n identical sites and we can look at the microscopic equilibrium constants (k_R and k_T) for each.

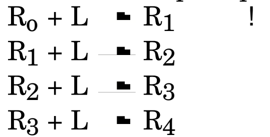
Let's look at a system with four sites (hemoglobin?). Using nomenclature similar to above, the microscopic states are given by:

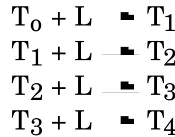




$$k_r = \frac{[r_1]}{[r_o][L]} = \frac{[r_2]}{[r_1][L]} = \frac{[r_3]}{[r_2][L]} = \frac{[r_4]}{[r_3][L]} \qquad k_t = \frac{[t_1]}{[t_o][L]} = \frac{[t_2]}{[t_1][L]} = \frac{[t_3]}{[t_2][L]} = \frac{[t_4]}{[t_3][L]}$$

The macroscopic equivalents are:





$$K_{R_i} = \frac{[R_i]}{[R_{i-1}][L]} = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k_r \qquad K_{T_i} = \frac{[T_i]}{[T_{i-1}][L]} = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k_t$$

remember $\Omega_{n,i} = \frac{n!}{(n-i)!i!}$

so $K_{R_i} = \frac{[R_i]}{[R_{i-1}][L]} = \frac{\Omega_{n,i}}{\Omega_{n,i-1}} k_r = \frac{(n-i+1)!(i-1)!}{n!} \frac{n!}{(n-i)!i!} k_r = \frac{(n-i+1)}{i} k_r$

and $K_{T_i} = \frac{[T_i]}{[T_{i-1}][L]} = \frac{(n-i+1)}{i} k_t$

so that for the above, $K_{R1} = 4k_r$, $K_{R2} = (3/2)k_r$, $K_{R3} = (2/3)k_r$, $K_{R4} = (1/4)k_r$ etc...

Again, think about this result. Does it make sense?

and ultimately (just as before): $T_i = \left[\frac{n!}{(n-i)!i!}\right] k_t^i L^i T_o$ and $R_i = \left[\frac{n!}{(n-i)!i!}\right] k_r^i L^i R_o$

From the definition of $\overline{\nu}$, we have $\overline{\nu} = \frac{\text{total L bound}}{\text{total \# sites}} = \frac{\sum_{i=1}^{n} iR_i + \sum_{i=1}^{n} iT_i}{\sum_{i=1}^{n} R_i + \sum_{i=1}^{n} T_i}$

$$\bar{\nu} = \frac{\sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!i!}\right]k_r^i L^i R_o + \sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!i!}\right]k_t^i L^i T_o}{\sum_{i=1}^{n}\left[\frac{n!}{(n-i)!i!}\right]k_r^i L^i R_o + \sum_{i=1}^{n}\left[\frac{n!}{(n-i)!i!}\right]k_t^i L^i T_o} = \frac{R_o \sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!i!}\right]k_r^i L^i + T_o \sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!i!}\right]k_t^i L^i}{R_o \sum_{i=1}^{n}\left[\frac{n!}{(n-i)!i!}\right]k_r^i L^i + T_o \sum_{i=1}^{n}\left[\frac{n!}{(n-i)!i!}\right]k_t^i L^i}$$

we know that the free forms T_o and R_o are related by the equilib constant L, that is $T_o = L_{RT} R_o$.
and playing the same manipulation that we did previously relating this to $\bar{\nu}$, we have

$$\bar{\nu} = \frac{nk_r L(1+k_r L)^{n-1} + L_{RT} nk_t L(1+k_t L)^{n-1}}{(1+k_r L)^n + L_{RT}(1+k_t L)^n}$$

now by convention let the ratio of k_t and k_r equal c $\quad c = \frac{k_t}{k_r}$

$$\bar{\nu} = nk_r L\frac{(1+k_r L)^{n-1} + cL_{RT}(1+ck_r L)^{n-1}}{(1+k_r L)^n + L_{RT}(1+ck_r L)^n}$$

Rearranging this to the form for a Hill Plot, we have

$$\frac{\bar{\nu}}{n-\bar{\nu}} = k_r L\frac{1+cL_{RT}\left(\frac{1+ck_r L}{1+k_r L}\right)^{n-1}}{1+L_{RT}\left(\frac{1+ck_r L}{1+k_r L}\right)^{n-1}}$$

As is always a good idea, let's look at the limits:

As $L \to 0$ $\quad \frac{\bar{\nu}}{n-\bar{\nu}} \longrightarrow k_r L\frac{1+cL_{RT}}{1+L_{RT}}$

$$\ln\frac{\bar{\nu}}{n-\bar{\nu}} \longrightarrow \ln L + \ln\left(k_r \frac{1+cL_{RT}}{1+L_{RT}}\right) \quad \text{(Hill plot)}$$

As $L \to \infty$ $\quad \frac{\bar{\nu}}{n-\bar{\nu}} \longrightarrow k_r L\frac{1+cL_{RT}\left(\frac{ck_r}{k_r}\right)^{n-1}}{1+L_{RT}\left(\frac{ck_r}{k_r}\right)^{n-1}} = k_r L\frac{1+L_{RT}c^n}{1+L_{RT}c^{n-1}}$

$$\ln\frac{\bar{\nu}}{n-\bar{\nu}} \longrightarrow \ln L + \ln\left(k_r \frac{1+L_{RT}c^n}{1+L_{RT}c^{n-1}}\right) \quad \text{(Hill plot)}$$

On a Hill Plot,we see that we predict a straight line with a slope of 1 for each case.
Finally, again going to limits, if T is very highly favored in the absence of ligand then

As $L \longrightarrow 0$ and $L_{RT} \longrightarrow \infty$ then $\ln\frac{\bar{\nu}}{n-\bar{\nu}} \longrightarrow \ln L + \ln(k_r c) = \ln L + \ln k_t$

As expected, **early** in the titration the system behaves just like a simple **T** state.
If we additionally assume that c is small ($k_r >> k_t$ ligand binding is much stronger to the R state), then

As $L \longrightarrow \infty$ and $c << 1$ then $\ln\frac{\bar{\nu}}{n-\bar{\nu}} \longrightarrow \ln L + \ln k_r$

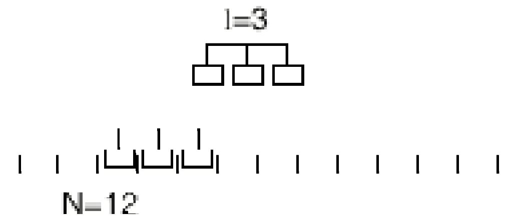
As expected, **late** in the titration the system behaves just like a simple **R** state.

KNF - Koshland, Nemethy, Filmer

Although the MWC model does explain hemoglobin and various other systems very well, the MWC model makes the dramatic assumption that the protein can only exist all R or all T. This is most certainly not true for all systems.
The KNF model allows for mixed states of the subunits within a single protein and so is more general. It is also called the "sequential model" because subunit can sequentially (one at a time) convert

from T to R and vice versa. It is also much more complicated mathematically and solutions depend on the exact form of the intersubunit energetics.

Lattice Site Binding



We can also examine a multi-site ligand binding to a multi-site lattice (the first examples that come to mind include proteins or drugs binding to DNA).

As before, we can take ν as the number of moles of L bound per mole of lattice.

In this case, N is the number of monomeric binding sites, however , N/*l* is the maximum number of multimeric ligands which can bind at saturation.. However, before any ligand has bound, there are N-*l*+1 *potential* binding sites. To understand intermediate states, we must invoke statistical arguments as before.

$\nu = N_{fl} L k$ where N_{fl} is the average number of free ligand sites of length *l* per lattice and k is the intrinsic microscopic association constant, just as before.

Cantor & Schimmel derive (pp 878-881) the following expression

$$\frac{\nu}{L} = N\left(1-\frac{l\nu}{N}\right)k\left[\frac{\left(1-\frac{l\nu}{N}\right)}{1-\frac{(l-1)\nu}{N}}\right]^{l-1}$$

there is the caveat that this is strictly only true for large N

As always, let's look at this in its limits:

For *l*=1 we have $\frac{\nu}{L} = N\left(1-\frac{\nu}{N}\right)k\left[\frac{\left(1-\frac{\nu}{N}\right)}{1-\frac{(1-1)\nu}{N}}\right]^{1-1} = N\left(1-\frac{\nu}{N}\right)k = Nk - \nu k$

Rearranging the general lattice equation we have $\frac{\nu}{L} = Nk\gamma - \nu k\frac{\gamma l}{N}$ where $\gamma = \left[\frac{\left(1-\frac{l\nu}{N}\right)}{1-\frac{(l-1)\nu}{N}}\right]^{l-1}$

For large *l* and small ν we have

$$\frac{\nu}{L} = Nk\gamma - \nu\frac{k}{N}\gamma l \quad \text{where} \quad \frac{k}{N}\gamma l = \frac{k}{N}l\left[\frac{\left(1-\frac{l\nu}{N}\right)}{1-\frac{(l-1)\nu}{N}}\right]^{l-1} \xrightarrow{l\to\infty} l\frac{k}{N}(1)^{l-1} \longrightarrow \infty$$

so that slope in a Scatchard plot goes to -∞, or more generally the slope gets more steep in a negative sense.

For large *l* and large ν (as ν approaches N/*l*), we have

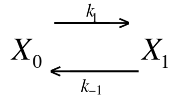
$$\frac{\nu}{L} = Nk\gamma - \nu\frac{k}{N}\gamma l \quad \text{where} \quad \frac{k}{N}\gamma l = \frac{k}{N}l\left[\frac{\left(1-\frac{l\nu}{N}\right)}{\left(1-\frac{l\nu}{N}\right)-\frac{\nu}{N}}\right]^{l-1} = \frac{k}{N}l\left[\frac{0}{0-\frac{\nu}{N}}\right]^{l-1} \longrightarrow 0$$

So we see an initial steep negative slope which eventually levels out and approaches 0 as we are saturated.

Early in the titration, binding is strongly favored. This is a result of the increase in entropy of having ligand bound in many possible different sites. However, as the lattice becomes saturated there are fewer and fewer sites available and now entropy pushes against binding.

Kinetics of Ligand Binding / Reaction Kinetics

Unimolecular reactions (C&S Chapt. 16-2)



The rate equation is $-\frac{dX_o}{dt} = k_1 X_o - k_{-1} X_1$

How might one follow such a reaction? One example is to perturb a system at equilibrium. An example is a temperature jump experiment. At the original temperature, the system is at equilibrium. If we "instantaneously" change the temperature to a new temperature, a new equilibrium distribution will be defined, but the system is sitting at the old distribution.

Take $\overline{X_o}$ as the new equilibrium concentration of X_0. Then

Kinetics of Protein Folding

Simple Two-State Unfolding

Kinetics

NOTE: Convention in kinetics is to use lower case k for kinetic constants and upper case K for equilibrium constants (except for microscopic equilibrium constants).

Important: Note that the stoichiometry of a reaction does not tell you anything about the kinetic mechanism of a reaction! This is very often overlooked.

Example:

For the reaction whose stoichiometry is: $aA + bB \xrightarrow{k} cC + dD$

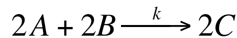
The potential kinetic mechanisms could lead to almost any velocity equation, including:

$V = kA^a B^b$ or $V = kAB^2$ or $V = kA$ or $V = kB^2$ or $V = k$ or ...

Remember: the upper equation simply describes the *stoichiometry* of the reaction.

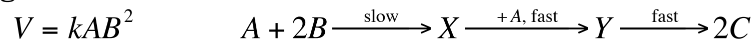
Why is this?

Because the underlying *kinetic* mechanism for the overall reaction

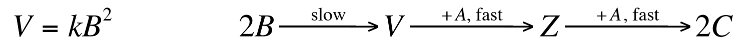


$$2A + 2B \xrightarrow{k} 2C$$

might be:

$V = kAB^2$  $A + 2B \xrightarrow{\text{slow}} X \xrightarrow{+A,\text{ fast}} Y \xrightarrow{\text{fast}} 2C$

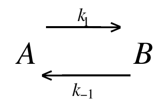
$V = kA$ $A \xrightarrow{\text{slow}} A^* \xrightarrow{+A,\text{ fast}} W \xrightarrow{+2B,\text{ fast}} 2C$

$V = kB^2$  $2B \xrightarrow{\text{slow}} V \xrightarrow{+A,\text{ fast}} Z \xrightarrow{+A,\text{ fast}} 2C$

and many other possibilities.

Simple kinetic mechanisms

Unimolecular, one-step reactions



e.g. protein folding

$$-\frac{dA}{dt} = k_1 A - k_{-1} B \qquad \text{let } \Delta A = A - A_{eq} \qquad \Delta B = B - B_{eq}$$

then

$$-\frac{d\Delta A}{dt} = k_1\left(\Delta A + A_{eq}\right) - k_{-1}\left(\Delta B + B_{eq}\right)$$

note that from the stoichiometry, you know that ΔA=-ΔB

$$-\frac{d\Delta A}{dt} = k_1\Delta A + k_1 A_{eq} + k_{-1}\Delta A - k_{-1}B_{eq} = \left(k_1 + k_{-1}\right)\Delta A + k_1 A_{eq} - k_{-1}B_{eq}$$

At equilibrium, $k_1 A_{eq} = k_{-1}B_{eq}$

$$-\frac{d\Delta A}{dt} = \left(k_1 + k_{-1}\right)\Delta A \quad \text{which then leads simply to} \quad -\int\frac{d\Delta A}{\Delta A} = \int\left(k_1 + k_{-1}\right)dt$$

finally

$$-\ln \Delta A_t + \ln \Delta A_o = \ln \frac{\Delta A_o}{\Delta A_t} = (k_1 + k_{-1})(t - 0)$$

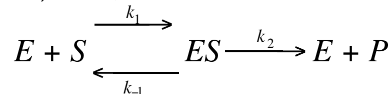
$$\Delta A_t = \Delta A_o e^{-(k_1 + k_{-1})t}$$

$$A_t = A_{eq} + (A_o - A_{eq}) e^{-(k_1 + k_{-1})t}$$

This tells us that A approaches exponentially its equilibrium value. Make sense?
A simple example of a unimolecular, one-step reaction is protein folding (see later).

Pre-equilibrium kinetics

(Leonor Michaelis and Maude Menten, 1913)



The simplest kinetic formalism falls directly from our equilibrium studies. If we assume that our reaction (k_2, above) is very slow relative to both the on (k_1) and off (k_{-1}) rates for binding of substrate to enzyme, then we can simply treat substrate binding as an equilibrium binding (using the equations above). In other words, the first step is always at equilibrium, unperturbed by the second step. The velocity of the reaction under the simplest of conditions is then V = k_2[ES], where [ES] is determined as before for ligand binding (also remember that [ES] is proportional to $\bar{v}$, so that V!=!k_2K_a[E][S].
Solve for the equilibrium in terms of $[E]_t$ and $[S]_t$

$$K_a = \frac{[ES]}{[E][S]} \qquad [E]_t = [E] + [ES] \qquad [S]_t = [S] + [ES]$$

$$K_a = \frac{[ES]}{([E]_t - [ES])([S]_t - [ES])} = \frac{x}{(E_t - x)(S_t - x)}$$

$$K_a(E_t - x)(S_t - x) = x$$

This can be solved exactly very simply, but for simplicity, *if* we can assume substrate in excess, then:

$$K_a(E_t - x)S_t = x$$

$$K_a E_t S_t = K_a x S_t + x$$

$$x = [ES] = \frac{K_a E_t S_t}{K_a S_t + 1} = \frac{E_t S_t}{S_t + 1/K_a} = \frac{E_t S_t}{S_t + K_d}$$

Finally,

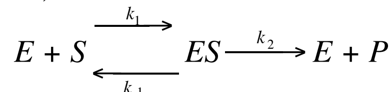
$$V = k_2[ES] = \frac{k_2 E_t S_t}{S_t + K_d}$$

Figuring that the reaction is fastest as $S_t \rightarrow \infty$, calculate V_{max} (try it!).
This looks very similar to the equation for simple ligand binding (as we would expect):

$$\bar{v} = \frac{K_a[L]}{1 + K_a[L]} = \frac{[L]}{1/K_a + [L]} = \frac{[L]}{K_d + [L]}$$

Steady state kinetics

(G. E. Briggs & J. B. S. Haldane, 1925)



If pre-equilibrium cannot be assumed, then we can take a slightly different approach

As before, we have the equations for mass conservation:

$$[E]_t = [E] + [ES] \qquad [S]_t = [S] + [ES]$$

We then *assume* that after the reaction has been running for some time, the rate for formation of [ES] equals the rate of its loss - we *assume* that [ES] has reached *steady state*:

$$-\frac{d[ES]}{dt} = 0 = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

$$(k_{-1} + k_2)[ES] = k_1[E][S]$$

$$\frac{k_1}{k_{-1} + k_2} = \frac{1}{K_m} = \frac{[ES]}{[E][S]}$$

We can see that this is just the same equation we dealt with above, except that $1/K_m$ substitutes for K_a (note that the latter is simply $1/K_d$).

It should be obvious that "pre-equilibrium" kinetics is simply a special case of the more general steady state kinetics (in pre-equilibrium kinetics, $k_{-1} >> k_2$).

The form of the solution for these reaction equations is the same as the form of the solution for the related binding equations. In the same way that we devised Scatchard and other plots for binding, we can do exactly the same for kinetic analyses. They now adopt new names.

Note that the same caveats apply as before, regarding the manipulation of raw data and the resulting effects on error analysis.

Similarly, the same kinds of analyses can be brought in to consider multiple sites, independent or dependent.

Finally, a reminder that the above analyses assume substrate in excess. For an enzymatic reaction, note that substrate is depleted with time, so that what is in excess initially may not be in excess near the end of the reaction. Also, since steady state is assumed, the equations are not valid for the very beginning of the reaction (pre-steady state kinetics).

Single Site Binding

$$\frac{\bar{v}}{[L]} = \frac{K_a}{1 + K_a[L]} = -K_a\bar{v} + K_a$$

Plot $\frac{\bar{v}}{[L]}$ vs. $\bar{v}$, slope = $-K$, y-intercept = K (Scatchard)

Single Site Kinetics

$$\frac{v}{[S]} = \frac{\frac{1}{K_m} k_2 E_t}{1 + \frac{1}{K_m}[L]} = \frac{\frac{1}{K_m} V_{\max}}{1 + \frac{1}{K_m}[L]}$$

$$\frac{v}{[S]} = -\frac{1}{K_m} v + \frac{V_{\max}}{K_m}$$

Plot $\frac{v}{[L]}$ vs. v, slope = $-1/K_m$, y-intercept = $Vmax/K_m$ (Eadie-Hofstee)

Exact solutions (numerical integration) of complex rate equations