L. RECEPTOR-LIGAND DISSOCIATION

In biology, structure is meaningless absent function. Among the most fundamental functions in biochemistry is binding. We commonly discuss **receptor-ligand** interactions. The receptor is commonly a macromolecule (protein or nucleic acid) and the ligand is typically a smaller molecular species, though it is possible for two proteins to engage in receptor-ligand interactions, so the definition of "receptor" and "ligand" is pretty vague except to say that they are two molecules that associate via **non-covalent** interactions.

Common examples of receptor-ligand complexes are:

- Drugs bound to proteins, such as AZT binding to the HIV reverse transcriptase enzyme.
- Hormones bound to hormone receptors, such as estrogen to the estrogen receptor.
- Antibody proteins binding to antigens, such as virus coat proteins.
- Protein bound to DNA, such as the lac repressor bound to its DNA operator sequence.
- Amino acids to riboswitches, small RNA sequences that fold around small molecules to regulate gene expression.


A common, yet striking feature of these interactions is the specificity with which the complexes form. The ideal drug will only bind to one target protein in the cell. If the wrong hormone binds to the wrong receptor all sorts of problems, physical and emotional, ensue. Antibodies that attack self-proteins lead to autoimmune diseases like rheumatoid arthritis. A protein that binds to the wrong piece of DNA can shut down an essential metabolic pathway, and a riboswitch that binds the wrong amino acid will cause the cell to stop making the wrong amino acid just when it's needed most.

Explaining the structural origins of receptor-ligand specificity is an on-going project of biochemistry. While we have already laid out some of the tools to describe structure, we now need to quantify specificity. That will be done using the dissociation constant, K_d.

Simple Equilibrium Binding of Receptors to Ligands

The Dissociation Constant

Receptor-ligand interactions are equilibrium phenomena. A free receptor (**R**) associates non-covalently with the free ligand (**L**) to form the receptor ligand complex (**R•L**; note that the dot, •, will commonly be used this semester to indicate a non-covalent association). Although we are primarily concerned with the formation of the R•L complex, for reasons that will become apparent, we typically discuss the interaction in terms of the dissociation of the complex:
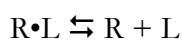
$$R•L \rightleftarrows R + L$$

Accordingly, the following equilibrium constant holds.

$$K_d = \frac{[R][L]}{[R \bullet L]}$$ (Eq. L.1)

Where $\mathbf{K_d}$ is the so-called "dissociation constant", reflecting the degree of dissociation of the receptor and ligand from each other.

The numerical values of dissociation constants are typically given in units of concentration (though Dan Gerrity will tell you, quite rightly, that equilibrium constants are unitless).[1] The reason for this convenience will appear shortly, but for now it's worth noting that in biology, the value of K_d can vary dramatically from one receptor ligand pairing to another. Typical values range from 10^{-15} M to 10^{-6} M, which reflect free energies of dissociation (ΔG_{dissoc}) from +21 kcal/mol (positive and unfavorable) to +8 kcal/mol (still unfavorable, but not as much so). However, binding can be as tight as to give zeptomolar K_d values (10^{-21} M) and weak enough to be in the millimolar range (10^{-3} M).

Graphical Determination of the Dissociation Constant

The most common means of determining the K_d for a given R•L complex is to vary the concentration of ligand in a solution containing a fixed, low concentration of the receptor.[2] At each concentration of ligand, the fraction of bound receptor is measured. That fraction (Y) is represented as:

$$Y = \frac{[R \bullet L]}{[R]_{total}}$$ (Eq. L.2)

Where $[R]_{total}$ is the total amount of receptor present in the assay – typically at much lower concentrations than the total amount ligand. Note that the total amount of receptor can be expressed as the sum of the concentrations of free ([R]) and bound ([R•L) receptor.

$$Y = \frac{[R \bullet L]}{[R]_{total}} = \frac{[R \bullet L]}{[R] + [R \bullet L]}$$ (Eq. L.3)

The algebraic expansion in equation L.3 is the most important algebraic trick to learn in biochemistry. Almost every derivation we will do this semester starts with a fraction of some form of a biological molecule divided by all forms present. The first step is always to expand the total amount into all those forms. From here we proceed to substitute for [R•L], using equation L.2, to obtain the fraction of bound receptor as a function of free ligand concentration.

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[1] For details on why that is the case, see Appendix L1.

[2] The low concentration of receptor is key to a simplifying assumption that the concentration of free ligand is equal to the total concentration of added ligand. That is, the amount of ligand actually bound to the receptor should be negligible in comparison to the total in solution. See Appendix L.2 for the algebra when that isn't true.

$$Y = \frac{[R \cdot L]}{[R] + [R \cdot L]} = \frac{[R][L]/K_d}{[R] + [R][L]/K_d} \qquad \text{(Eq. L.4)}$$

Note that in equation L.4, the numerator and denominator are both multiples of [R], which may be factored out.

$$Y = \frac{[R][L]/K_d}{[R] + [R][L]/K_d} = \frac{[R][L]/K_d}{[R]\left(1 + [L]/K_d\right)} = \frac{[L]/K_d}{1 + [L]/K_d} \qquad \text{(Eq. L.5)}$$

When both numerator and denominator are multiplied by K_d, the following form of the equation is obtained.

$$Y = \frac{[L]/K_d}{1 + [L]/K_d} \cdot \frac{K_d}{K_d} = \frac{[L]}{K_d + [L]} \qquad \text{(Eq. L.6)}$$

Equation L.6 allows Y to range from a value of zero (when the concentration of free ligand is zero) to one, but that is only approached at very, very high concentrations of ligand (Figure L.1). Of particular interest is that when [L] is equal to K_d, the fraction of bound receptor is ½.

$$Y = \frac{[L]}{K_d + [L]} = \frac{K_d}{K_d + K_d} = 0.5 \qquad \text{(Eq. L.7)}$$

K_d can therefore be interpreted as *the ligand concentration that leads to 50% occupancy of the receptor's binding site.* The lower the value of K_d, the less ligand required to achieve 50% occupancy, indicating a higher affinity between receptor and ligand.
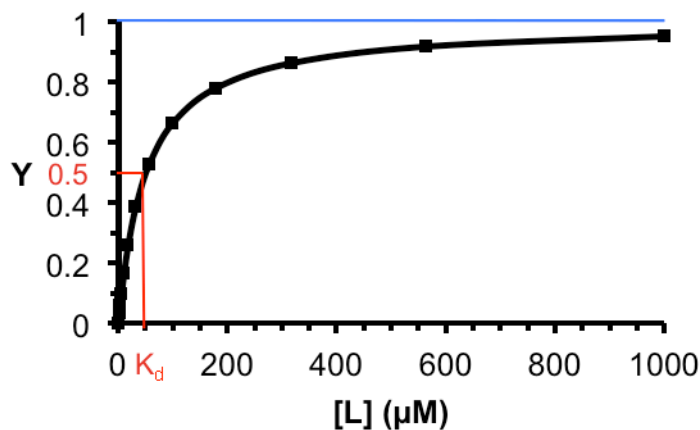
Figure L.1. Plot of fraction bound receptor (Y) vs. the concentration of ligand. Note
that the K_d can be identified from this plot as the concentration of ligand that yields
50% bound receptor. Note also that even at ligand concentrations 20-fold higher
than the K_d, only 95% of the receptor is bound.

Equation L.6 provides an algebraic relationship between Y and [L] that is often visualized graphically
(Figure L.1). The plot of Y vs. [L] yields a rectangular hyperbola that asymptotically approaches a
value of 1 as the concentration of L increases. From a visual perspective, it's worth noting that the
approach to the asymptote is somewhat slower than one might guess. In Figure L.6, the K_d can be
identified as the [L] that gives 50% bound receptor, or 50 µM L in this instance. At 500 µM L, the
fraction of bound receptor is only about 90% of the total (prove this to yourself by some simple
arithmetic) and even at 20-fold excess L above the K_d, 95% of the receptor is bound. Because of
the large changes in bound fraction taking place at low concentrations of L and the slow, dreary rise
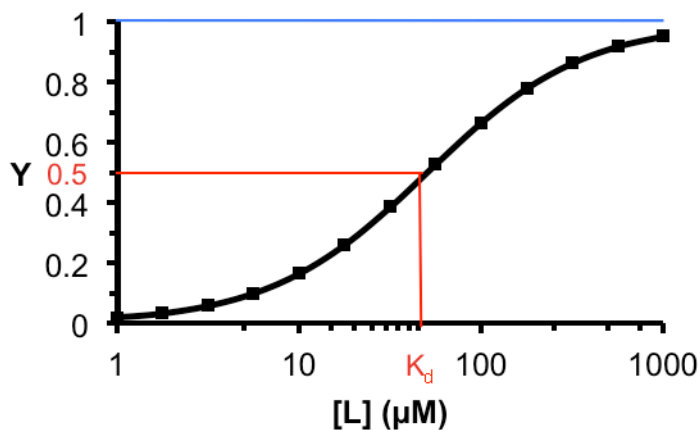after K_d, it is common to find these plots depicted with a logarithmic x-axis (Figure L.2).

Figure L.2. Plot of fraction bound receptor (Y) vs. the concentration of ligand, with
logarithmic x-axis. Note that the K_d can be identified from this plot as the
concentration of ligand that yields 50% bound receptor, which also happens to occur
at the inflection point. This depiction of the data provides a better view of the curve
fit at low and high ligand concentration.

Free Energy of Dissociation

As noted above, the dissociation constant provides a convenient measure of affinity. The lower the
value of the equilibrium constant, the tighter the binding. Importantly, equilibrium constants can be
linked to thermodynamics via the equation:

$$\Delta G_{diss}° = -RTln(K_d) \qquad\qquad\qquad (Eq.\ L.8)$$

The free energy of dissociation takes the equilibrium constant into the land of kcal/mol (or kJ/mol
if you must) and allows simple thermodynamic comparisons between different ligands or different
receptors. One **important caveat** to this equation is the assumption that all equilibrium constants

must be in **units of Molar** (or bar), since the standard state assumes all solutes to be at 1 M concentration (or 1 bar pressure). In the example above, where the K_d is 50 μM, it must be expressed as 50 x 10^{-6} M before use in Eq. L.8:

$$\Delta G_{diss}° = = -(0.001987\ ^{kcal}/_{molK})(298\ K)(-9.9) = +5.9\ ^{kcal}/_{mol} \qquad (Eq.\ L.9)$$

Note that the positive change in free energy is sensible, since the dissociation constant is less than one; dissociation is non-spontaneous at the standard state.

Cooperative Binding of Ligands

Figure L.3 Schematic representation of cooperative binding. A dimeric receptor in a low affinity state (also called the "tense" state) binds a ligand (green triangle) and changes conformation to the "relaxed" state, which has higher affinity for a second ligand.

In most instances, the simple binding model for receptor-ligand interactions works fine. However, more complex patterns of binding exist. For example, some receptors are multimeric – dimers, trimers, tetramers, etc. While each subunit of the receptor may individually bind one ligand molecule, it is possible for the binding of the first ligand to the first subunit to affect binding of subsequence ligands to other subunits in the complex. Figure L.3 illustrates the phenomenon, called **cooperative binding**. In this instance, there is **positive cooperativity**, since the second binding event is more favorable than the first. Note that this is due to **allostery** (Greek for "other place"); a ligand binding event changes the structure of a remote part of the receptor.
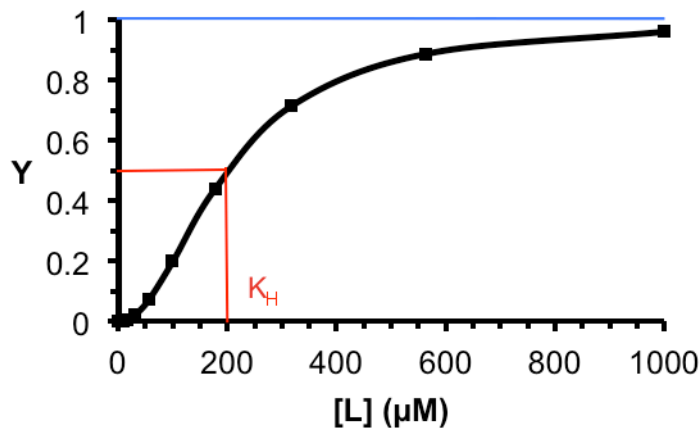
Figure L.4 Graphical representation of ligand binding to a receptor with positive cooperativity. Note the sigmoidal shape of the plot, even with a linear x-axis. Affinity is low initially, but rises sharply as some of the dimeric receptor becomes bound to the first ligand and changes to the *relaxed* state, with greater affinity for the ligand. K_H is the Hill coefficient (see below); 200 µM in this case.

From a graphical perspective, cooperative binding is in evidence when a plot of fraction of bound receptor (Y) vs. ligand concentration gives a sigmoidal plot. Note that the x-axis is linear in Figure L.4; the weak binding observed at low [L] gradually accelerates at moderate concentrations. This is **positive cooperativity**, where receptor multimers bind ligand weakly at first, but as bound subunits come to exist, subsequent binding events occur with higher affinity. On the other hand, if binding to the second subunit is made less favorable, that's **negative cooperativity**. If there is no effect; if each subunit acts independently, then no cooperativity is observed.

There are a few common ways of treating cooperativity algebraically. Two of them are discussed below.

The Hill Coefficient

The first treatment of cooperativity is simplistic, but powerful – it assigns a single value to the degree of cooperativity being observed, called the **Hill coefficient, n**. The Hill coefficient is an imaginary value that sort of describes the number of ligands that simultaneously bind to a multimeric receptor:

$$R + n\,L \Leftrightarrow R{\bullet}L_n \qquad\qquad\qquad\qquad\text{(Eq. L.***)}$$

This value is essentially an abstraction that helps fit the curve. Imagine a <u>dimeric receptor</u> – there are three general scenarios that could arise:

•   Positive cooperativity, n > 1. The affinity of the second ligand for the dimer is greater than the first. It will bind more readily. In the extreme case, both ligands appear to bind simultaneously, since the affinity of the second is so great. In that extreme case, n=2.

•   No cooperativity, n = 1. The affinity of the second ligand is the same as the first. In that case, the two subunits essentially behave independently and can be treated by the simple model described in Figure L.1 and Equations L.1 and L.6.

•   Negative cooperativity, n < 1. The affinity of the second ligand is less than that of the first. To determine the value of the Hill coefficient, one simply uses a modified form of equation L.6, in which K_H, the Hill constant, replaces K_d, the dissociation constant. As with K_d, K_H is the concentration of ligand that gives half-maximal binding of the receptor.

$$Y = \frac{[L]^n}{\left(K_H\right)^n + [L]^n} \qquad\qquad\qquad\qquad\text{(Eq. L.***)}$$
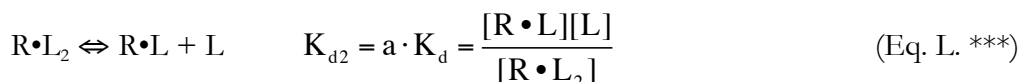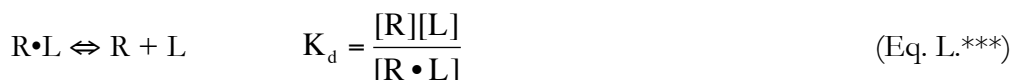
The Hill coefficient, n, is a way of roughly measuring how much the binding of one ligand influences binding of another. In a multimeric protein, the maximum value of n is equal to the

number of subunits present. In a dimer, the maximum Hill coefficient is two – in a tetramer, it is four. Etc.

If one has a dimeric receptor with a Hill coefficient of 1.5, then that means there is significant increase in affinity for the second ligand, but not enough to guarantee that the second equivalent will bind immediately.

The KNF Model

Daniel Koshland, in collaboration with George Nemethy and D. Filmer, developed an algebraic model for how ligand binding can allosterically affect the behavior of complex receptors (and enzymes). Their model describes the modulation of affinity at some receptor binding site by ligand binding to a more remote site.[3] Imagine, again, our dimeric receptor. The assumption is that the dissociation constant of the second ligand is different from the first.

$$R \bullet L \Leftrightarrow R + L \qquad K_d = \frac{[R][L]}{[R \bullet L]} \qquad\qquad \text{(Eq. L.***)}$$

$$R \bullet L_2 \Leftrightarrow R \bullet L + L \qquad K_{d2} = a \cdot K_d = \frac{[R \bullet L][L]}{[R \bullet L_2]} \qquad\qquad \text{(Eq. L. ***)}$$

Note that the second dissociation constant can easily be related to the other by a constant factor, **a**. When a is less than one, the binding of the second equivalent is tighter than the first. If a is greater than one, binding of the 2nd equivalent is weaker. The nice thing about "a" is that it has physical meaning where "n" from the Hill analysis is a little make-believe.

The algebraic analysis by the KNF model is relatively simple, but it needs to be done with an eye to the number of subunits in the receptor. In this case, I will do it for a dimer. The derivation starts just like it did in Eq. L.2:

$$Y = \frac{2[R \bullet L] + [R \bullet L_2]}{[R]_{total}} = \frac{2[R \bullet L] + [R \bullet L_2]}{[R] + 2[R \bullet L] + [R \bullet L_2]} \qquad\qquad \text{(Eq. L.***)}$$

The one wrinkle here is that the definition of the fraction of bound receptor is altered by the fact that there are different states of the receptor that may be considered "bound". For example, if we labeled the two subunits A and B, we have two possible singly bound states, $R \bullet L_A$ and $R \bullet L_B$, where the subscript ID's the subunit in which the ligand is bound. Since they are chemically indistinguishable, the concentrations are lumped together as 2[R•L]. Of course there is only one way to have an empty receptor, R, and completely occupied receptor, $R \bullet L_2$, so in Eq. L.*** they appear in multiples of one only. The remainder of the derivation is pretty straightforward.

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[3] I'm being a little simplistic in this section. KNF deals with a variety of scenarios that I don't feel like covering.

$$Y = \frac{\dfrac{2[R][L]}{K_d} + \dfrac{[R \bullet L][L]}{a \cdot K_d}}{[R] + \dfrac{2[R][L]}{K_d} + \dfrac{[R \bullet L][L]}{a \cdot K_d}} = \frac{\dfrac{2[R][L]}{K_d} + \dfrac{[R][L]^2}{a \cdot K_d^2}}{[R] + \dfrac{2[R][L]}{K_d} + \dfrac{[R][L]^2}{a \cdot K_d^2}} = \frac{\dfrac{2[L]}{K_d} + \dfrac{[L]^2}{a \cdot K_d^2}}{1 + \dfrac{2[L]}{K_d} + \dfrac{[L]^2}{a \cdot K_d^2}}$$

(Eq. L.*)

It's not easy to see what concentration of ligand gives a Y of 1/2, but the nice thing is that it gives discrete values of dissociation constants for each of the two dissociation events.

Appendix L.1 - Units in the Equilibrium Constant

The Physcial Chemist's Perspective

Strictly speaking, equilibrium constants are unitless. That is because they are always referenced to a **standard state** in which (a) liquids and solids are pure, (b) gasses are at 1 bar pressure and (c) solutes are at 1 M concentration.

When we write equilibrium expressions, that truth is generally ignored, but a physical chemist would insist that the correct way to write the dissociation equilibrium constant (K_d) of a ligand from a receptor is as follows:

$$R \cdot L \Leftrightarrow R + L \qquad K_d = \frac{\gamma_R \left({[R]_{eq}} \big/ {[R]_{ss}} \right) \cdot \gamma_L \left({[L]_{eq}} \big/ {[L]_{ss}} \right)}{\gamma_{RL} \left({[RL]_{eq}} \big/ {[RL]_{ss}} \right)}$$

Where γ_X is the unitless activity coefficient for species X, $[X]_{eq}$ is its equilibrium concentration (in Molar) and $[X]_{ss}$ is its standard state concentration (1 M). In dilute solutions, γ_X is close to one, and the standard state concentrations are one (molar), so the only numerical value that really matters is the equilibrium concentration, but because each species is present as a ratio of concentrations, the overall value of K_d is unitless, but its value is normally defined from concentrations calculated in units of Molar, and that matters, as we'll see.

The Biochemist's Perspective

Biochemists write equilibrium constants with units attached because we are calculating them using an incorrect equation for the equilibrium constant:

$$R \cdot L \Leftrightarrow R + L \qquad K_d = \frac{[R]_{eq}[L]_{eq}}{[RL]_{eq}}$$

This presents a problem when it comes time to calculate $\Delta G°$ from K_d because now the equilibrium constant has units and you can't take the logarithm of values with units:

$$\Delta G° = -RT\ln(K_d)$$

Moreover, we often don't calculate the equilibrium constant from concentrations expressed in Molar. Sometimes it is μM, sometimes nM, etc. So to make yourself right with the physical chemistry community, before calculating $\Delta G°$, recalculate K_d in units of Molar and then remember that the true formula for K_d would have those units cancel out. Now you're ready to calculate $\Delta G°$.

Appendix L.2 – When $[R]_{tot}$ is close to K_d

The algebra described for simple ligand binding in equations L.1-L.6 assumes that the concentration of receptor is vanishingly small in comparison to the amount of ligand present. In that way, it takes a very small fraction of the ligand present in the test tube to bind the receptor completely. For example, if there is 1 nM receptor and the K_d of the R•L complex is 50 μM, then the concentration of free ligand is:

$$[L] = 50\ \mu M - \tfrac{1}{2}(1\ nM) \approx 50\ \mu M \qquad\qquad (Eq.\ L.A1)$$

Thus the amount of the total ligand is roughly the same as the amount of free ligand, and we can use equation L.6 without jeopardy.

In some instances, however, the receptor is present at concentrations near K_d. For example, imagine a system in which the total concentration of the receptor is 1 nM and the K_d is 2 nM. When 50% of the receptor is bound there is 2 nM of free ligand in the solution and 0.5 nM of the bound ligand, meaning that the total amount of ligand in solution is 2.5 nM. Thus the total ligand concentration that gives 50% bound receptor is *not the K_d*. We can no longer make the assumption that total ligand concentration and free ligand concentration are the same. The following algebra fixes that.

$$K_d = \frac{[R][L]}{[R\bullet L]} = \frac{\left([R]_{tot} - [R\bullet L]\right)\left([L]_{tot} - [R\bullet L]\right)}{[R\bullet L]}$$

$$K_d = \frac{[R]_{tot}\cdot[L]_{tot} - \left([L]_{tot} + [R]_{tot}\right)[R\bullet L] + [R\bullet L]^2}{[R\bullet L]}$$

$$K_d[R\bullet L] = [R]_{tot}\cdot[L]_{tot} - \left([L]_{tot} + [R]_{tot}\right)[R\bullet L] + [R\bullet L]^2$$

$$0 = [R]_{tot}\cdot[L]_{tot} - \left([L]_{tot} + [R]_{tot} + K_d\right)[R\bullet L] + [R\bullet L]^2$$

The last equation is a second order polynomial in which one can solve for "x", [R•L], using the quadratic equation.

$$[R\bullet L] = \frac{\left([L]_{tot} + [R]_{tot} + K_d\right) - \sqrt{\left([L]_{tot} + [R]_{tot} + K_d\right)^2 - 4\cdot1\cdot[R]_{tot}\cdot[L]_{tot}}}{2\cdot1}$$

And then we can get Y in a quick little bit of division!

$$Y = \frac{[R\bullet L]}{[R]_{tot}} = \frac{\left([L]_{tot} + [R]_{tot} + K_d\right) - \sqrt{\left([L]_{tot} + [R]_{tot} + K_d\right)^2 - 4\cdot[R]_{tot}\cdot[L]_{tot}}}{2\cdot[R]_{tot}}$$

So, when to use it? As a rule, any time K_d is less than ten times the total receptor concentration.