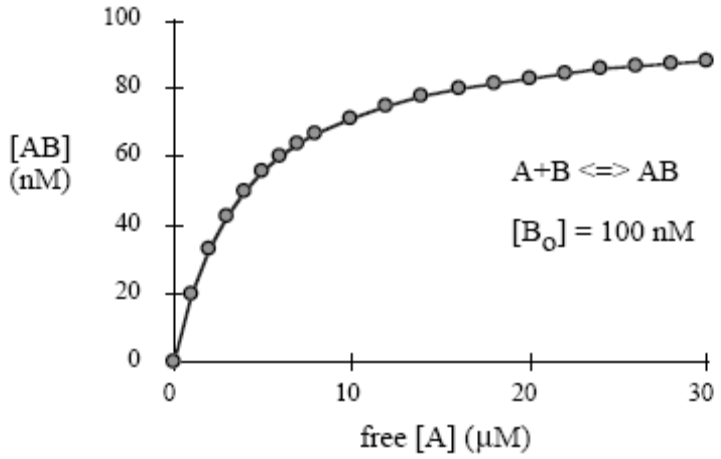
Review of Chemical Equilibrium

Equilibrium experiments study how the concentration of reaction products change as a function of reactant concentrations and/or reaction conditions. For a typical bimolecular equilibrium reaction such as A+B ⇔ AB, increasing amounts of reactant [A] might be titrated against a fixed amount of the reactant [B] and the equilibrium concentration of the product [AB] determined.



The shape of the equilibrium curve depends upon the reaction mechanism and can be used to decide between different equilibrium models.

Equilibrium constants

An **equilibrium constant**, designated by a upper case K, is the ratio of the **equilibrium concentrations** of reaction products to reactants or vice versa.

For the bimolecular reaction, A+B ⇔ AB, we can define an **equilibrium dissociation constant** (K_d) or an **equilibrium association constant** (K_a), which are reciprocally related, as shown below:

$$K_d = \frac{[A][B]}{[AB]} \qquad K_a = \frac{[AB]}{[A][B]}$$

For bimolecular reactions, the units of K_d are concentration (M, mM, μM, etc.) and the units of K_a are concentration $^{-1}$ (M^{-1}, mM^{-1}, μM^{-1}, etc.).

For a unimolecular protein folding reaction, U ⇔ N, we can define an **equilibrium unfolding constant** (K_u) or an **equilibrium folding constant** (K_f), which are reciprocally related:

$$K_u = \frac{[U]}{[N]} \qquad K_f = \frac{[N]}{[U]}$$

These equilibrium constants, like all those for unimolecular reactions, are unit less.

For any equilibrium expression, the direction of the reaction (i.e., dissociation vs. association; folding vs. unfolding) is defined by going from the molecular species on the bottom of the right side of the expression to those on the top.

The equilibrium constants for a reaction such as nA + mB ⇔ A_nB_m are:

$$K_d = \frac{[A]^n[B]^m}{[A_nB_m]} \qquad K_a = \frac{[A_nB_m]}{[A]^n[B]^m}$$

The value of any equilibrium constant will be c onstant only for a given temperature, pressure, etc. Thus, the equilibrium constants for the same reaction at different temperatures (e.g., 20 °C vs. 37 °C) could be very different.

Why reactions come to equilibrium

Irrespective of mechanism, all reversible reactions reach an equilibrium distribution of reactants and products when the rates of the forward and back reactions become equal. Consider the overall rate at which [AB] changes for the reaction A+B ⇔ AB.

$$d[AB]/dt = k_{assn}[A][B] - k_{diss}[AB]$$

If we initiated the reaction by mixing free A and free B, then the association rate ($k_{assn}[A][B]$) would dominate the reaction and the dissociation rate ($-k_{diss}[AB]$) would be small because there would be very little AB complex. As more complexes formed, however, the association rate would begin to decrease and the dissociation rate would increase because the concentrations of [A] and [B] would decrease and that of [AB] would increase. At some point the rates of the opposing reactions would become equal and the there would no longer be any change in the concentrations of [AB], [A], and [B].

$$d[AB]/dt = -d[A]/dt = - d[B]/dt = k_{assn}[A][B] - k_{diss}[AB] = 0$$

Under these conditions

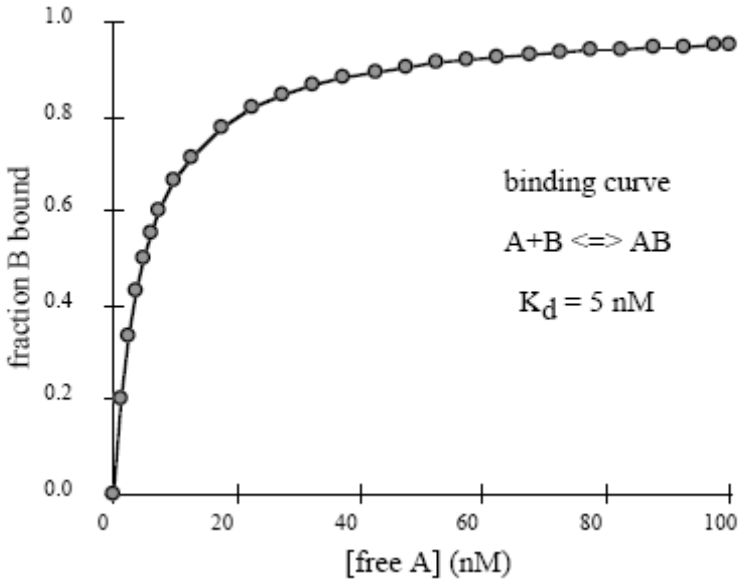
$$\frac{[A][B]}{[AB]} = \frac{k_{diss}}{k_{assn}} = \mathbf{K_d}$$

This expression shows that the equilibrium concentrations of reactants and products will have a constant ratio (K_d) that is equal to the ratio of the reverse and forward rate constants. Kd is called an equilibrium dissociation constant. The equilibrium concentrations of reactants and products could also be characterized by an equilibrium association constant (K_a) which is simply the reciprocal of K_d.

Determining Kd or Ka for bimolecular reactions

To study a bimolecular equilibrium reaction (A+B ⇔ AB) experimentally, one would start by mixing free A and free B, or alternatively by diluting the AB complex, and then waiting until there was no further change in the concentrations of [A], [B], and [AB]. The ratio of the equilibrium concentrations as shown above then determines the value of K_d or K_a. In practice, equilibrium experiments are performed using many different initial concentrations to ensure that the equilibrium model is correct and thus that the same value of K_d or K_a is measured irrespective of the initial concentrations. Typically, one fixes the initial concentration of one reactant (e.g., $[B_0]$) and then, in different experiments, adds increasing initial concentrations of the other reaction $[A_0]$. For each set of concentrations, one waits until equilibrium is reached and then assays [A], [B], and [AB]. There are three common ways to plot equilibrium data of this kind.

The first is a simple binding curve in which the fraction of B bound is plotted vs. the concentration of free [A]



The mathematical relationship between the fraction of B bound and the free concentration of [A] is straightforward.

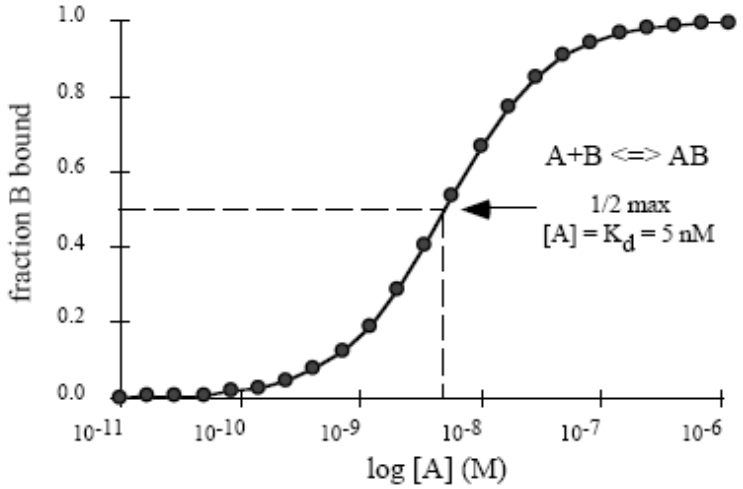
$$\text{fraction B bound} = \frac{[AB]}{[B]+[AB]} = \frac{1}{\frac{[B]}{[AB]} + 1} = \frac{1}{\frac{K_d}{[A]} + 1} = \frac{[A]}{K_d + [A]}$$

This is an equation for a rectangular hyperbola and bimolecular binding curves are often referred to as hyperbolic binding curves.

The fraction of B bound is often designated Θ_b, so that

$$\Theta_b = \frac{[A]}{K_d + [A]}$$

It's easy to see from this expression that if [A] = K_d, then $\Theta_b = 0.5$. Thus, half-maximal binding of B occurs when the free A concentration is equal to K_d. This makes it simple to estimate K_d simply by inspection of the binding curve. The same binding data is shown below but plotted against log [A].



For any bimolecular reaction, 10% of B will be bound when [A] = Kd/9 and 90% of B will be bound when [A] = 9Kd. Thus, 80% of the binding reaction occurs over a concentration range of about 80-fold in [A] centered around the K_d.

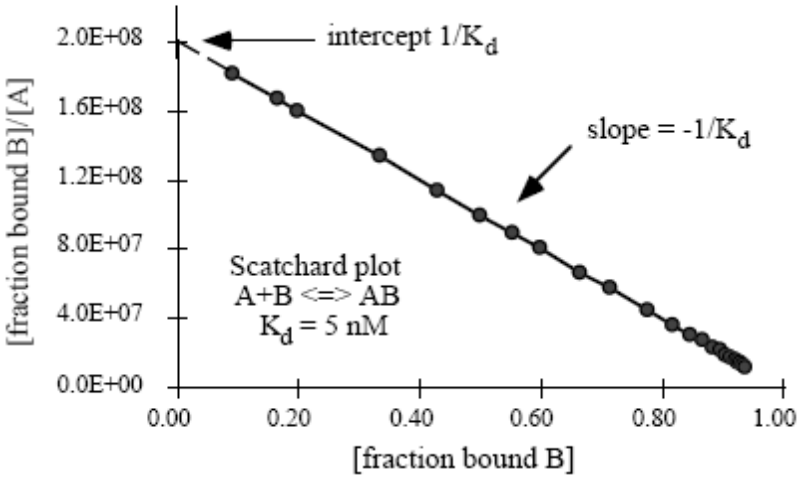
$$\Theta_b = \frac{[A]}{K_d + [A]} \quad \text{rearranges to} \quad \Theta_b K_d + \Theta_b[A] = [A]$$

$$\Theta_b K_d/[A] + \Theta_b = 1$$

$$\Theta_b K_d/[A] = 1 - \Theta_b$$

$$\frac{\Theta_b}{[A]} = -\frac{\Theta_b}{K_d} + \frac{1}{K_d}$$

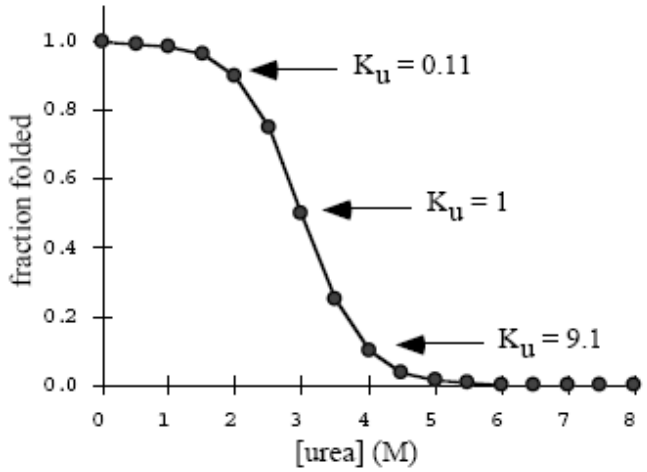
Thus, plotting $\Theta_b/[A]$ as a function of Θ_b should give a straight line with a slope of $-1/K_d$. The Scatchard plot shown below is for the same data plotted in the binding curves above.



Hyperbolic binding curves and linear Scatchard plots are diagnostic of simple bimolecular reactions. Later on, we'll show that higher-order reactions such as 2A+B $\Leftrightarrow$ A_2B and 3A+B $\Leftrightarrow$ A_3B give Scatchard plots that are concave downward and binding curves with sigmoid shapes.

Unimolecular equilibria

Equilibrium reactions, such as protein folding, that involve a conformational change in a single molecule are generally studied by determining the concentrations of reactant and product as a function of some environmental change that perturbs the equilibrium. Perturbants might include pH, temperature, pressure, chemical denaturants, etc. In the example shown below, urea is used as a denaturant to study the unfolding of a monomeric protein.



$$N \rightleftharpoons U$$

$$K_u = \frac{[U]}{[N]}$$

Here, equilibrium constants (K_u) for protein unfolding can be calculated for each of the urea concentrations from roughly 2 to 4.5 M where appreciable concentrations of both folded and unfold protein are present. For example, at 3 M urea, there are equal amounts of native and denatured protein and thus $K_u = 1$. To calculate K_u in the absence of urea or at 8 M urea, however, we would need a model for how K_u changed as a function of [urea]. Generally, it is found that ln K_u varies linearly with [urea] concentration, allowing determination of K_u in the absence of urea.

Common problems in measuring equilibrium constants

There are several common mistakes that can lead to the calculation of incorrect equilibrium constants.

(1) Using concentrations of species that are not equilibrium concentrations. Any equilibrium experiment has a kinetic component. One waits a certain amount of time after initiating the reaction before assaying products. If this time is too short, the concentrations of the reactants and products may still be changing. A good test for whether a reaction is at equilibrium is to see if the same final state is reached irrespective of whether the reaction is started by adding reactants or products.

(2) Using **total** concentrations not **free** concentrations in the equilibrium expression.

(3) Using the wrong equilibrium model. The ratio [A][B]/[AB] will only be constant at equilibrium for the model A+B $\Leftrightarrow$ AB.

Reactions involving changes in oligomeric form

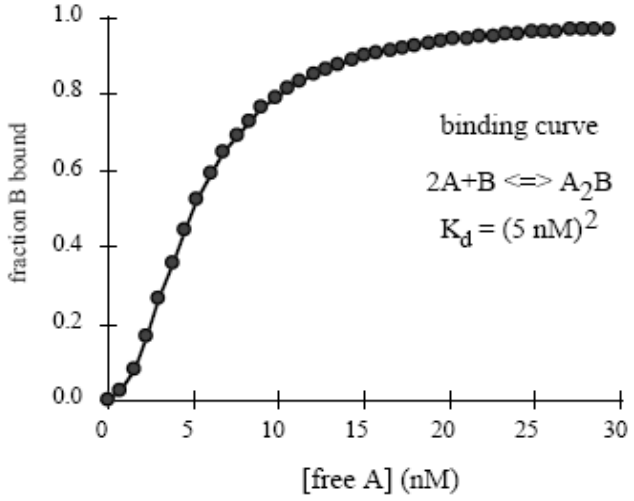
Oligomeric proteins are very common in biology. Consider a reaction in which two molecules of free A combine with B to form an A_2B complex without detectable intermediates.

$$2A + B \Leftrightarrow A_2B \qquad\qquad K_d = [A]^2[B]/[A_2B]$$

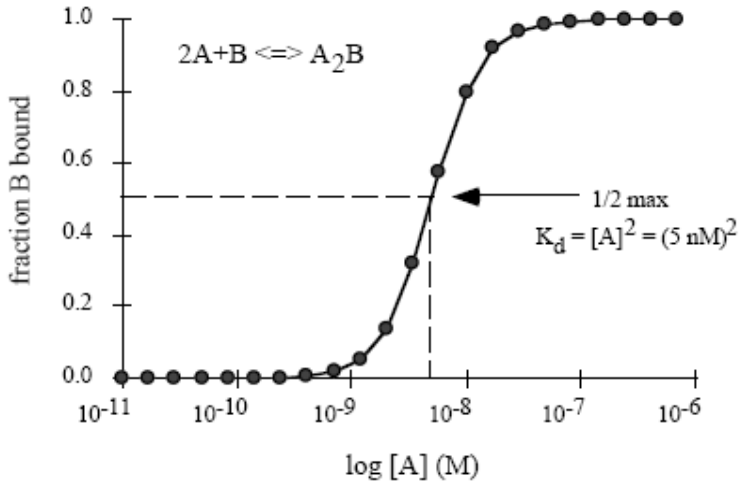
To measure K_d, which has units of M^2 for this reaction, we titrate increasing concentrations of [A] against a fixed quantity of [B] and assay the fraction of bound B. The equation relating fraction B bound to [A] for this equilibrium model is:

$$\text{fraction B bound} = \frac{[A_2B]}{[B]+[A_2B]} = \frac{1}{\frac{[B]}{[A_2B]}+1} = \frac{1}{\frac{K_d}{[A]^2}+1} = \frac{[A]^2}{K_d+[A]^2}$$

The binding curve for this equilibrium model has a sigmoidal or S-shape as shown below.

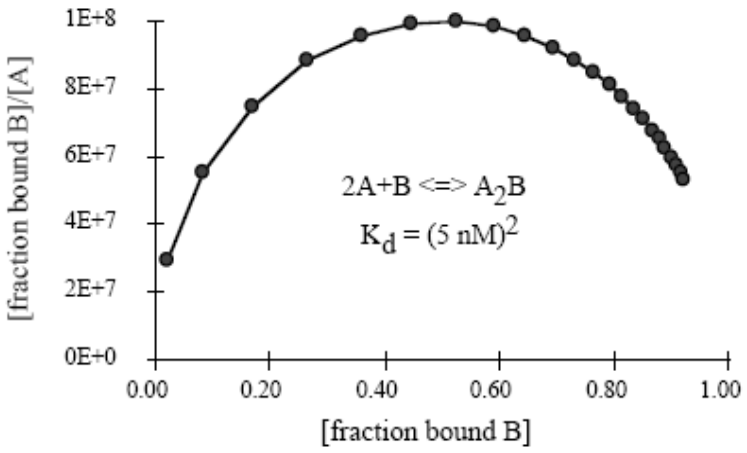


Notice also that $K_d = [A]^2$ when half of the available B is bound. Plotting this data on a log scale emphasizes that the binding curve is steeper than for the simple A+B ⇔ AB case. Now 90% of the binding reaction occurs within a 9-fold concentration range of [A] centered around half-maximal binding.



The Scatchard plot for this reaction is concave downward, which is diagnostic of a reaction showing positive cooperativity[1]. In this case, the cooperativity arises because A dimerizes in the bound A_2B complex.

[1] For a reaction such as 2A+B ⇔ A_2B, positive cooperativity means that potential intermediates in the reaction (e.g., A_2 or AB) are poorly populated relative to the end states at equilibrium.



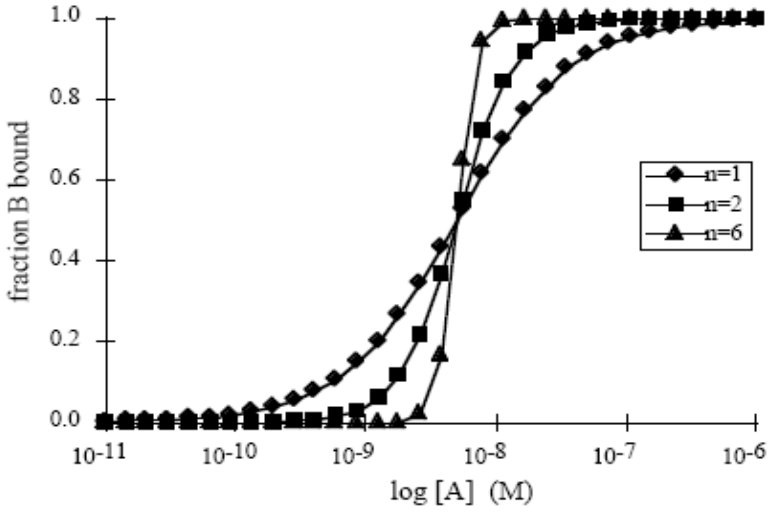
For the general case, where n molecules of A combine with one molecule of B to form a complex:

$$nA + B \Leftrightarrow A_nB \qquad K_d = [A]^n[B]/[A_nB]$$

If intermediates are not populated, then:

$$\text{fraction B bound} = \frac{[A]^n}{K_d + [A]^n}$$

Thus, for a fully cooperative reaction, as the number of subunits (n) increases, the plot of fraction B bound vs. [A] becomes steeper and steeper. The graph below shows plots for n=1, n=2, and n=6 subunits.



Binding in cooperative systems with large changes in oligomeric state can be extraordinarily sensitive to small changes in ligand concentration.

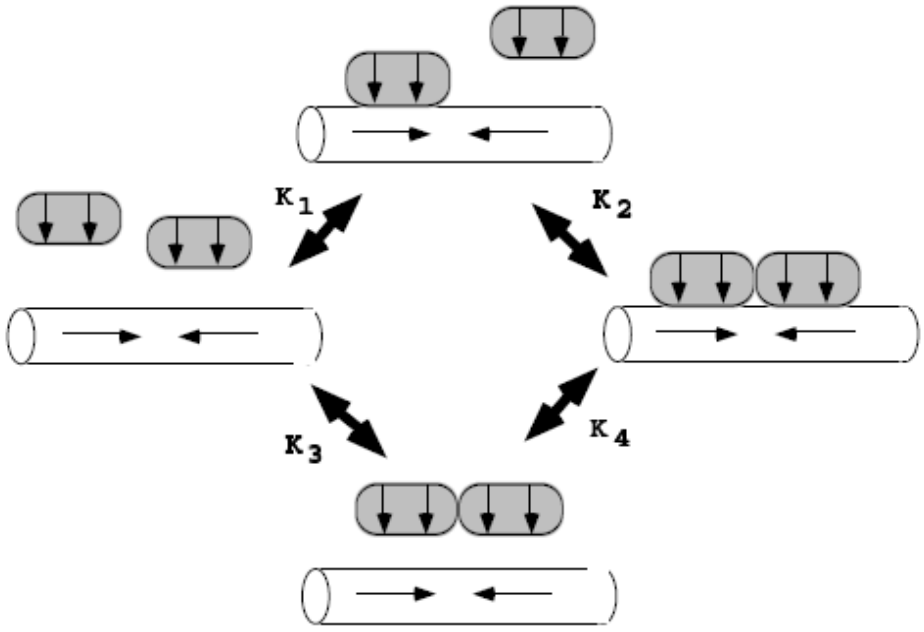
Intermediates in Equilibrium Reactions

Complexes containing three molecules or more are unlikely to form in a single step in which all of the molecules collide simultaneously. Such reactions are much more likely to proceed by successive bimolecular reactions. For the $2A+B \Leftrightarrow A_2B$ reaction, A might dimerize first and then bind to B. Alternatively, a single molecule of A could bind to B in reactions would be written as:

$$2A + B \Leftrightarrow A_2 + B \Leftrightarrow A_2B$$

$$2A + B \Leftrightarrow AB + B \Leftrightarrow A_2B$$

and are shown schematically for a DNA binding protein in the diagram below.



Either assembly pathway consists of two **coupled** equilibria. In any set of coupled reactions, one step affects the adjacent step only by changing the concentration of a common participant through mass action. If we consider the top K_1K_2 pathway, then the coupled reactions are:

$$2A + B \Leftrightarrow \mathbf{AB} + A \qquad K_1 = [A][B]/[\mathbf{AB}]$$

$$\mathbf{AB} + A \Leftrightarrow A_2B \qquad K_2 = [\mathbf{AB}][A]/[A_2B]$$

AB is the common species in the K_1 and K_2 equilibria. Thus, increasing the concentrations of free [A] or [B] in the K_1 reaction would lead to an increased concentration of [**AB**]. This, in turn, would result in a higher concentration of A_2B in the K_2 reaction. As shown below, $\mathbf{A_2}$ is the common molecular species in the K_3 and K_4 reactions.

$$2A + B \Leftrightarrow \mathbf{A_2} + B \qquad K_3 = [A]2/[\mathbf{A_2}]$$

$$\mathbf{A_2} + B \Leftrightarrow A_2B \qquad K_4 = [\mathbf{A_2}][B]/A_2B]$$

Notice that product of the equilibrium constants for the K_1 and K_2 steps is equal to the product of the equilibrium constants for the K_3 and K_4 steps and that both products give the equilibrium constant for the overall reaction.

$$K_1K_2 = K_3K_4 = K_d = [A]^2[B]/[A_2B]$$

This is always true in coupled equilibria because the concentration of the common species drops out when the equilibrium constants for each step are multiplied.

In coupled equilibrium reactions, one question is whether the intermediate species will be significantly populated relative to the end states. The answer will depend on the relative values of the equilibrium constants for each step. If both steps are bimolecular, then intermediate species would not be expected to be significantly populated if the first equilibrium dissociation constant is significantly larger than the second equilibrium dissociation constant and vice versa.

For the case described above, assume that the reaction proceeds by the K_1K_2 pathway with $K_1 = 5 \bullet 10^{-7}$ M and $K_2 = 5 \bullet 10^{-11}$ M. Thus, binding of the second A is much stronger than binding of the first. Intuitively, any concentration of [A] where [AB] would be expected to form would be far in excess of the concentration required for binding of the second A. Thus, the [AB] intermediate would not be expected to be significantly populated. We can also show this mathematically. When $[A] = 5 \bullet 10^{-9}$ M,

$$[AB] = [A][B]/K_1 = 0.01 \bullet [B] \textbf{ and } [A_2B] = [A]^2[B]/K_1K_2 = [B]$$

Thus, the concentration of [AB] will only be 1% of the concentration of either $[A_2B]$ or [B] and this intermediate is poorly populated relative to the end states.

When intermediates are present at <u>low</u> levels compared to end-states, they can effectively be ignored for purposes of calculating the equilibrium constant for the overall reaction. In the case discussed, this is equivalent to making the approximations:

$$[B]_{total} = [B] + [AB] + [A_2B] \approx [B] + [A_2B]$$

$$[A]_{total} = [A] + [AB] + 2[A_2B] \approx [A] + 2[A_2B]$$

which is clearly justified if $K_1 >> K_2$

What would happen if $K_1 = K_2 = 5 \bullet 10^{-9}$ M. Now, when $[A] = 5 \bullet 10^{-9}$ M,

$$[AB] = [A][B]/K_1 = [B] \textbf{ and } [A_2B] = [A]^2[B]/K_1K_2 = [B]$$

[AB] and $[A_2B]$ are now present at the same concentration and the presence of the intermediate could not be ignored in equilibrium calculations. In such a case, if one had an assay that could distinguish AB from A_2B (perhaps a gel-shift or footprinting assay), then values of K_1 and K_2 could be calculated directly.

If, however, intermediates are substantially populated and the assay can not distinguish AB from A_2B, then both species would have to be included in calculating Θ_b, the fraction of bound B.

$$\Theta_b = \frac{[AB]+[A_2B]}{[B]+[AB]+[A_2B]} = \frac{\frac{[A]}{K_1} + \frac{[A]^2}{K_1K_2}}{1 + \frac{[A]}{K_1} + \frac{[A]^2}{K_1K_2}}$$

Equilibria involving buffer components

It is common to assay the binding of two macromolecules or the binding of a small ligand to a macromolecule in a buffer that contains components that, in principle, might participate in the reaction. Assume, for example, that 2 chloride ions bind at a dimer interface and are required for stable dimerization.

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$$(A \bullet Cl)_2 \Leftrightarrow 2A + 2Cl$$

Intuitively, increasing the chloride concentration should result in more complex formation. The proper equilibrium expression for this reaction is:

$$K_d = [A]^2[Cl]^2/[(A \bullet Cl)_2]$$

In such circumstances, however, an apparent equilibrium constant for dimerization would often be written without explicit consideration of the chloride.

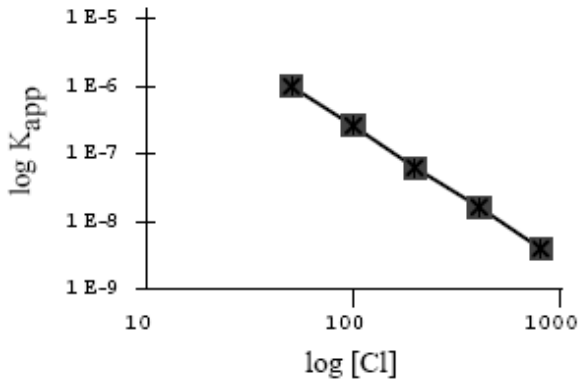
$$K_{app} = [A]^2/[A_2]$$

A bit of algebra yields

$$K_{app} = K_d/[Cl]^2$$

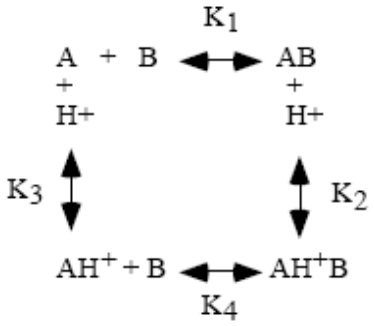
$$\log (K_{app}) = \ln (K_d) - 2 \bullet \log [Cl]$$

Thus, if K_{app} were measured in buffers with different concentrations of NaCl, one would expect a plot of log (K_{app}) vs. log [Cl] to be linear with a slope of -2. Experiments of this type can be very useful in detecting the participation and stoichiometry of buffer components in a reaction.



Note, however, that because chloride ion and sodium ion increase together when we increase the NaCl concentration, the experiment shown above does not show that chloride rather than sodium is the buffer component involved in the reaction. Control experiments examining dimerization in buffers with different concentrations of KCl, KF, NaF, Na_2SO_4, Na_2PO_4, etc. could be performed to test whether the anion or cation is important and whether the reaction is specific for a particular anion or cation.

Proteins contain numerous ionizable groups and protons or hydrogen ions are frequently involved in folding reactions, conformational change reactions, and binding reactions. Thus, it is common for K_{app} for a reaction to change as a function of $[H^+]$ concentration. pH is just -log$[H^+]$ and thus plotting log K_{app} against pH should reveal whether one or more protons is required for the reaction. Sometimes, however, a bound proton is not absolutely required for the reaction but its presence does change the equilibrium constant. We might write this set of reactions as follows:



Imagine that we assay A binding to B at low pH where all species are protonated and find that $[AH^+][B]/[AH^+B] = 10^{-9}$ M. This gives us an estimate of K_4. Now we repeat the experiment at high pH where all the species are unprotonated and get $[A][B]/[AB] = 10^{-7}$ M. Thus, protonation of A makes the binding of B 100-fold stronger. Because $K_1K_2 = K_3K_4$, we know that $K_3 = 100 \bullet K_2$ (this simply says that the proton binds more tightly to the AB complex than to free A).

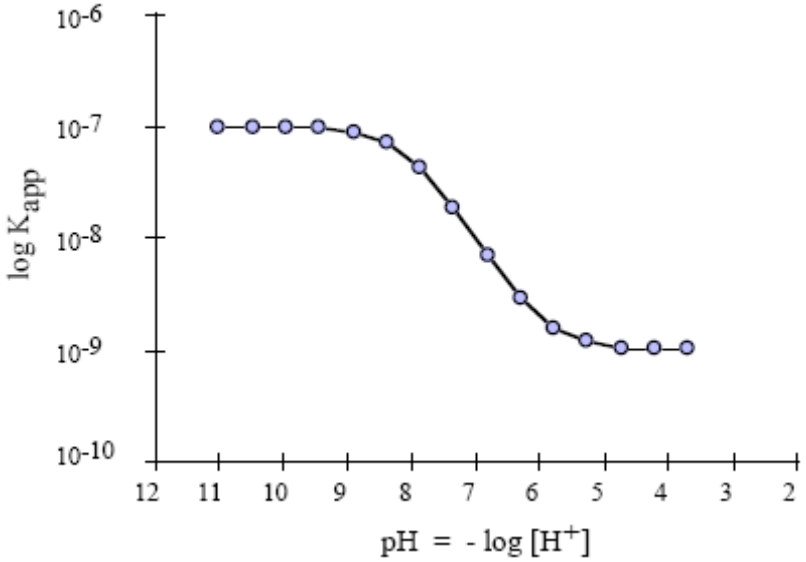
We now measure K_{app} at a series of different pH's. Because we don't distinguish protonated from unprotonated species in our binding assay,

$$K_{app} = \frac{[A + AH^+][B]}{[AB + AH^+B]}$$

To get K_{app} as a function of $[H^+]$, we substitute $[A][[H^+]/K_3$ for AH^+ and substitute $[AB][H^+]/K_2$ for AH+B. Rearranging and some more substitution gives:

$$K_{app} = \frac{1+[H^+]/K_3}{1+[H^+]/K_2} K_1$$

The data in the plot below was generated using $K_3 = 10^{-6}$ M ($pK_a = 6$) which might be expected for a histidine side chain.



If this were experimental data, we could fit it to get pKa's for proton binding to the free protein ($pK_a = -\log (K_3) = 6$) and to the complex ($pK_a = -\log (K_2) = 8$).