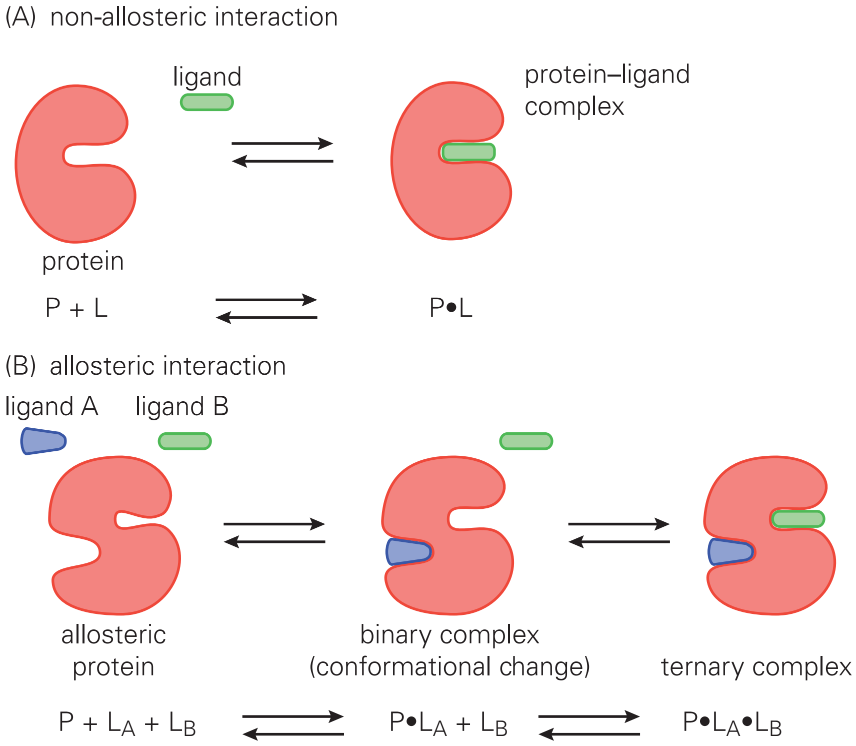


Figure 12.2 Allosteric and non-allosteric binding interactions. (A) In a simple binding interaction, each encounter between the protein molecule and a ligand molecule is independent of other binding interactions. (B) An allosteric protein has more than one ligand binding site, and the binding of a ligand to one site influences the affinity of the other site for its ligand. Allosteric binding sites are often symmetry related sites in oligomeric proteins. Allosteric interactions are discussed in Chapter 14.

12.1 The affinity of a protein for a ligand is characterized by the dissociation constant, K_D

We begin our analysis of noncovalent complexes by restating some thermodynamic relationships that are familiar to us from Chapter 10, but which we now place explicitly in the context of a ligand, L, binding noncovalently to a protein, P.

The general binding equilibrium for the interaction of a protein, P, with a ligand, L, can be written as follows:

$$\mathrm{P} + \mathrm{L} \rightleftharpoons \mathrm{P} \bullet \mathrm{L} \tag{12.1}$$

In Equation 12.1, P•L represents the noncovalent protein–ligand complex. The equilibrium constant, K, for the reaction shown in Equation 12.1, is given by Equation 12.2 (see Equation 10.51):

$$K = \frac{[\mathrm{P} \bullet \mathrm{L}]}{[\mathrm{P}][\mathrm{L}]} \tag{12.2}$$

In Equation 12.2, [P•L] is the concentration of the liganded protein, [P] is the concentration of the free protein, and [L] is the concentration of the free ligand. Because the binding reaction (Equation 12.1), as read from left to right, is in the direction of association, the equilibrium constant as defined in Equation 12.2 is referred to as the **association constant**, K_A:

$$K_A = \frac{[\mathrm{P} \bullet \mathrm{L}]}{[\mathrm{P}][\mathrm{L}]} \tag{12.3}$$

The standard free-energy change, ΔG^o, for the binding reaction is given by Equation 12.4 (see Equation 10.52):

$$\Delta G^o = -RT \ln K_A \tag{12.4}$$

Recall that ΔG^o is the change in free energy upon converting one mole of reactants into a stoichiometric equivalent of products (**Figure 12.3**). In this case, ΔG^o is the change in free energy when 1 mole of protein binds to 1 mole of ligand under

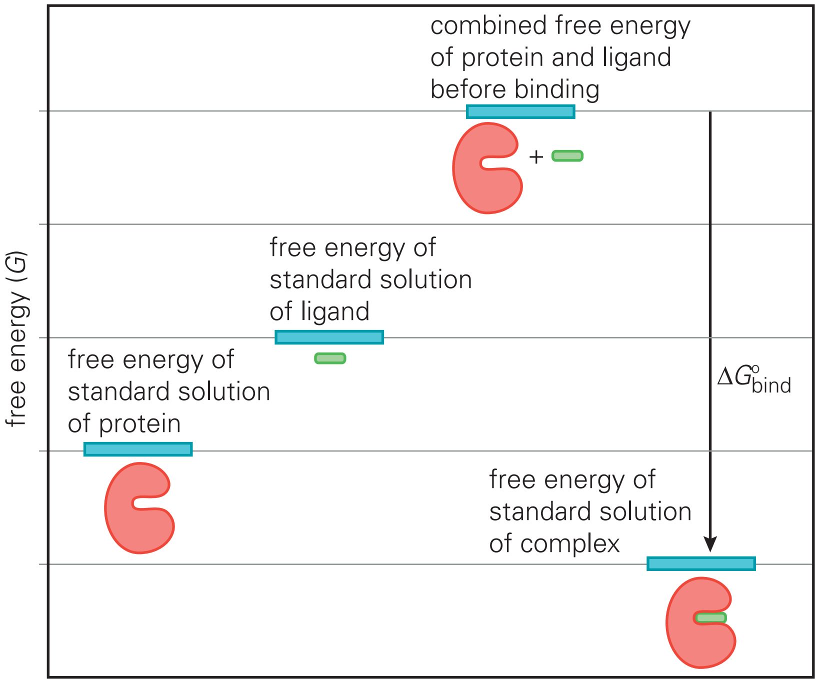


Figure 12.3 Schematic diagram showing the changes in free energy upon ligand binding. The standard free-energy change for binding, $\Delta G^{\circ}_{\text{bind}}$, refers to the conversion of one mole of protein and one mole of ligand to a complex under standard conditions.

standard conditions (1 molar solution of each). The standard free-energy change upon complex formation is called the **binding free-energy change** or, more simply, just the **binding free-energy**, $\Delta G^{\circ}_{\text{bind}}$:

$$\Delta G^{\circ}_{\text{bind}} = -RT \ln K_{\text{A}} \tag{12.5}$$

The value of $\Delta G^{\circ}_{\text{bind}}$ is a measure of the **affinity** of the interaction, that is, how strongly the molecules bind to each other. It is common practice to characterize the affinity of a binding interaction in terms of the equilibrium constant for the dissociation reaction, K_{D}, rather than the association constant, K_{A}. The dissociation reaction is simply the reverse of the association reaction:

$$\text{P}\bullet\text{L} \rightleftharpoons \text{P} + \text{L} \tag{12.6}$$

As a result, the **dissociation constant**, K_{D}, is the inverse of the association constant:

$$K_{\text{D}} = \frac{[\text{P}][\text{L}]}{[\text{P}\bullet\text{L}]} = \frac{1}{K_{\text{A}}} \tag{12.7}$$

It follows from Equations 12.5 and 12.7, then, that the binding free-energy is given by:

$$\Delta G^{\circ}_{\text{bind}} = +RT \ln K_{\text{D}} \tag{12.8}$$

Although the dissociation constant is a dimensionless number, it is usually discussed as if it has molar units of concentration (see Section 12.3). Biologically important nonconvalent interactions have dissociation constants that range from picomolar to nanomolar (that is, 10^{-12}–10^{-9} M) for the tightest interactions, to millimolar (that is, 10^{-3} M) for the weakest ones (**Table 12.1**). These correspond to standard free-energy changes upon binding of approximately –50 kJ•mol^{-1} for the tightest interactions to approximately –17 kJ•mol^{-1} for the weaker ones. Small-molecule drugs usually bind very tightly to their target proteins, with dissociation constants in the nanomolar (10^{-9} M) to picomolar (10^{-12} M) range. The value of the dissociation constant is sometimes simply referred to by biochemists as the "affinity" of an interaction; an interaction with a dissociation constant of 1 nanomolar is described as having a *nanomolar affinity*.

Affinity

The affinity of a molecular interaction refers to its strength. The greater the decrease in free energy upon binding, the greater the affinity. Another important concept is the specificity of the interaction, which refers to the relative strength of the interactions made between one protein and alternative ligands. Biologically relevant interactions are usually highly specific, as discussed in more detail in Chapter 13.

Table 12.1 Typical strengths of different kinds of interactions.

Type of interaction	K_D (molar)	ΔG^o_{bind} (at 300 K) (kJ•mol^{-1})
Enzyme–ATP	~1×10^{-3} to ~1×10^{-6} (millimolar to micromolar)	–17 to –35
Signaling protein binding to a target	~1×10^{-6} (micromolar)	–35
Sequence-specific recognition of DNA by a transcription factor	~1×10^{-9} (nanomolar)	–52
Small molecule inhibitors of proteins (drugs)	~1×10^{-9} to ~1×10^{-12} (nanomolar to picomolar)	–52 to –69
Biotin binding to avidin protein (one of the strongest known noncovalent interactions)	~1×10^{-15} (femtomolar)	–86

12.2 The value of K_D corresponds to the concentration of free ligand at which the protein is half saturated

The reason that the dissociation constant, K_D, is more commonly referred to than the association constant, K_A, is that the value of K_D is equal in magnitude to the concentration of free ligand at which half the protein molecules are bound to ligand (and half are unliganded) at equilibrium (**Figure 12.4**). The value of K_D is therefore determined readily if we have some way of measuring the fraction of protein molecules that are bound to ligand.

It is straightforward to see why the value of K_D corresponds to the ligand concentration at which the protein is half saturated. Let us define a parameter, *f*, which is the **fractional saturation** or **fractional occupancy** of the ligand binding sites in the protein molecules. If we assume that each protein molecule can bind to one ligand molecule, then *f* is the ratio of the number of protein molecules that have ligand bound to them to the total number of protein molecules (see Figure 12.4). In terms of concentrations, *f* can be expressed as:

$$f = \frac{\text{concentration of protein with ligand bound}}{\text{total protein concentration}} = \frac{[\mathrm{P\bullet L}]}{[\mathrm{P}]+[\mathrm{P\bullet L}]} \qquad (12.9)$$

Using Equation 12.7, we can relate [P•L] to the dissociation constant as follows:

$$[\mathrm{P\bullet L}] = \frac{[\mathrm{P}][\mathrm{L}]}{K_\mathrm{D}} \qquad (12.10)$$

Substituting the expression for [P•L] from Equation 12.10 into Equation 12.9, we get:

$$f = \frac{[\mathrm{P}][\mathrm{L}]}{K_\mathrm{D}\left([\mathrm{P}]+\dfrac{[\mathrm{P}][\mathrm{L}]}{K_\mathrm{D}}\right)}$$

$$\Rightarrow f = \frac{[\mathrm{L}]}{K_\mathrm{D}\left(1+\dfrac{[\mathrm{L}]}{K_\mathrm{D}}\right)} = \frac{[\mathrm{L}]}{K_\mathrm{D}+[\mathrm{L}]} = \frac{\dfrac{[\mathrm{L}]}{K_\mathrm{D}}}{1+\dfrac{[\mathrm{L}]}{K_\mathrm{D}}} \qquad (12.11)$$

By using Equation 12.11, we can calculate the value of the fractional saturation, *f*, when the ligand concentration is equal in magnitude to the value of the dissociation constant. That is, if

$$f = \frac{[\mathrm{L}]}{[\mathrm{L}]+K_\mathrm{D}}$$

Fractional saturation, *f*

The fractional saturation is the extent to which the binding sites on a protein are filled with ligand. For a protein with a single ligand binding site, the value of *f* is given by the ratio of the concentration of the protein with ligand bound to the total protein concentration. The fractional saturation is an important parameter, because experimentally measurable responses to ligand binding usually depend directly on the fractional saturation.

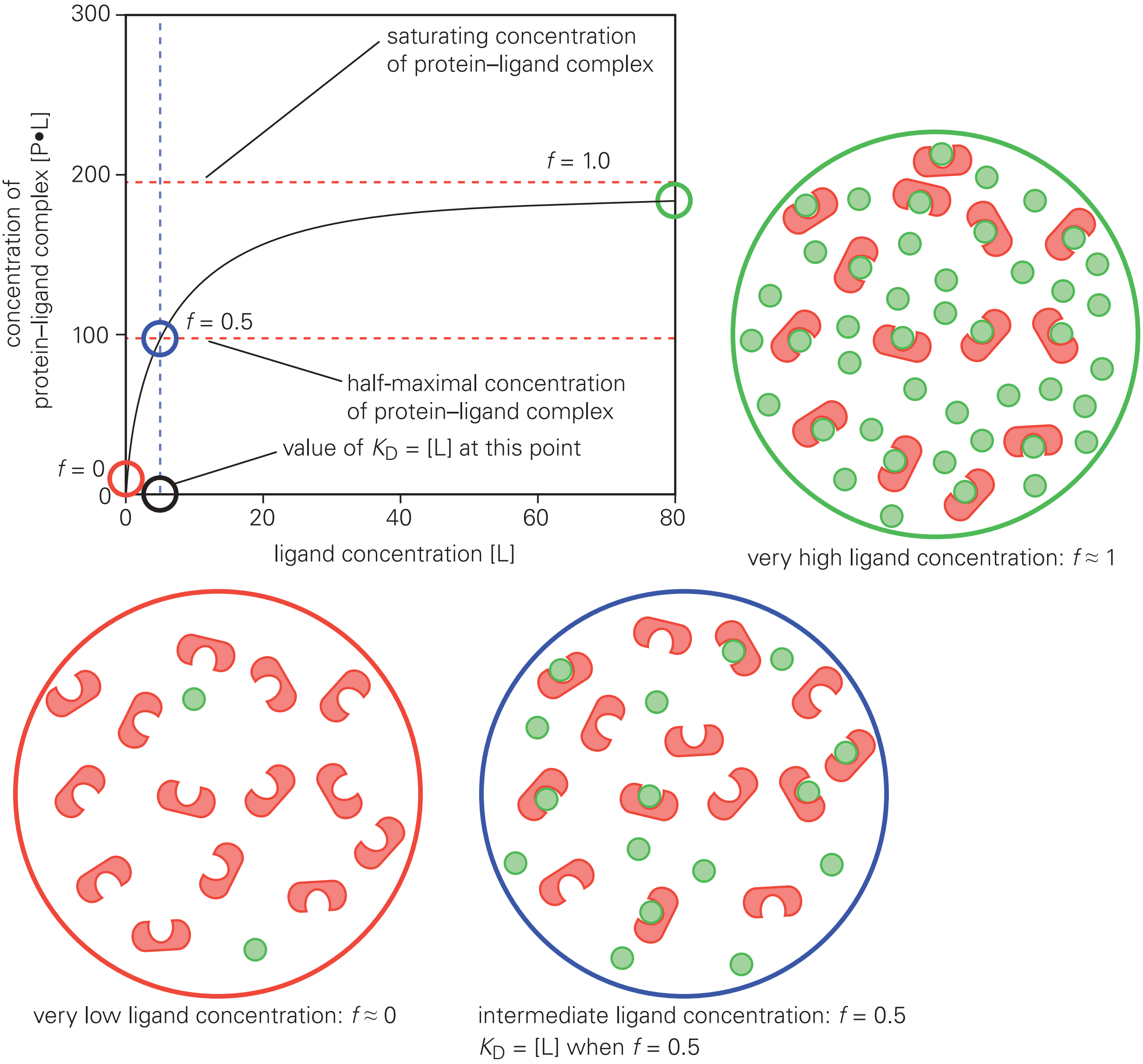


Figure 12.4 The fractional saturation, *f*, as a function of free ligand concentration, [L]. The value of *f* ranges from zero (no binding) to 1.0 (all protein molecules are bound to ligand). The graph of *f* versus [L] shown here is known as a binding isotherm. The shape of the binding curve is that of a rectangular hyperbola.

then when [L] = K_D

$$f = \frac{K_D}{K_D + K_D} = \frac{1}{2} \tag{12.12}$$

According to Equation 12.12, when the protein is half saturated (that is, when half the protein molecules in the solution have ligand bound to them), then the value of the ligand concentration is equal to the dissociation constant (see Figure 12.4).

A plot of fractional saturation, *f*, as a function of ligand concentration, measured at constant temperature, is known as a **binding isotherm** or **binding curve**. The term “isotherm” refers to the fact that all of the measurements have to be made at a constant temperature in order for the binding curve to be meaningful. Note that the fractional occupancy, *f*, at a given concentration of free ligand, [L], depends on the dissociation constant, K_D (Equation 12.7). The value of K_D depends, in turn, on the temperature (see Equation 12.8). That is, the dissociation “constant” is a constant only if the temperature is maintained at a constant value. If the temperature is allowed to fluctuate while a series of binding measurements are made then the results will make little sense.

The shape of the binding isotherm shown in Figure 12.4 is referred to as a *rectangular hyperbola*, which is a curve traced out by a cone when it intersects a plane. The binding isotherm for the simple equilibrium represented by Equation 12.1 is sometimes referred to as a **hyperbolic binding isotherm**.

12.3 The dissociation constant is a dimensionless number, but is commonly referred to in concentration units

The dissociation constant, like all equilibrium constants, is a dimensionless number. This has to be true, as we can see by considering Equation 12.8:

$$\Delta G^{o}_{\text{bind}} = RT \ln K_{\text{D}}$$

$\Delta G^{o}_{\text{bind}}$ has units of energy (for example, kJ•mol^{-1}), as does RT on the right-hand side of the equation. Hence, for the units to balance, K_D must be a pure number. If K_D is dimensionless, how is it that we equate K_D with ligand concentration in Equation 12.12? The apparent discrepancy in the units of K_D arises because we customarily omit the values of the standard state concentrations in the definition of the equilibrium constants (see Section 10.7).

If we write out the complete expression for the dissociation constant, then we have the following expression (see Equation 10.54):

$$K_{\text{D}} = \frac{\dfrac{[\text{P}]}{[\text{P}]^{o}}\dfrac{[\text{L}]}{[\text{L}]^{o}}}{\dfrac{[\text{P}\bullet\text{L}]}{[\text{P}\bullet\text{L}]^{o}}} \qquad (12.13)$$

where $[\text{P}]°$, $[\text{L}]°$, and $[\text{P}\bullet\text{L}]°$ are standard state concentrations and are numerically equal to 1 M, and are therefore usually not written out explicitly.

We can rewrite equation 12.13 as:

$$K_{\text{D}} = \left(\frac{[\text{P}\bullet\text{L}]^{o}}{[\text{P}]^{o}[\text{L}]^{o}}\right)\frac{[\text{P}][\text{L}]}{[\text{P}\bullet\text{L}]} = \left(\frac{[\text{P}\bullet\text{L}]^{o}}{[\text{P}]^{o}[\text{L}]^{o}}\right)K^{*}_{\text{D}} \qquad (12.14)$$

where K^{*}_{D}, a pseudo equilibrium constant, is given by:

$$K^{*}_{\text{D}} = \left(\frac{[\text{P}][\text{L}]}{[\text{P}\bullet\text{L}]}\right) \qquad (12.15)$$

K^{*}_{D} has units of concentration, and its value is equal to the ligand concentration at which the protein is half saturated. Because the value of the term $\left(\dfrac{[\text{P}\bullet\text{L}]^{o}}{[\text{P}]^{o}[\text{L}]^{o}}\right)$ in Equation 12.14 is 1.0, the numerical values of K_D and K^{*}_{D} are the same, even though they have different units. We use K_D and K^{*}_{D} interchangeably in practice, and will often use molar units when referring to K_D.

12.4 Dissociation constants are determined experimentally using binding assays

Dissociation constants are derived experimentally from binding isotherms, which rely on methods for measuring the amount of ligand bound to the protein. There are many different ways of making such a measurement, known as a **binding assay**. Exactly how a binding assay is carried out depends on the details of the interaction being monitored and the ingenuity of the biochemical investigator. Here we discuss an example in which radioactivity is used to monitor the amount of ligand bound to the receptor for the hormone estrogen (**Figure 12.5**).

Estrogen is a hormone in females, and its receptor is a site-specific DNA binding protein. The estrogen receptor belongs to a large family of closely related transcription factors known as the **nuclear** or **steroid hormone receptors**. The estrogen receptor consists of two important domains, one that binds to the hormone

Binding isotherm

A series of measurements of the extent of binding or the fractional saturation, *f*, as a function of ligand concentration is known as a binding isotherm. Such a measurement can be analyzed to yield the dissociation constant only if all of the measurements are made at the same temperature and, hence, the series of measurements is called an *isotherm*.

Hyperbolic binding curve or isotherm

The simple non-allosteric binding of a ligand to a protein results in a hyperbolic relationship between the fractional saturation, *f*, and the ligand concentration [L]. Because of this relationship, a simple ligand-binding equilibrium is referred to as hyperbolic binding. Deviation from the hyperbolic shape of the binding curve is evidence for more complicated phenomena, such as allostery or multiple binding sites with different affinities.

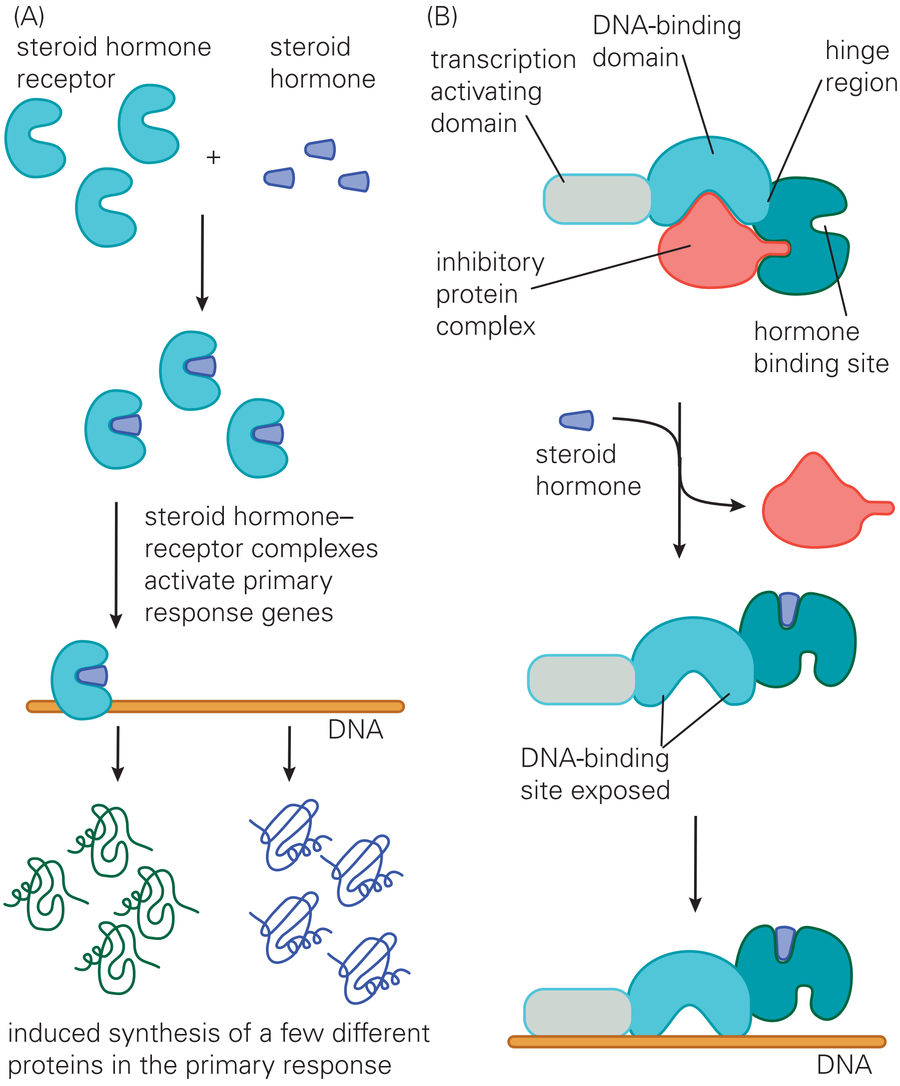


Figure 12.5 Mechanism of steroid receptors, such as the estrogen receptor. (A) Simplified functional diagram: these receptors bind to specific sites on DNA and activate transcription, but only when bound to their specific ligand (for example, estrogen). (B) Structural mechanism: the binding of the hormone to its receptor causes a conformational change, exposing the DNA-binding domain of the receptor, allowing it to interact with target DNA sequences. The active conformation is actually a dimer (second molecule not shown). The activated receptor also binds to proteins that are responsible for recruiting the transcriptional machinery (not shown here). In this complicated pathway, the amount of signaling can be modified by many other factors, such as phosphorylation of the receptor. (Adapted from B. Alberts et al. Molecular Biology of the Cell, 5th ed. New York: Garland Science, 2008.)

and one that binds to DNA (see Figure 12.5). When estrogen binds to the receptor, it promotes the dimerization of the receptor, which facilitates the binding of the receptor to sites on DNA that contain specific recognition sequences. Binding of estrogen to the receptor also induces a conformational change in the ligand-binding domain, which results in the recruitment of proteins known as transcriptional co-activators to the receptors. The co-activator proteins are responsible for turning on transcription from the gene.

In the binding assay shown in **Figure 12.6**, the estrogen sample contains a known amount of radioactively labeled estrogen that has been synthesized separately and mixed in with the normal estrogen (see Figure 12.6C). It is assumed that the presence of the radioactive isotope in the labeled estrogen molecule does not affect its ability to bind to the receptor. This allows us to assume that the amount of radioactivity that remains associated with the receptor after the free ligand is removed is proportional to the total amount of ligand bound by the receptor.

In order to measure the amount of ligand bound by the receptor, we need a way to separate the bound ligand from the unbound ligand. In the experiment shown in Figure 12.6, a negatively charged resin is added to the solution. The estrogen receptor binds to this resin, and a centrifugation step separates the bound from

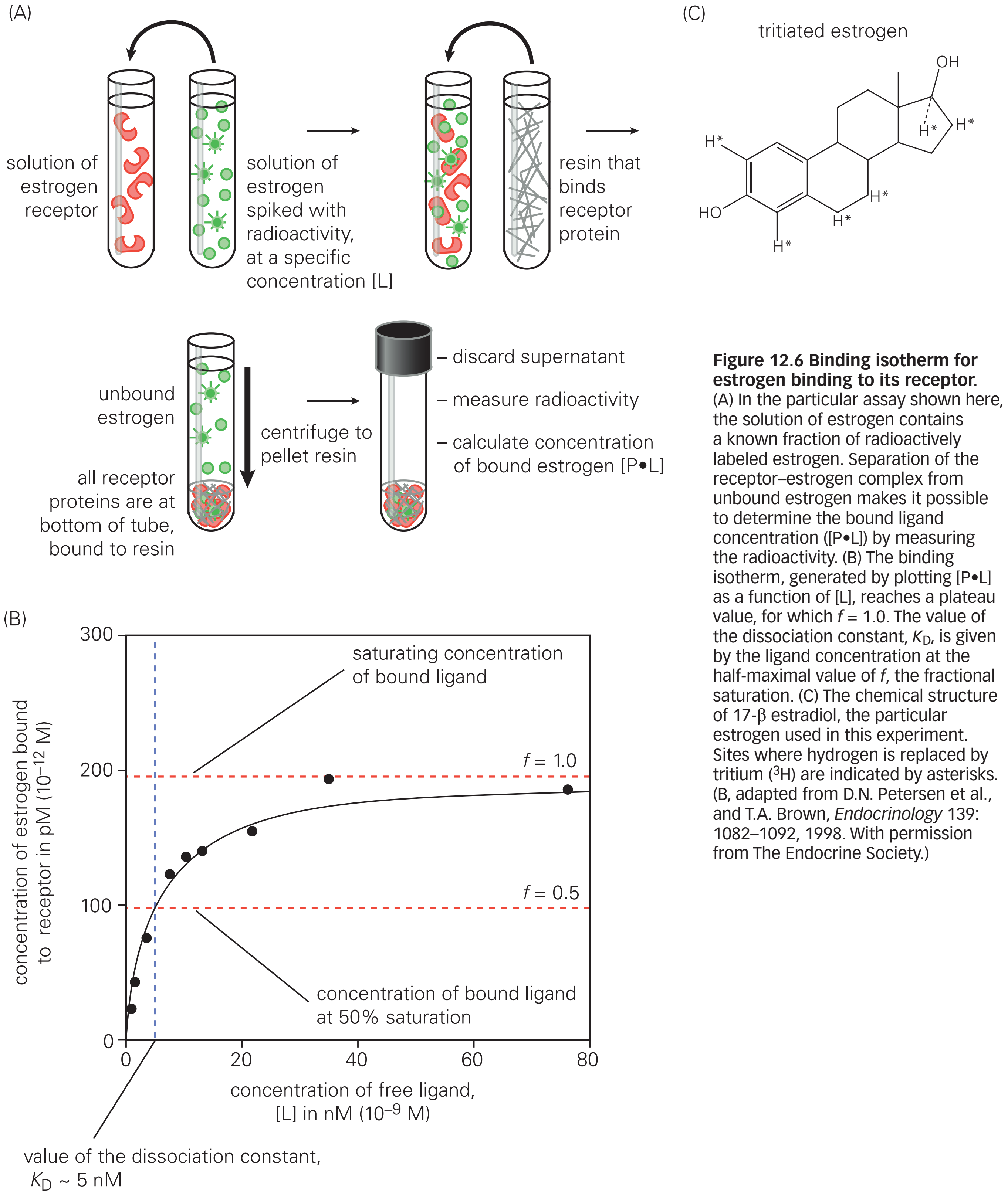


Figure 12.6 Binding isotherm for estrogen binding to its receptor. (A) In the particular assay shown here, the solution of estrogen contains a known fraction of radioactively labeled estrogen. Separation of the receptor–estrogen complex from unbound estrogen makes it possible to determine the bound ligand concentration ([P•L]) by measuring the radioactivity. (B) The binding isotherm, generated by plotting [P•L] as a function of [L], reaches a plateau value, for which $f = 1.0$. The value of the dissociation constant, K_D, is given by the ligand concentration at the half-maximal value of f, the fractional saturation. (C) The chemical structure of 17-β estradiol, the particular estrogen used in this experiment. Sites where hydrogen is replaced by tritium (^3H) are indicated by asterisks. (B, adapted from D.N. Petersen et al., and T.A. Brown, *Endocrinology* 139: 1082–1092, 1998. With permission from The Endocrine Society.)

the unbound ligand. The pelleted resin with the protein is transferred to a vial, and the total amount of radioactivity in it is estimated by using a liquid scintillation counter. Another common way to separate the bound ligand from the free ligand is to pass the solution through a filter that allows the solution to flow through but

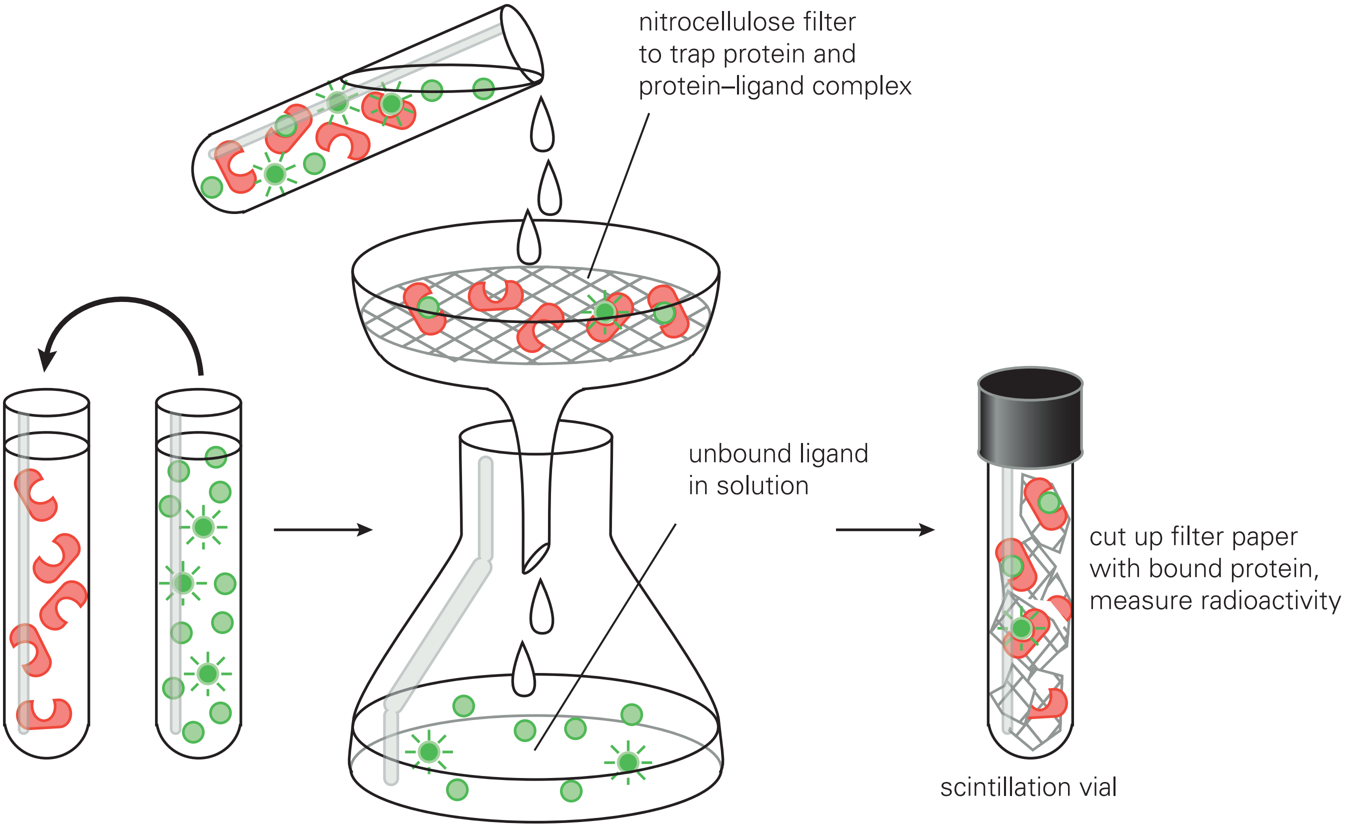


Figure 12.7 Filter binding assays. Proteins stick to filters made of material such as nitrocellulose, which allows ligand bound to the protein to be separated from unbound ligand. The presence of ligand bound to protein is then detected using a readout such as radioactivity.

to which the protein molecules (and the ligands bound to them) adhere. Filters made of nitrocellulose are commonly used for this purpose, and such a **filter binding assay** is illustrated in Figure 12.7.

The amount of bound ligand is plotted as a function of the total ligand concentration (usually assumed to be equal to the free ligand concentration, see Section 12.6) to obtain a binding isotherm, as shown in Figure 12.6B In this experiment, the radioactively labeled estrogen contains tritium atoms instead of normal hydrogens at several positions (Figure 12.6C). The tritium atoms emit β particles (that is, high-velocity electrons), which are detected by the scintillation counter. The amount of estrogen bound to the protein is proportional to the level of radioactivity detected and is plotted as a function of estrogen concentration to yield the binding isotherm.

Notice in Figure 12.6B that the amount of bound estrogen in the binding isotherm reaches a maximum or plateau value. This occurs when all of the estrogen receptor molecules are bound to estrogen—that is, when the receptor is saturated. The concentration of estrogen at which the amount of bound estrogen is half that of the saturating value gives us the dissociation constant. The value of K_D for estrogen binding to estrogen receptor is ~5 nM (5×10^{-9} M), according to the data in Figure 12.6.

12.5 Binding isotherms plotted with logarithmic axes are commonly used to determine the dissociation constant

A binding curve or isotherm, such as the one shown in Figure 12.8A, is a graph of the value of f, the fractional saturation of the protein, as a function of the ligand concentration, [L]. The value of K_D can be estimated by reading off the concentration at which the value of f is equal to 0.5. As we can see in Figure 12.8A, the range of concentrations over which the value of f changes from low to high is relatively narrow, and the most informative data points are crowded together on the left side of the graph. This can make it difficult to estimate the value of f by visual inspection of the binding isotherms. We could expand this region of the graph to more easily read out the value of K_D. Alternatively, we could switch to a graph with logarithmic axes, which would spread the data out more conveniently.

One such logarithmic graph involving fractional saturation and ligand concentration is shown in Figure 12.8B. This kind of graph turns out to be particularly useful for analyzing allostery in binding, and so we introduce it here and apply it to the analysis of allostery in Chapter 14.

To understand the nature of the graph in Figure 12.8B, we start with the expression for the fraction, f, of the protein that is bound to the ligand (that is, Equation 12.11):

$$f = \frac{[\mathrm{L}]}{[\mathrm{L}] + K_\mathrm{D}}$$

The fraction of the protein that is *not* bound to ligand is given by $1 - f$:

$$1 - f = 1 - \frac{[\mathrm{L}]}{[\mathrm{L}] + K_\mathrm{D}} = \frac{[\mathrm{L}] + K_\mathrm{D} - [\mathrm{L}]}{[\mathrm{L}] + K_\mathrm{D}} = \frac{K_\mathrm{D}}{[\mathrm{L}] + K_\mathrm{D}} \qquad (12.16)$$

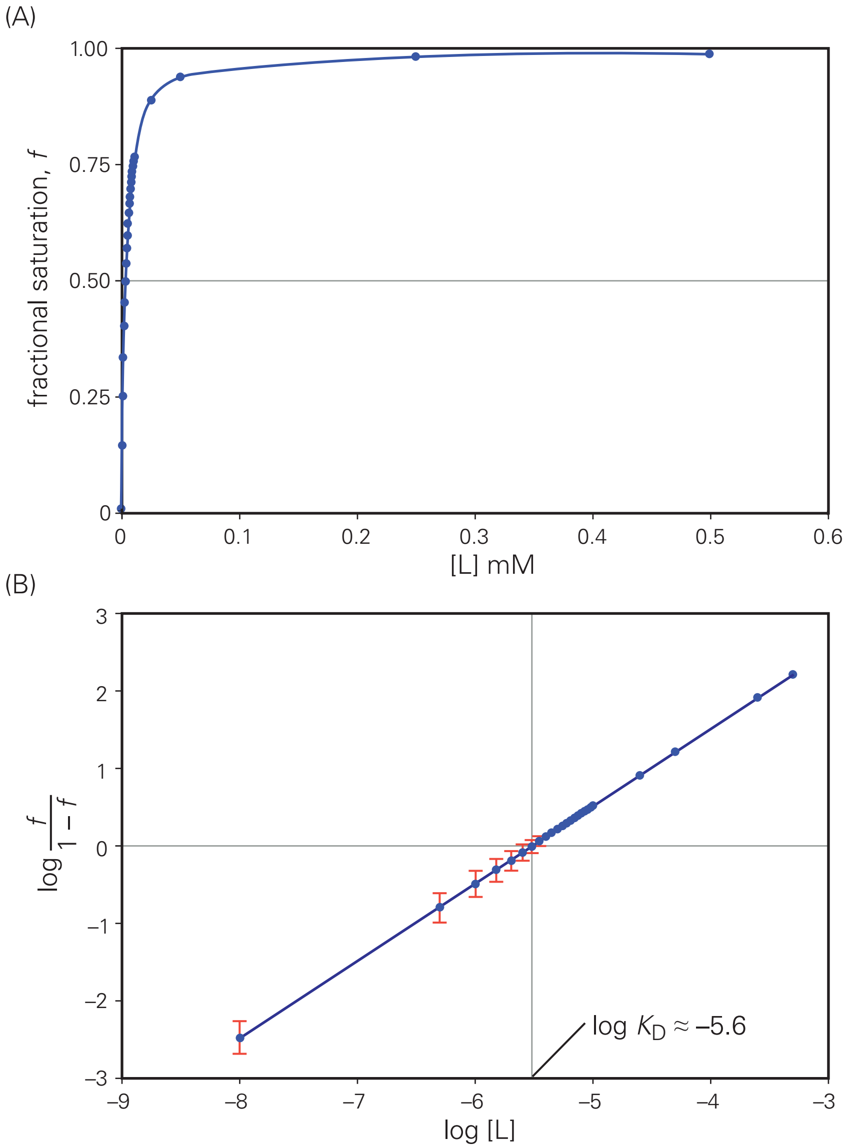


Figure 12.8. Binding isotherms with logarithmic axes. (A) A normal binding isotherm. The binding of ligand to protein is measured over a wide range of ligand concentration, which leads to some of the data being compressed into a small region of the graph. (B) The data shown in (A) are graphed using logarithmic axes. A linear plot is obtained when $\log\left(\frac{f}{1-f}\right)$—that is, when $\log\left(\frac{\text{fraction bound}}{\text{fraction unbound}}\right)$—is graphed versus log [L]. Note that the values of $\log\left(\frac{f}{1-f}\right)$ associated with low values of log [L] often have large errors associated with them because of errors in measurement when the ligand concentration is very low.

Using Equations 12.11 and 12.16, we can calculate the ratio of the fraction of protein that is bound to the fraction that is unbound:

$$\frac{\text{fraction bound}}{\text{fraction unbound}} = \frac{f}{1-f} = \frac{[\mathrm{L}]}{[\mathrm{L}]+K_\mathrm{D}}\frac{[\mathrm{L}]+K_\mathrm{D}}{K_\mathrm{D}} = \frac{[\mathrm{L}]}{K_\mathrm{D}} \tag{12.17}$$

Taking the logarithm of both sides of Equation 12.17, we get:

$$\log\left(\frac{f}{1-f}\right) = \log\left(\frac{[\mathrm{L}]}{K_\mathrm{D}}\right) = \log[\mathrm{L}] - \log K_\mathrm{D} \tag{12.18}$$

As shown in Figure 12.8B, graphing the value of $\log\left(\frac{f}{1-f}\right)$ as a function of log [L] yields a straight line. When the protein is half saturated—that is, when $\left(\frac{f}{1-f}\right)=1$, then $\log\left(\frac{f}{1-f}\right)$ is zero. The intercept of the line on the horizontal axis is therefore equal to log K_D.

A logarithmic graph such as the one shown in Figure 12.8B is a convenient way of checking the assumption that the protein binds to the ligand in a simple way, as described by Equation 12.1. As we shall see in Chapter 14, if the actual binding isotherm is not linear, or has a slope that is not unity, then this could be an indication that the actual binding process is more complex, and might include factors such as allostery.

The slope of the binding isotherm and the value of its intercept on the horizontal axis can be difficult to determine accurately if the binding data have errors in them. Values of the fractional saturation determined at low ligand concentration are particularly error prone, because the detection signal (for example, fluorescence or radioactivity) is correspondingly weak at low ligand concentration. Care must therefore be taken to ensure that errors associated with very weak signals do not unduly bias the analysis of the binding isotherm.

12.6 When the ligand is in great excess over the protein, the free ligand concentration, [L], is essentially equal to the total ligand concentration

In Figure 12.8, the critical parameter is the free ligand concentration, [L]—that is, the concentration of the ligand that is not bound to the protein. In most situations we are more concerned with the total ligand concentration, $[\mathrm{L}]_\mathrm{total}$, because this is something we know directly from the total amount of ligand added to the system under study. For example, if a patient takes a pill that contains 500 mg of a drug, the total concentration of the drug in the blood can be estimated by knowing the volume of blood in a typical human body (~5 liters). The free ligand concentration, [L], is a different matter, and can only be determined, in principle, by making a measurement.

In many biochemical applications, including the study of drug binding, a simplification occurs because the number (or concentration) of protein molecules is usually very small compared to that of the drug (**Figure 12.9**). The maximum concentration of bound ligand, $[\mathrm{L}]_\mathrm{bound}$, is therefore very small compared to the total ligand concentration, $[\mathrm{L}]_\mathrm{total}$, if protein concentration is very low compared to total ligand concentration:

$$[\mathrm{L}]_\mathrm{bound} << [\mathrm{L}]_\mathrm{total} \tag{12.19}$$

Because the total ligand concentration is the sum of the free ligand concentration, [L], and the bound ligand concentration, $[\mathrm{L}]_\mathrm{bound}$, it follows that the free ligand concentration is essentially the same as the total ligand concentration when $[\mathrm{L}]_\mathrm{bound} << [\mathrm{L}]_\mathrm{total}$:

$$[\mathrm{L}]_\mathrm{total} = [\mathrm{L}] + [\mathrm{L}]_\mathrm{bound} \approx [\mathrm{L}] \tag{12.20}$$

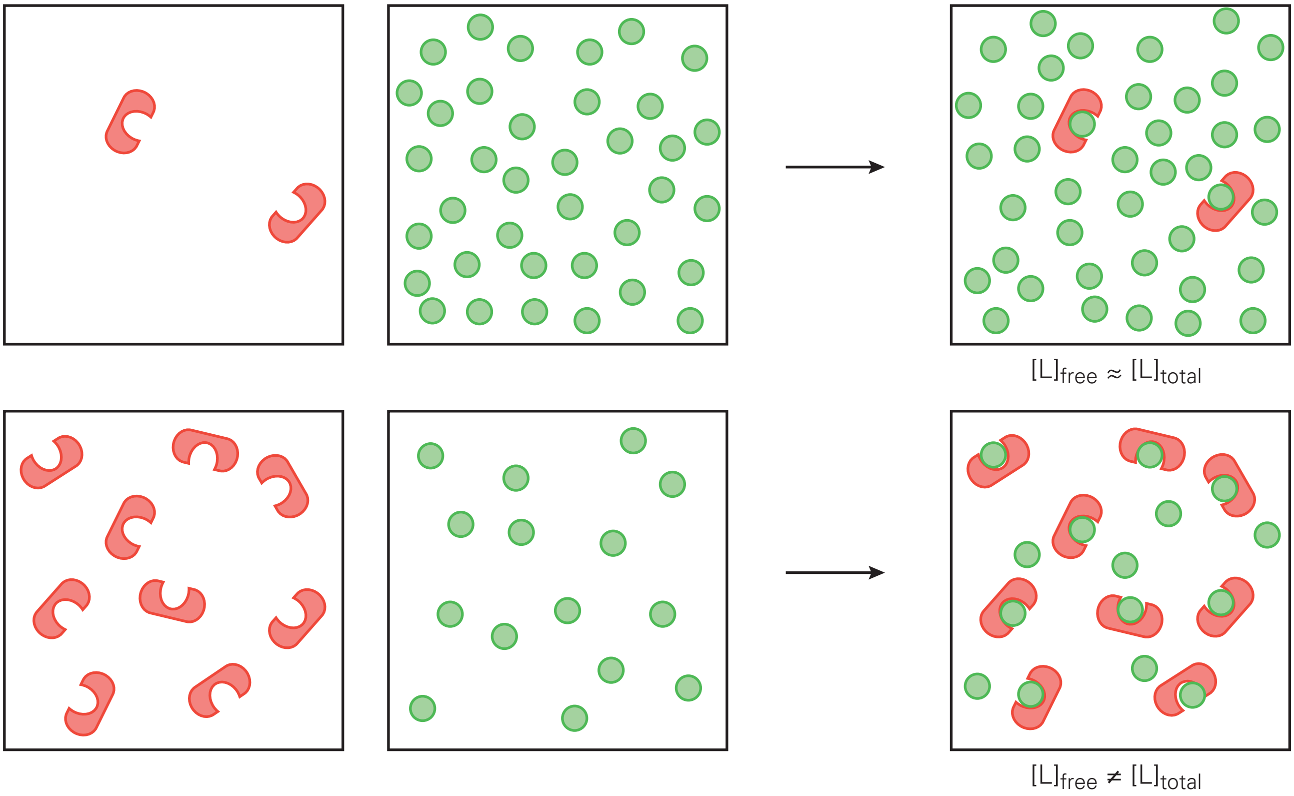


Figure 12.9 The free ligand concentration. (A) When the concentration of ligand is much greater than that of the protein, then the concentration of the unbound (free) ligand, [L], does not change much as ligand binds to protein. (B) When the concentrations of ligand and protein are comparable, the free ligand concentration is affected by how much ligand is bound to protein.

In calculations involving the saturation of protein binding sites by a ligand, we often assume that the amount of bound ligand is very small compared to the total amount of ligand available, and in such cases we use the free ligand concentration and the total ligand concentration interchangeably.

12.7 Scatchard analysis makes it possible to estimate the value of K_D when the concentration of the receptor is unknown

In the preceding analysis of binding isotherms, we assumed that both the protein and ligand concentrations were known. Without knowing the protein concentration, we cannot calculate the fractional saturation, *f*, knowledge of which is critical for determining the value of K_D. There are many situations in biology where it is straightforward to determine the concentration of bound and unbound ligand, but the protein concentration is not directly measurable. This is the case, for example, when we study the binding of a ligand to a cellular protein, without fractionation or purification.

When the assumption of single-site binding is valid, a method known as **Scatchard analysis**, named after physical chemist George Scatchard, allows us to determine the dissociation constant as well as the total protein concentration.

Let us return to the definition of the dissociation constant, K_D, given originally in Equation 12.7:

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

Scatchard analysis

A simple binding equilibrium between a protein and a ligand results in the hyperbolic binding curve shown in Figure 12.9B. Deviations from the hyperbolic curve, however, can be difficult to detect visually. Scatchard analysis involves rearranging the basic binding equation to yield the following form, known as the Scatchard equation:

$$\frac{[L]_{bound}}{[L]} = -\frac{1}{K_D}[L]_{bound} + \frac{[P]_{total}}{K_D}$$

The Scatchard equation, which is an alternative form of the hyperbolic binding equation, tells us that the ratio of bound to free ligand concentration is related linearly to the bound ligand concentration (Figure 12.10C).

12.10C. The slope of the line is equal to $-\frac{1}{K_D}$, making it possible to determine the value of the dissociation constant, which is ~0.2 nM in this case. The intercept of the line on the vertical axis is $\frac{[P]_{total}}{K_D}$. The Scatchard analysis, therefore, allows us to determine the concentration of the receptor protein in the cell lysates, even though the receptor protein was not purified.

12.9 Saturable binding is a hallmark of specific binding interactions

Saturable binding

In a situation where a ligand binds to a single binding site on a protein and no other, all of the protein molecules are bound to ligand at very high ligand concentrations. Increasing the ligand concentration beyond this point does not lead to any increase in protein binding. Such a binding interaction is referred to as being saturable. When a binding isotherm does *not* saturate, even at very high ligand concentrations, then it usually indicates that the ligand is also binding to things other than the protein of interest.

In any binding measurement, there is usually a need to correct the data for systematic effects that lead to distortions in the apparent values of the amount of ligand bound to the protein. In the case of the retinoic acid receptor, for instance, it turns out that there is a significant amount of nonspecific binding of retinoic acid to something other than its receptor. This can be seen by adding a 100-fold excess of unlabeled retinoic acid to each of the binding reactions (see Figure 12.10B). We would now expect all of the receptor molecules to bind predominantly to the unlabeled retinoic acid. Nevertheless, when this is done, a certain amount of labeled retinoic acid is still seen to be retained in the fraction left behind after the charcoal is removed (see Figure 12.10B). This binding occurs, presumably, to other proteins in the cell lysate or to the material (such as plastic) that makes up the reaction chamber. Whatever the nonspecific target may be, it offers so many binding sites that the addition of 100-fold excess of unlabeled retinoic acid still leaves sufficient binding sites to capture some labeled retinoic acid. If we subtract this nonspecific binding from the total binding measured in the absence of unlabeled retinoic acid, then we get a corrected binding isotherm (see Figure 12.10B).

Notice that the corrected binding isotherm in Figure 12.10B shows saturation of the binding—that is, the amount of bound retinoic acid reaches a maximum plateau value and then does not increase further. A plateau value in the binding isotherm, referred to as **saturable binding**, is a hallmark of the binding of a ligand to a defined binding site on a specific protein whose availability is limited. In contrast, nonspecific binding often shows no evidence of reaching a plateau value.

12.10 The value of the dissociation constant, K_D, defines the ligand concentration range over which the protein switches from unbound to bound

A question we are often concerned with in biochemistry or pharmacology is the extent to which a particular protein is bound to a ligand at a specific concentration of the ligand. For example, if a patient is to take a pill that delivers an inhibitor for an enzyme, what should be the concentration of the inhibitor in the blood in order to have most of the enzyme bound to the inhibitor? A related question concerns the specificity of the interaction. Most ligands will bind to more than one protein in the cell. Can we choose a ligand concentration such that one protein is bound to the ligand and another is not?

For a simple binding equilibrium involving one ligand and one protein, it is straightforward to estimate what the ligand concentration has to be in order to saturate the protein. The protein goes from having very little ligand bound to being almost saturated within a concentration range that extends from ~$0.1K_D$ to ~$10K_D$—that is, over a concentration range that spans two orders of magnitude. For example, if the concentration of the free ligand, [L], is 10 times the value of K_D, then the value of the fractional saturation, *f*, is given by:

$$f = \frac{[L]}{[L]+K_D} = \frac{10K_D}{10K_D + K_D} = \frac{10}{11} = 0.91 \qquad (12.25)$$

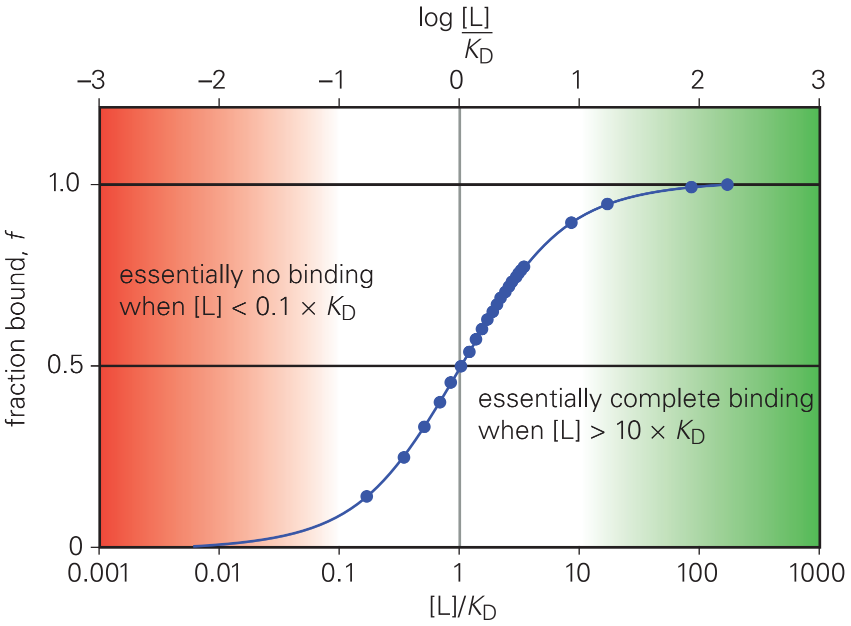


Figure 12.11 A "universal" binding isotherm. This graph expresses concentration in terms of the dimensionless number $\frac{[L]}{K_D}$. This graph is "universal" in the sense that it applies to any simple binding equilibrium.

Hence, the protein is 91% saturated when the ligand concentration is 10 times greater than the value of K_D. Likewise, when $[L] = 0.1K_D$, then the fractional saturation is given by:

$$f = \frac{0.1K_D}{K_D + 0.1K_D} = \frac{0.1}{1.1} = 9\% \tag{12.26}$$

At this lower concentration, only 9% of the protein is bound to the ligand. Thus, the ligand concentration range that is within a factor of 10 on either side of the value of the dissociation constant is the range in which the protein switches from being essentially unbound to nearly completely bound.

We can appreciate the way in which the population of protein molecules switches from bound to unbound by plotting the ligand concentration on a logarithmic scale, since in practice the concentrations of ligand under consideration span several orders of magnitude [for example, picomolar (that is, 10^{-12} M) to millimolar (that is, 10^{-3} M)]. It is particularly useful to plot the fractional saturation, *f*, as a function of $\log\left(\frac{[L]}{K_D}\right)$. By expressing the ligand concentration in terms of the dissociation constant, we get a "universal" binding curve (**Figure 12.11**) that is helpful in the discussion of ligands binding to alternative target proteins (see Chapter 13).

As an example, consider a drug that binds to a target protein, A, with a dissociation constant of 1 nM (10^{-9} M). The interaction between the drug and protein A is critical for treatment of a disease. Now imagine that the drug also binds to another protein, B, and that this interaction has undesirable side effects. Let us suppose that the binding of the drug to protein B occurs with a dissociation constant of 10 μM (10^{-5} M). How do we determine a concentration at which to deliver the drug so that protein A is essentially shut down by the drug, while protein B is essentially unaffected?

Using the universal binding curve in Figure 12.11, we look for a concentration range within which binding to A is maximal while binding to B is minimal. As the value of $\frac{[L]}{K_D}$ approaches 100, the value of *f* approaches 1.0. Since the value of K_D for protein A is 1×10^{-9} M (0.001 μM), protein A will be essentially saturated if the drug is delivered at a concentration of 0.1 μM (note that we assume that Equation

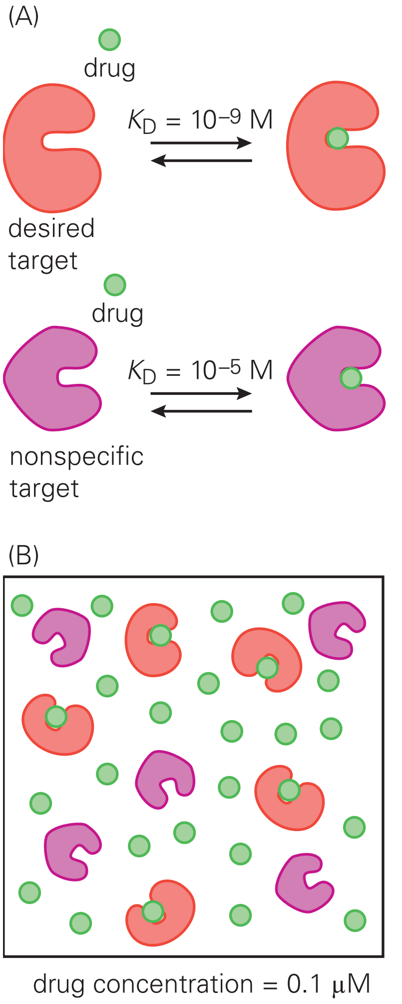


Figure 12.12 Affinity and specificity in drug binding. (A) A drug binds tightly to a desired protein and weakly to an undesired target. (B) The drug is delivered at a concentration that is much below the value of K_D for the undesired target. Very little binding to the undesired target occurs. Binding to the desired target is maintained if the concentration is above the value of K_D for that target.

Evolutionary relationship between dissociation constants and physiological concentrations of ligands

The dissociation constant of a protein for a naturally occurring ligand is usually close to the physiological concentration of the ligand. Much tighter interactions are unnecessary, because the protein is 99% saturated when the ligand concentration is 100 times greater than the dissociation constant.

12.20 holds true). At this concentration of the drug, the value of $\frac{[\mathrm{L}]}{K_\mathrm{D}}$ for protein B is $\frac{0.1\times10^{-6}\,\mathrm{M}}{1\times10^{-5}\,\mathrm{M}}$, which is 0.01. From the universal binding curve (see Figure 12.11), we can see that if the value of $\frac{[\mathrm{L}]}{K_\mathrm{D}}$ is 0.01, then the value of f is very small. Thus, if the drug is delivered at a concentration of 0.1 μM, then we expect protein B to be essentially unaffected (**Figure 12.12**). Thus, one way to avoid unwanted side effects in the action of a drug is to make its interaction with its desired target protein as tight as possible (that is, the dissociation constant should be as low as possible).

12.11 The dissociation constant for a physiological ligand is usually close to the natural concentration of the ligand

The fact that proteins switch from being empty to fully bound when the ligand concentration is close to the value of the dissociation constant has implications for the way in which evolution "tunes" the strength of the interaction between a protein and its natural ligands. In most cases, the dissociation constant for a natural binding interaction is lower by no more than a factor of 10–100 than the physiological concentration of the ligand. For example, the concentration of ATP in the cell is approximately 1 mM (10^{-3} M). Later in the chapter we discuss enzymes known as protein kinases, which bind to ATP and transfer the terminal phosphate group to the sidechains of proteins. The dissociation constant of ATP for protein kinases is typically ~10 μM (10^{-5} M)—that is, approximately one-hundredth that of the physiological ATP concentration. Certain motor proteins known as kinesins, which utilize ATP as a fuel to power the movement of organelles and other objects inside the cell, also bind to ATP with a similar dissociation constant, even though kinesins are completely unrelated to the protein kinases in terms of structure and mechanism.

It is easy to understand why the dissociation constant of a protein for its natural ligand is relatively close to the physiological concentration of the ligand. Suppose that a protein accumulates mutations that lead to an increased affinity for the ligand, such that the dissociation constant is much smaller than the natural concentration of the ligand. If a value of $\frac{[\mathrm{L}]}{K_\mathrm{D}} = 100$ is reached, then the saturation (f) is given by (see Equation 12.11):

$$f = \frac{\frac{[\mathrm{L}]}{K_\mathrm{D}}}{1+\frac{[\mathrm{L}]}{K_\mathrm{D}}} = \frac{100}{101} = 99\%$$

At this point the protein is essentially saturated, and further increases in affinity will not lead to any appreciable increases in ligand binding to the protein. As a consequence, mutations that do lead to higher affinity will not have an evolutionary advantage, and will likely disappear due to evolutionary drift. Furthermore, if the dissociation constant becomes too small, the protein would always have ligand bound. Signaling systems in cells use ligand binding to proteins as a way to turn proteins on and off, and always having the ligand bound would prevent normal "on"–"off" signaling in such systems. Even if the ligand concentration drops to very low levels, the tighter binding may make the rate of dissociation of the ligand very slow (see Chapter 15), which may interfere with function.

Mutations that weaken the binding, so that the value of K_D increases much beyond the physiological level of the ligand, will result in a failure of the protein to bind to the ligand. Because of the loss of function, such mutations will also be selected against.

CHAPTER 13

Specificity of Macromolecular Recognition

Almost every process in a living cell involves the dynamic interactions of biological macromolecules with one another. Cellular signaling, the control of metabolic activity, and the transport of material from one location in the cell to another are just a few of the essential processes that rely on protein–protein interactions. Both protein–protein and protein–DNA interactions are critical for the assembly of the required sets of transcription factors that initiate transcription of specific genes. Once mRNA is synthesized, its modification, including splicing, requires further sets of very specific protein–RNA interactions.

In order to understand how protein molecules recognize each other, and how proteins recognize specific nucleic acid sequences, we need to understand how residues arranged on protein surfaces interact with target molecules to provide both affinity and specificity. For the simplified analysis of binding in Chapter 12, we assumed that just two kinds of interacting molecules were present (the receptor and its ligand). In real systems there are many possible binding partners for any ligand, and the relative probabilities of forming different complexes determine the functional outcome. Thus, in addition to considering the affinity of a ligand for one receptor, we must also develop the concept of specificity—that is, the preference of a ligand for one particular target over others.

In this chapter, we begin by formally defining specificity in intermolecular interactions, and then discuss the molecular basis by which affinity and specificity are generated in protein–protein, protein–DNA, and protein–RNA interactions (**Figure 13.1**).

A. AFFINITY AND SPECIFICITY

13.1 Both affinity and specificity are important in intermolecular interactions

The **affinity** of an intermolecular interaction reflects its strength. The affinity of two molecules for each other is given by the dissociation constant, K_{D}, for the complex, with lower values of K_{D} corresponding to higher affinity. Alternatively, we can describe the affinity in terms of the free energy of formation of the complex, ΔG^{o} (where the more negative the value of ΔG^{o}, the higher the affinity; see Section 12.1). If a complex is functionally important, then the interaction of the ligand and its target receptor must be sufficiently strong that they bind one another at their physiologically relevant concentrations (see Section 12.10).

Affinity and specificity

The *affinity* of a particular molecular interaction refers to its strength. The *specificity* of an interaction refers to the preference for a molecule to bind one particular target relative to all others. Interactions between biological macromolecules usually have high specificity.

High affinity between a molecule and its target is by itself insufficient to ensure activity *in vivo*. If a ligand were simply "sticky" and bound tightly to any receptor, then most of it would be bound to off-target receptors and not to the real target. In order to achieve a particular activity through binding, the ligand must have a preference for binding to a particular receptor—that is, the interaction needs to be specific.

The **specificity** of an interaction reflects the affinity of a ligand for one particular receptor relative to all other possible kinds of receptors, as described

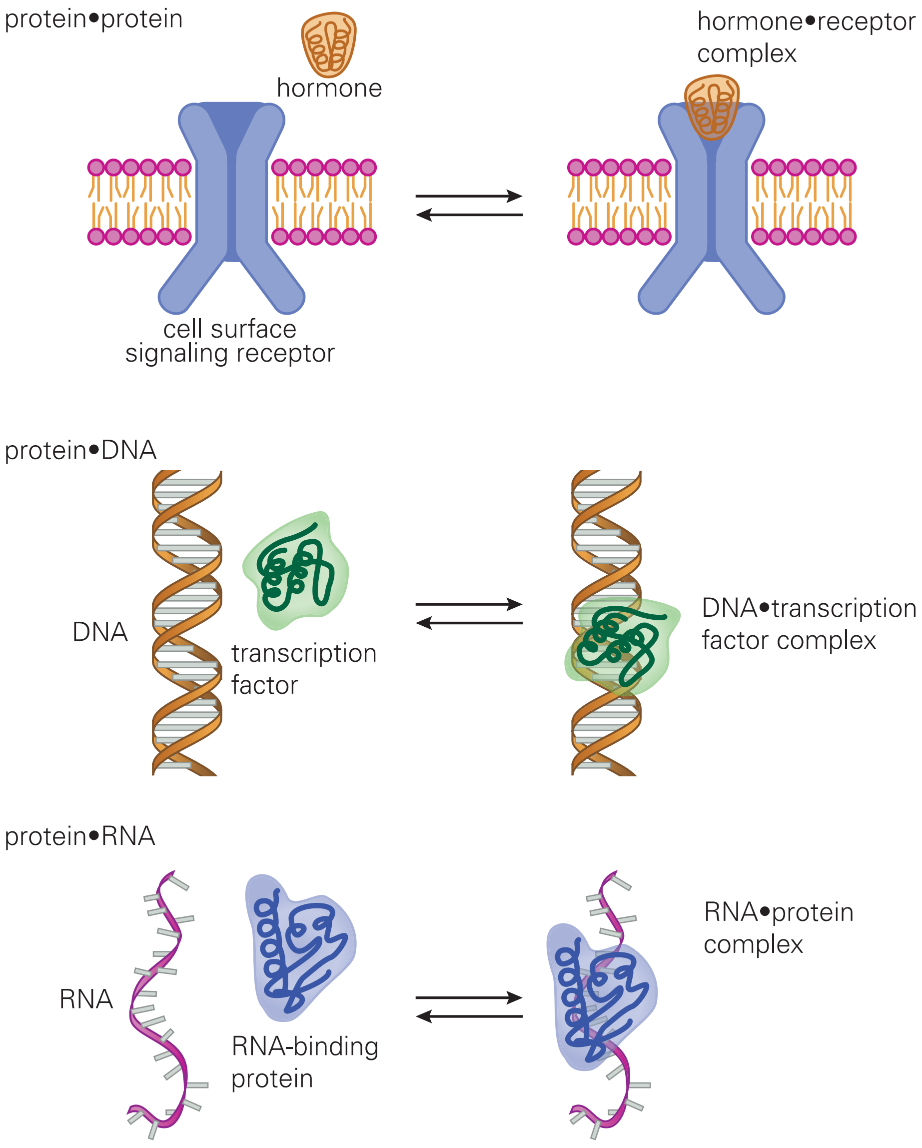
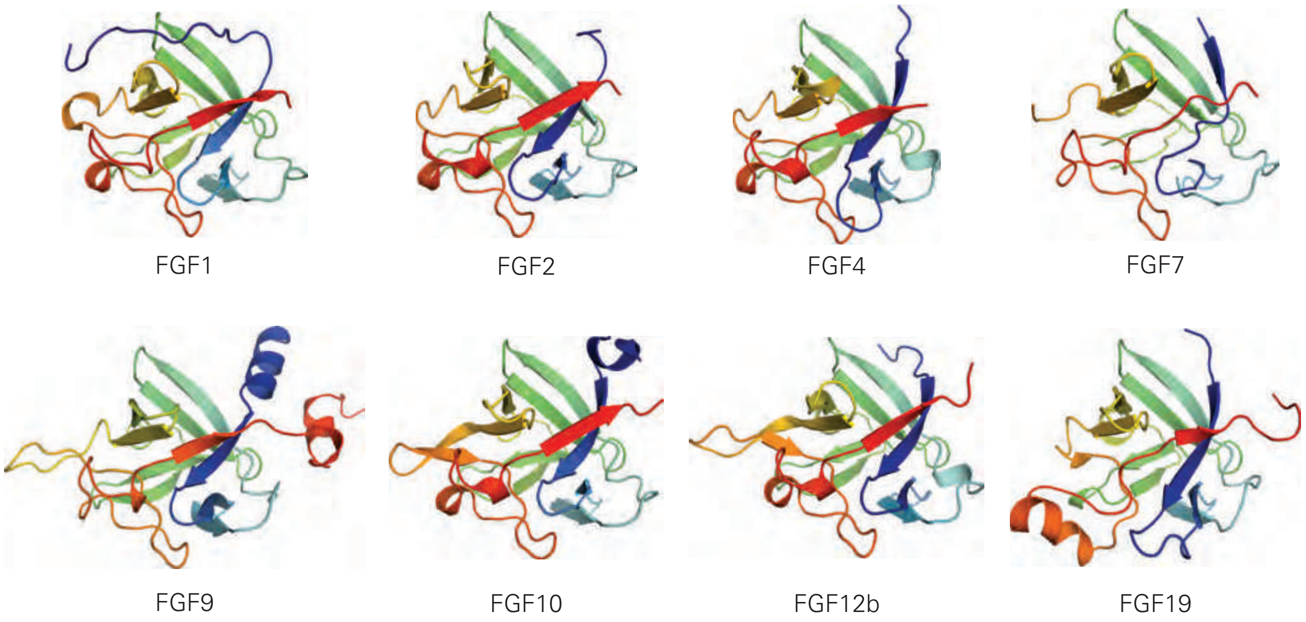


Figure 13.1 Molecular recognition. Examples of the three kinds of macromolecular interactions discussed in this chapter.

mathematically below. For many interactions in cells, the **affinity** of a ligand for one particular receptor is higher than for binding to other receptors (that is, functionally important binding usually has high specificity). The examples shown in Figure 13.1 are interactions with high specificity.

13.2 Proteins often have to choose between several closely related targets

The specificity of the interaction between a protein and its receptor is particularly important when the protein has to choose between many closely related targets in the cell. This is very common during cell signaling, when protein hormones can activate multiple receptors, leading to different outputs from each receptor. One such example is provided by a family of protein hormones known as the fibroblast growth factors (FGFs) and their receptors. The FGFs constitute a family of closely related proteins that control processes such as the growth of new blood vessels, wound healing, and embryonic development (**Figure 13.2**). The primary targets of these hormones are the fibroblast growth factor receptors (FGFRs), as shown in **Figure 13.3**. The FGF receptors are transmembrane tyrosine kinases with general similarity to the epidermal growth factor receptor that was described in Chapter 12 (see Figure 12.15).


FGF1 FGF2 FGF4 FGF7

FGF9 FGF10 FGF12b FGF19

There are 18 known FGFs in humans, which are related in sequence and structure (some of these are shown schematically in Figure 13.2). There are four genes for FGF receptors in humans, but the resulting mRNA can be spliced alternatively, giving three more variants. In principle, any FGF can bind to any FGF receptor, so there are 126 possible FGF–FGFR combinations.

In order for this signaling system to have the required selective responses to FGF signals, a few of the interactions are strong (corresponding to the primary signaling pathways), while many others are very weak, as indicated in **Figure 13.4**. A given tissue may express only one or two of the receptors, and the binding affinity may be modified by glycosylation of the receptor (see Section 3.11), as well as binding to heparin sulfate on cell surfaces.

Figure 13.2 Fibroblast growth factors. The structures of eight related mammalian fibroblast growth factors (FGFs) are shown. They share the same core fold, with some differences in peripheral structures. The chains are shaded in the color of the rainbow, from N-terminus to C-terminus. (Adapted from M. Mohammadi, S.K. Olsen, and O.A. Ibrahimi, *Cytokine Growth Factor Rev.* 16: 107–137, 2005; PDB codes: 1RG8, 1CVS, 1IJT, 1IHK, 1QQK, 1NUN, 1Q1U, and 2P23.)

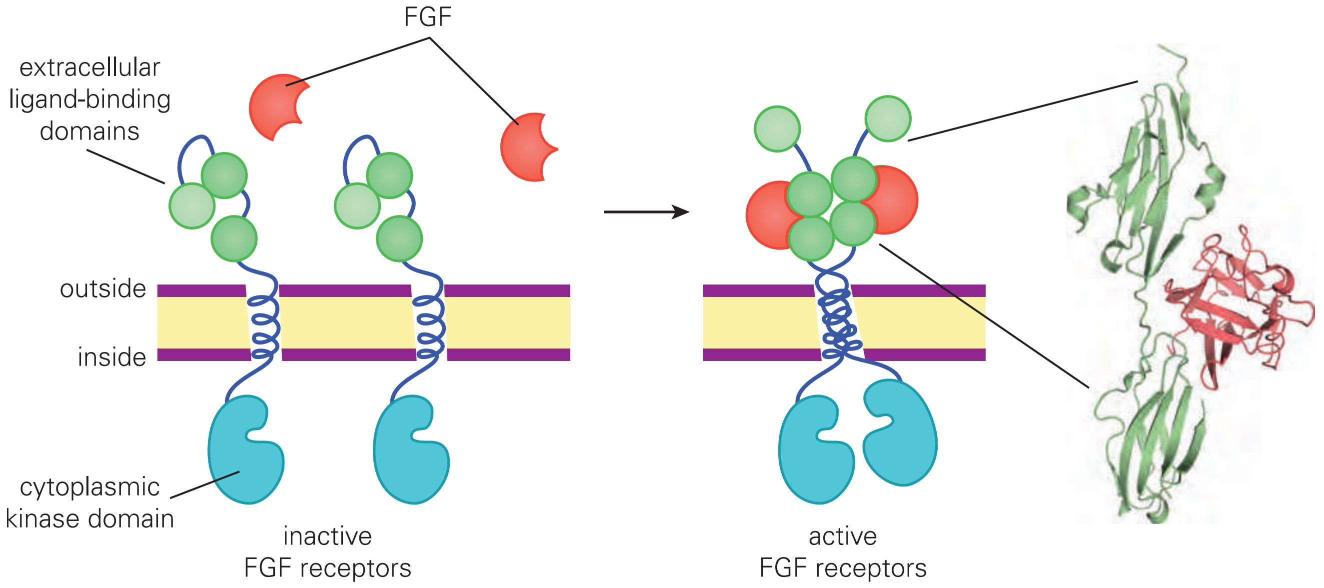


Figure 13.3 Interaction between fibroblast growth factor (FGF) and its receptor. FGF binding displaces an autoinhibition domain in the receptor, which blocks dimerization in the absence of FGF. The kinase domains then phosphorylate each other, which activates the receptor. To the right, the crystal structure of the complex of FGF1 with two domains of the FGF1 receptor is shown. Not shown in this diagram is heparin, a sulfated glycan that promotes receptor dimerization by bridging two FGF molecules. (PDB code: 1DJS.)

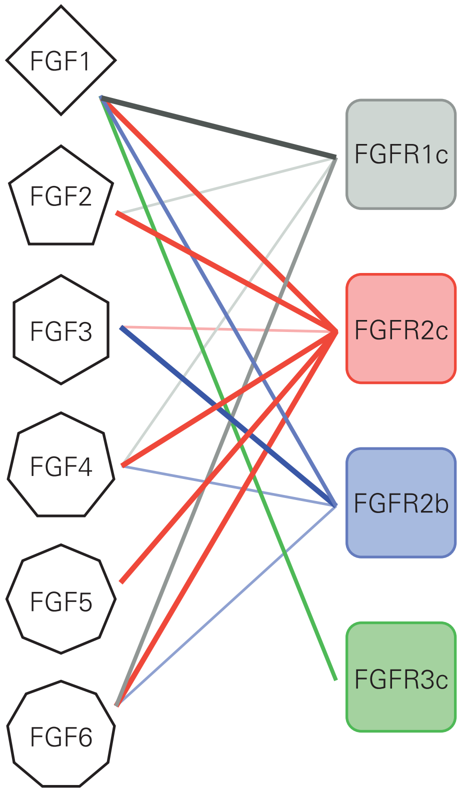


Figure 13.4 Schematic representation of some FGF–FGFR interactions. Each color reflects a different receptor, and the intensity of the line from each FGF reflects the affinity (relative to the tightest interaction for each). Missing lines correspond to interactions with very low affinity.

To understand the specificity of FGF signaling, it is necessary to understand how the occupancies of the various receptors change in response to altered levels of FGFs. We will return to a quantitative analysis of the FGF system after first defining more precisely what we mean by specificity.

13.3 Specificity is defined in terms of ratios of dissociation constants

In Section 12.10 we discussed the idea that substantial binding of a ligand to a receptor occurs when the concentration of the ligand exceeds the value of the dissociation constant. For the binding to be specific, two conditions must be satisfied. First, the dissociation constant for the primary target receptor (we will denote this receptor by R_0) must be smaller than the ligand concentration. Second, the dissociation constants for secondary or off-target receptors, denoted by R_i, must be larger than the ligand concentration. We use this idea to come up with a numerical value for the specificity of binding.

The binding equilibrium between the ligand and the primary receptor, R_0, is given by Equation 13.1:

$$R_0 \bullet L \rightleftharpoons R_0 + L \tag{13.1}$$

The strength of this interaction is characterized by the dissociation constant, K_{D,R_0}:

$$K_{D,R_0} = \frac{[R_0][L]}{[R_0 \bullet L]} \tag{13.2}$$

Suppose that the ligand can bind to a set of N secondary receptors, R_i, in addition to the primary one. Each of these interactions is governed by a binding equilibrium:

$$R_i \bullet L \rightleftharpoons R_i + L \tag{13.3}$$

The dissociation constant for each of these binding equilibria is given by:

$$K_{D,R_i} = \frac{[R_i][L]}{[R_i \bullet L]} \tag{13.4}$$

Thus, when both R_0 and R_i are present in the same sample, the concentration of free ligand, [L], will be identical in Equations 13.2 and 13.4, which must both be satisfied simultaneously.

We will define the **specificity factor**, α, as the ratio of the concentration of ligand bound to the specific receptor, R_0, divided by the total concentration of ligand bound to all of the secondary receptors, R_i:

$$\alpha = \frac{[R_0 \bullet L]}{\sum_{i=1}^{N} [R_i \bullet L]} \qquad (13.5)$$

It is useful to rewrite Equation 13.5 in terms of the dissociation constants and the total ligand concentration. To do this, we use the following set of relationships, where R_i refers to any one of the several receptors:

Specificity factor

The ratio of the concentration of ligand bound to the target receptor to the total concentration of ligand bound to all other receptor is known as the specificity factor, α.

$$[R_i]_{\text{total}} = [R_i] + [R_i \bullet L]$$

so:

$$[R_i] = [R_i]_{\text{total}} - [R_i \bullet L] \qquad (13.6)$$

From Equation 13.4, the value of $[R_i \bullet L]$ is given by:

$$[R_i \bullet L] = \frac{[R_i][L]}{K_{D,R_i}}$$

Using Equation 13.6 we now get:

$$[R_i \bullet L] = \frac{([R_i]_{\text{total}} - [R_i \bullet L])[L]}{K_{D,R_i}} \qquad (13.7)$$

Rearranging Equation 13.7 we obtain the following relationship:

$$[R_i \bullet L]\left(1 + \frac{[L]}{K_D}\right) = \frac{[R_i]_{\text{total}}\,[L]}{K_{D,R_i}}$$

and so:

$$[R_i \bullet L] = \frac{[R_i]_{\text{total}}}{1 + \dfrac{K_{D,R_i}}{[L]}} \qquad (13.8)$$

Equation 13.8 holds for each of the receptor–ligand interactions separately. Putting these relationships into the numerator and denominator of Equation 13.5, we get:

$$\alpha = \frac{\dfrac{[R_0]_{\text{total}}}{1 + \dfrac{K_{D,R_0}}{[L]}}}{\displaystyle\sum_{i=1}^{N} \left(\dfrac{[R_i]_{\text{total}}}{1 + \dfrac{K_{D,R_i}}{[L]}} \right)} \qquad (13.9)$$

Equation 13.9 allows us to calculate the specificity, α (that is, how much of the ligand is bound to the specific receptor), if we know the dissociation constants and the ligand concentration. A high value of α means that the primary target receptor is preferentially bound over the secondary receptors, shown schematically in **Figure 13.5**.

13.4 The specificity of binding depends on the concentration of ligand

What conditions favor high specificity in binding? From Equation 13.9, the value of α is large when the numerator is large but the denominator small. If we assume that the target and secondary receptors are at similar concentrations, α will be large when $K_{D,R_0} \leq [L] \leq K_{D,R_i}$ (see Figure 13.5A). In this range the target receptor is occupied significantly, but the secondary receptors are not.

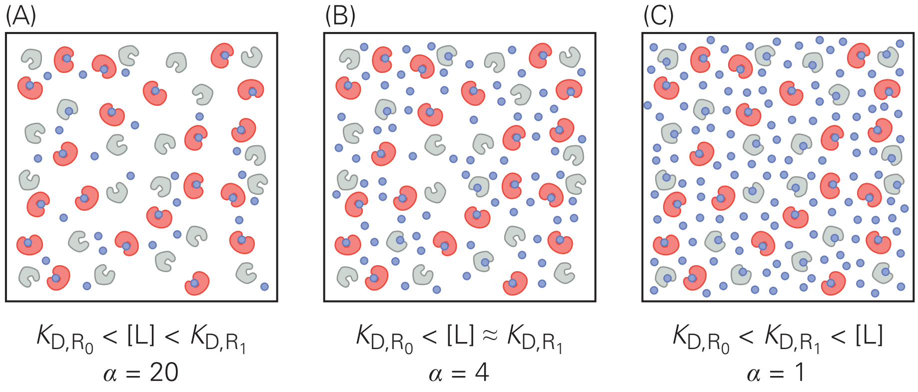


Figure 13.5 Specificity of a ligand for two receptors. The two receptors are denoted R_0 for a primary, high-affinity, receptor (*red*) and R_1 for a secondary one (*gray*). (A) When ligand is present at concentrations greater than K_{D,R_0} but less than K_{D,R_1}, most of the ligand will be associated with R_0. The binding is highly specific under these circumstances. If [L] equals (B) or exceeds (C) K_{D,R_1}, then both receptors are occupied by ligand, thus lowering specificity.

If the ligand concentration is increased so that $[L] \approx K_{D,R_i}$ for some of the secondary receptors, then the value of α starts to decrease (see Figure 13.5B). When free ligand is present at high concentration, meaning that $[L]/K_{D,R_0} \gg 1$ and $[L]/K_{D,R_i} \gg 1$, then the value of α is small. If we assume that the primary target and secondary receptors are present at the same concentration, $[R_0]_{total} = [R_i]_{total}$, then the value of α approaches $1/N$, where N is the number of receptor types. Under these conditions, both the target and the secondary receptors are fully occupied and the specificity factor is just the ratio of the number of target receptors to the total number of alternative receptors.

The value of α as a function of ligand concentration is graphed in **Figure 13.6** for the simple case when there are just two kinds of receptors, R_0 and R_1. At low ligand concentration, the primary receptor "wins," and the specificity is high. But, as the ligand concentration increases, an increasing amount of ligand binds to the secondary receptor, and the specificity decreases. Two situations are shown in this figure. In one case, the affinity of the primary receptor is much higher than that of the secondary receptor. In the other case, the difference between the affinities is not so large. When the values of K_{D,R_0} and K_{D,R_i} are very similar, then it is not possible to have high specificity (unless off-target receptors are only present at very low concentration), as can be seen in Figure 13.6.

This analysis also helps us understand the potential effects of a structural change in the ligand, L. If L is a protein whose sequence is altered through a mutation, for example, then binding to both target and secondary receptors may be modified. If the altered residue improves the steric fit to the primary receptor, R_0, or enhances the charge complementarity to it, then the affinity of the ligand for the R_0 receptor will increase (that is, K_{D,R_0} becomes smaller). Such a change may result in an increase in specificity if the altered interactions do not also increase the affinity of the ligand for the secondary receptors by an equal amount. Mutations that widen the gap between K_{D,R_0} and K_{D,R_i} enhance specificity and are more likely to be kept during natural selection, while those that reduce this gap lead to lower specificity and are likely to be discarded during evolution.

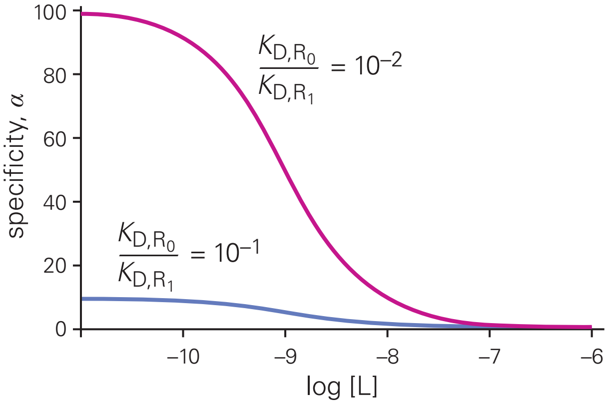


Figure 13.6 Specificity as a function of ligand concentration. In this example, there are two receptors, R_0 and R_1. The specificity, α, is shown as a function of ligand concentration for dissociation constants of 10^{-9} for R_0 and 10^{-7} for R_1 (upper curve) and 10^{-9} for R_0 and 10^{-8} for R_1 (lower curve). In both cases, the specificity decreases with increasing ligand concentration, reaching a value of 1 at high ligand concentration.

13.5 Fractional occupancy and specificity are important for activities resulting from binding

The activity of a receptor is usually determined by whether a ligand is bound to it or not. It is therefore useful to describe the activity of a receptor in terms of fractional occupancy, a concept that we developed in Section 12.2. In particular, with the ligand–receptor notation used in this chapter, the fraction of receptors occupied by ligand, f_{R_i}, is:

$$f_{\mathrm{R}_i}=\frac{[\mathrm{R}_i\bullet\mathrm{L}]}{[\mathrm{R}_i]+[\mathrm{R}_i\bullet\mathrm{L}]}=\frac{[\mathrm{R}_i\bullet\mathrm{L}]}{[\mathrm{R}_i]_{\mathrm{total}}} \qquad (13.10)$$

Hence, we can rewrite Equation 13.5 (the expression for the specificity, α) in terms of the fractional occupancies:

$$\alpha=\frac{f_{\mathrm{R}_0}[\mathrm{R}_0]_{\mathrm{total}}}{\sum_i f_{\mathrm{R}_i}[\mathrm{R}_i]_{\mathrm{total}}} \qquad (13.11)$$

As a result, a large fractional occupancy of the target receptor, R_0, and a low occupancy of off-target receptors, R_i, corresponds to high specificity.

It can be instructive to look at a simple case in which the ligand has just two different affinities for receptors, high for the primary receptor and 100 times weaker for all of the secondary receptors. For example, we assume that all receptors are present at equal total concentration, $[\mathrm{R}_0]_{\mathrm{total}} = [\mathrm{R}_i]_{\mathrm{total}}$, but with 10 different secondary receptors. In this case,

$$\alpha=\frac{f_{\mathrm{R}_0}[\mathrm{R}_0]_{\mathrm{total}}}{10f_{\mathrm{R}_i}[\mathrm{R}_i]_{\mathrm{total}}}=\frac{f_{\mathrm{R}_0}}{10f_{\mathrm{R}_i}} \qquad (13.12)$$

As makes intuitive sense, the specificity depends on the ratio of the fractional occupancies (**Figure 13.7**). When ligand concentration is low (that is, when $[\mathrm{L}]/K_{\mathrm{D,R}_0}\ll 1$), you can see from Equation 13.9 that:

$$\alpha=\frac{\dfrac{[\mathrm{R}_0]_{\mathrm{total}}}{1+\dfrac{K_{\mathrm{D,R}_0}}{[\mathrm{L}]}}}{\sum_{i=1}^{N}\dfrac{[\mathrm{R}_i]_{\mathrm{total}}}{1+\dfrac{K_{\mathrm{D,R}_i}}{[\mathrm{L}]}}}\approx\frac{\dfrac{[\mathrm{R}_0]_{\mathrm{total}}[\mathrm{L}]}{K_{\mathrm{D,R}_0}}}{\sum_{i=1}^{10}\dfrac{[\mathrm{R}_0]_{\mathrm{total}}[\mathrm{L}]}{100K_{\mathrm{D,R}_0}}}=10 \qquad (13.13)$$

As the concentration of ligand increases, the occupancy of R_i increases and the specificity will decrease, reaching a limiting value of 0.1.

13.6 Most macromolecular interactions are a compromise between affinity and specificity

The requirements for generating high affinity and high specificity can often be conflicting for interactions between macromolecules. As we discussed in Section

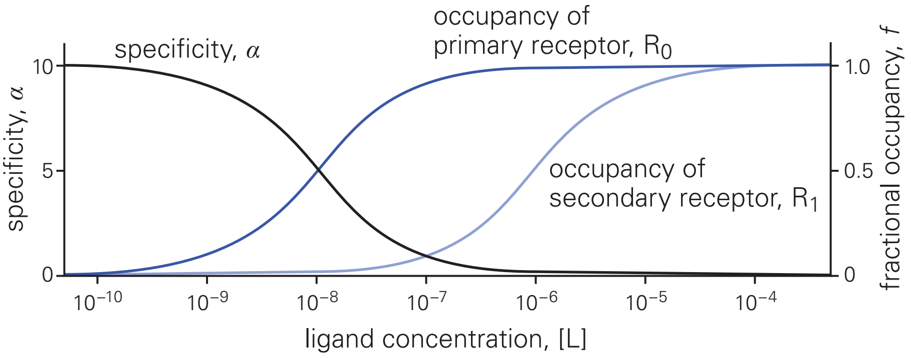


Figure 13.7 Graph of specificity and receptor occupancies as a function of ligand concentration. Curves are shown for the occupancy of both the primary receptor ($K_{\mathrm{D}} = 10^{-8}$) and 10 off-target receptors ($K_{\mathrm{D}} = 10^{-6}$), and the specificity, α. Near $[\mathrm{L}] = 10^{-7}$, there will be equal occupancy of the primary receptor and the set of secondary receptors; hence, $\alpha \approx 1$ at that point.

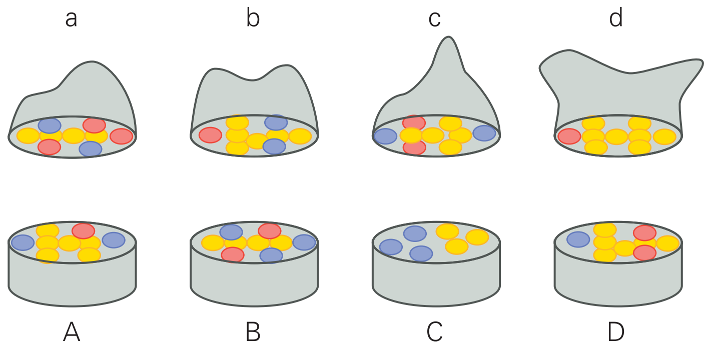


Figure 13.8 Charge interactions and specificity. Polar and electrostatic interactions, even if they do not provide net stabilization, can provide specificity by destabilizing off-target complexes. In this schematic, the ligands a, b, c, and d could potentially pair with receptors A, B, C, and D. The *yellow*, *blue*, and *red* regions are hydrophobic, positively charged, and negatively charged, respectively. The pairing b–D will be very specific.

12.19, the affinity of a noncovalent interaction is most easily increased by making the interface more hydrophobic. Hydrophobic interactions have lower specificity, however, because there are many hydrophobic amino acids, and many orientations of each one, that can make up a hydrophobic contact surface.

Polar or charged residues at an interface will only contribute favorably to the free energy of binding if there are complementary functional groups at specific positions on the opposite surface (**Figure 13.8**). For example, bringing a negatively charged residue into contact with a hydrophobic surface is unfavorable because the strong solvent interactions of the charged group are lost. But, if the contacted surface has an appropriately positioned positive charge, then the favorable electrostatic interaction compensates for the loss of solvent interactions. Many factors have entered into the evolution of specific interactions, including shape complementarity of surface hydrophobic patches, hydrogen bonding, and electrostatics.

The difference between on-target versus off-target affinities is the important factor for specificity, as shown in Figure 13.6. Interactions that lead to specificity quite often destabilize off-target complexes as often as they stabilize on-target ones. Because of this aspect of specificity, nonpolar contacts can play important roles in determining specificity, even when highly charged molecules such as nucleic acids are being recognized.

13.7 Fibroblast growth factors vary considerably in their affinities for receptors

Having now developed a precise definition of specificity, we return to the system of fibroblast growth factors (FGFs) and their receptors. As described in Section 13.2, there are many possible ligand–receptor pairings that can occur. The binding of several FGFs to different receptors has been studied quantitatively using purified ligands and receptors. Some of the dissociation constants that were determined are listed in **Table 13.1**. In addition to the natural receptors, folded RNA molecules have been developed that bind to FGFs with high affinity (RNAs do not normally bind to FGFs – these RNAs are artificial receptors for the FGFs). Binding of an FGF to the RNAs prevents binding to a real receptor, and hence blocks signaling. Data for the binding of one particular RNA to the FGFs are also given in Table 13.1.

Based on the data in this table, some FGFs, such as FGF1, bind to several receptors with similar affinity. In contrast, other FGFs are very selective (for example, FGF5). To make this distinction more quantitative, we can calculate values for the specificity, α, using these data. To do this, we assume that all of the receptors are present at the same concentration. We take the ligand concentration to be 100 nM, which is high enough to occupy high-affinity receptor binding sites. Considering FGF1 interacting with the natural receptors, we take the highest affinity receptor,