1

Theory of Ligand Binding to Monomeric Proteins

1.1 Importance of Ligand-Binding Phenomena in Biology

Reversible interactions between or among molecules underlie nearly every aspect of biology. To understand these interactions in a chemical way means to describe them quantitatively. To do so we must be able to determine their affinity, stoichiometry, and cooperativity by carrying out ligand-binding experiments. We use the term “ligand” in a way distinct from its use to discuss coordination complexes within inorganic chemistry. In ligand-binding theory we use this term to mean any interacting partner. Although many people consider a ligand to be a small molecule that interacts with a macromolecule, in fact, either partner can be considered to be the ligand of the other. In a typical experiment the concentration of one partner is held fixed while the concentration of the other partner is incremented. In ligand-binding theory and practice we define the ligand operationally as the partner whose concentration is incremented during the experiment. Such experiments resemble pH titrations both practically and theoretically, and thus are referred to as titrations. The partner whose concentration is held fixed is referred to as the “target,” and again the definition is strictly operational, that is, either a small molecule or a macromolecule can be the target, depending on how the titration is set up.

As we will show later, there are good reasons to carry out experiments with first one partner, then the other, treated as the ligand; however, depending on the chemical properties of the two partners in the reaction, there may be practical limitations to, or conceptual difficulties in, the possibility of interchanging their respective roles as the ligand and the target. In particular, if either of the reaction partners has multiple binding sites for the other, one may not obtain superimposable binding isotherms when exchanging the target and the ligand.

Affinity refers to the strength of interaction between partners. Affinity is quantitatively expressed by an equilibrium constant that we measure in our experiment or, equivalently, a free energy difference between the bound and free states of the system that we calculate from the equilibrium constant.

Stoichiometry refers to the number of molecules of each partner that participate in the binding process, and it must also be determined by our measurements. In practice, what we really mean by stoichiometry is more often molar ratio. For example, in a process involving four molecules of one kind with two of another kind, the stoichiometry...
is 4:2 but the molar ratio is 2:1. The determination of true stoichiometries usually requires additional information from sources other than a binding experiment (e.g., molecular weights, and state of aggregation of the target and ligand in solution).

Thermodynamic linkage is a general term that applies to ligand-binding experiments in which the same target binds two or more molecules of the same or different ligands, and each ligand modulates the affinity of the target for the other. There are at least three different types of linkage, called identical, homotropic, and heterotropic (Wyman and Gill, 1990). Identical linkage occurs when two different ligands compete for the same binding site on the target, and their binding is mutually exclusive. This type of linkage is discussed in Section 1.8 for single binding site targets, and in Section 4.8 for multiple binding site targets. Competitive enzyme inhibition is a very important case of identical linkage and is described in Section 8.6. Homotropic linkage occurs when the target can bind more than a single molecule of the same ligand, with different affinity. Homotropic linkage can occur only in targets with multiple binding sites, thus its analysis is deferred to Chapter 4. Finally, heterotropic linkage occurs when the target can bind two different ligands in a non-exclusive manner and the binding of one ligand alters the affinity of the other. It is described in Section 1.9, and for targets with multiple binding sites in Section 4.9. Important examples of heterotropic linkage are uncompetitive enzyme-inhibition (Section 8.7), and regulation of the oxygen affinity of hemoglobin by effectors, including protons (Bohr effect), diphosphoglycerate, or inositol hexaphosphate, dealt with in Chapter 7.

Homotropic and heterotropic linkage are typically regarded as an emergent property unique to proteins, but some non-protein molecules of ~500–1000 Da have been shown to exhibit cooperative binding of their ligands (Rebek, 1985). An interesting and biologically relevant example is provided by the axial ligands of iron-porphyrins (Traylor and Sharma, 1992).

Homotropic and heterotropic linkages may be either positive (if each ligand increases the affinity of the other) or negative (if each ligand decreases the affinity of the other). Cooperativity has often been used as a synonym of linkage, but unfortunately not always with the necessary precision. Often, cooperativity, or positive cooperativity, is used to indicate positive homotropic linkage, but the terms of negative cooperativity or anti-cooperativity may be used to indicate a negative homotropic or heterotropic linkage. The definition of cooperativity is sufficiently general to encompass cases in which even monomeric proteins can respond cooperatively to two different ligands, for example, the physiological ligand and an ionic component of the solution (Weber, 1992). Note that in such cases the ion must be also considered as a physiological effector. Because the general applicability of a definition is inversely related to its precision, in this book, we shall prefer the terms positive or negative homotropic or heterotropic linkage whenever precision is required. Positive cooperativity occurs in several proteins and has special relevance in physiology. For example, the binding of oxygen to hemoglobin is cooperative in that oxygen affinity becomes stronger as binding progresses, as described in detail in Chapter 7.

In this chapter we describe the theoretical bases of ligand binding under equilibrium conditions for protein:ligand complexes with 1:1 stoichiometry; in the next chapter we discuss the kinetics of the same system, and in Chapter 3 we consider some practical aspects of experimental design, and some common sources of errors.
1.2 Preliminary Requirements for Ligand-Binding Study

Whether or not a given ligand binds to a given macromolecular target may be known from prior experiment or inferred from physiological or chemical data. When no such information is available, or when the equilibrium constant for the reaction is required, then the interaction must be evaluated from titration, using a method of analysis that is suitable for the solution conditions of interest and the concentrations of partners that can be practically achieved. Although specific methods will not be detailed here, all useful methods have in common that they offer some observable that changes during, and thus reports on, the binding process. Any observable is referred to as the signal, and its change relates to a shift between the bound and free states of the system.

Whether or not a ligand binds reversibly and without any chemical transformation must also be established. Ligand-binding theory and practice generally refers only to such cases; but we shall cover in this book also some common non-conformant cases, for example, thiol reagents and enzyme-substrate reactions, that of course do begin with a ligand-binding process followed by a chemical transformation. The most common approach to determining reversibility is to simply assume it; this is not appropriate for rigorous scientific work. Reversibility is generally established by showing that the signal change is reversed when concentrations are reduced, for example, by dilution or dialysis. A more stringent criterion is to show that the separated ligand and target are recovered unchanged after their interaction, although it can be difficult to rule out a small extent or minor degree of change. One of the best ways to do so is to repeat the binding measurement itself with the recovered materials to evaluate whether the affinity is the same. However, this method can fail with labile partners.

Finally, the molar ratio and stoichiometry must be determined from the same kinds of binding experiments that are used to determine affinity and cooperativity, using strategies that will be outlined later. Without knowing the correct molar ratio the interpretation of the affinity may be plagued by high uncertainty. Another common, unfortunate, and sometimes untested, assumption is that an interaction has a 1:1 molar ratio. As we will show in Chapter 3, the experimental design required to establish molar ratio is not difficult conceptually, and usually is not practically difficult either. Thus there is no reason whatsoever to leave this very important feature to untested assumption. The analysis of binding data is simpler for the 1:1 stoichiometry case, thus we will treat this case first before expanding the treatment to cases of any molar ratio.

1.3 Chemical Equilibrium and the Law of Mass Action

Every chemical reaction, if allowed enough time, reaches an equilibrium condition in which the rate of product formation from reactants equals the rate of product degradation to reactants. When this condition is reached the concentrations of reactants and products undergo no further net change. The specific chemical composition of the reaction mixture at equilibrium depends on experimental conditions such as temperature, pH, solution composition, and so on, but at any given set of conditions it obeys the law of mass action. This law states that, under the equilibrium condition, the ratio between the product of product concentrations and the product of reactant concentrations,
each raised to a power corresponding to its stoichiometric coefficient, equals a constant, the *equilibrium constant*. Technically, *molar activities should be used*, but under most experimental conditions the concentrations of ligand and protein are low enough to allow the researcher to neglect this distinction.

The equilibrium constant of a chemical reaction is independent of the initial concentrations of reagents and products, but varies with the solution conditions, as discussed further below. The equilibrium constant of a chemical reaction has the units of molarity elevated to a positive or negative, usually integer, power factor that corresponds to the difference between the stoichiometric coefficients of the products and reagents. If the sum of the stoichiometric coefficients of the products equals that of reagents, the equilibrium constant is a pure number.

Ligand binding to proteins usually conforms to the above description, and can be described as:

\[ P + X \rightleftharpoons PX \]  

(eqn. 1.1)

where P represents the unliganded protein, X the molecule that binds to it and PX the protein-molecule complex. For practical reasons, it is often convenient to keep constant the protein concentration and to vary in the course of the experiment the concentration of the molecule X, in which case P is the target and X the ligand. Thus, unless differently specified, we shall assume that X is the ligand.

When the above reaction reaches its equilibrium condition, the law of mass action dictates:

\[ K_a = [PX] / [P][X] \]  

(eqn. 1.2)

where the square brackets indicate the molar concentrations of the chemical species involved, and the subscript “a” indicates the association direction of the reaction.

A typical example of the above reaction is that of respiratory proteins that reversibly bind oxygen, for example, myoglobin (Mb):

\[ \text{MbO}_2 \rightleftharpoons \text{MbO}_2 \]

Reversible chemical reactions may be written, and observed experimentally, in either forward or reverse direction. Thus one may write reaction 1.1 in the form of the dissociation of the protein-ligand complex:

\[ PX \rightleftharpoons P + X \]

with

\[ K_d = [P][X] / [PX] \]  

(eqn. 1.3)

where the subscript “d” refers to the dissociation direction. From equations 1.2 and 1.3 we observe that the equilibrium constant of the dissociation reaction is the reciprocal of that of the combination reaction. The dissociation equilibrium constant has units of molar concentration, whereas \( K_a \) has units of reciprocal concentration. Thus \( K_d \) can be directly compared to the concentration of the free ligand. Exceptions to this rule do exist (see Section 1.11) and can be a cause of confusion.

A consistent and unique formulation for ligand-binding experiments, using either the association or the dissociation reactions and equilibrium constants, would probably be
desirable, but is not likely to be universally adopted. Indeed, we usually design an experiment considering the association reaction, because adding the ligand to the unliganded protein is more obvious and easier to do than dissociating an already-formed complex (although dissociation is possible, e.g., by dilution, chemical transformation of the unbound ligand, or phase extraction). Once the association experiment has been carried out, however, we often switch our reasoning to the dissociation reaction because the dissociation constant, having the units of a molar concentration, corresponds to a point on the ligand concentration axis of the binding plot, and actually the ligand concentration itself can be expressed as a multiple or sub-multiple of $K_d$. Thus, in the literature one finds experiments, analyses, and models developed in both ways and must be familiar with both.

Unless one directly measures $[P]$, $[X]$, and $[PX]$ after the equilibrium condition has been reached, one only knows the total concentrations of the ligand and the protein, that is, $[X]_{\text{tot}} = [X] + [PX]$ and $[P]_{\text{tot}} = [P] + [PX]$ (for a 1:1 reaction). If this is the case, eqn. 1.3 should be rewritten as:

$$K_d = \frac{([P]_{\text{tot}} - [PX])([X]_{\text{tot}} - [PX])}{[PX]} \quad \text{(eqn. 1.4)}$$

Although eqn. 1.4 can be easily solved for $[PX]$, yielding a second-degree equation, the procedure is not completely straightforward. Indeed, the more $[PX]$ approaches $[X]_{\text{tot}}$, the greater the uncertainty in $[X]$ and, consequently, in $K_d$.

A great simplification can be achieved under conditions in which either (i) both the free and bound ligand (i.e., $[X]$ and $[PX]$) can be measured directly (e.g., by using equilibrium dialysis); or (ii) $[X]_{\text{tot}} \gg [PX]$. As we shall demonstrate in Section 1.4, the latter condition implies $[P]_{\text{tot}} \ll K_d$. If the experiment can be run under this condition, only a small fraction of the total ligand will be bound and $[X] \approx [X]_{\text{tot}}$, making the use of eqn. 1.4 unnecessary, and allowing direct use of eqns. 1.2 or 1.3. It may happen that, depending on $K_d$ and the experimental method chosen, this condition cannot be met, as it would require protein concentrations too low to be detected. We shall discuss experimental approaches that may overcome this limitation in Sections 1.8 and 1.9.

1.4 The Hyperbolic and Sigmoidal Representations of the Ligand-Binding Isotherms

The graphical representation of binding measurements is important because it is usually difficult to visualize equations like those in the above paragraph or the more complex ones we shall encounter in the following chapters. Some graphical representations may offer clear indications of some property of the system, but may distort or alter other properties. Thus some caution in their use is in order, especially when we want not only to look at them, but to use them for quantitative analysis, that is, to determine the values of the parameters describing the binding reaction. In the present section we shall describe the simplest graphical representations of the ligand-binding isotherm, that is, the hyperbolic plot of $[PX]$ versus $[X]$ and its variants. These representations do not usually distort the experimental error, and should be preferred for quantitative analysis. More complex, and distorting, plots will be considered in a following section.

The soundest and statistically least biased way to represent ligand-binding data is to plot the signal recorded in the experiment, whatever it may be, as a function of the free
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The signal will be discussed further in Chapter 3; it is a detectable physical property of the system that depends on the concentrations of P, PX, or both. Thus the graph of the signal is essentially equivalent to a plot of [P] or [PX] versus [X]. However, for the sake of clarity, the researcher may decide to calculate and report the concentration of the bound protein ([PX]) or its fraction ([PX]/[P]_tot) rather than the experimentally recorded signal. The graph of the fraction of bound protein, that is, the ratio between bound and total binding sites, versus the concentration of the free ligand, or total ligand if [X]_tot >> [PX], is probably the most commonly adopted representation in the biological context.

In order to define the relationship between [PX] and [X], we need to define the binding polynomial of the reaction. The binding polynomial expresses the sum of all species of the target as a function of one of them that is adopted as a reference species. For example, if the experiment conforms to eqn. 1.3 and we adopt as a reference the concentration of the unliganded protein P, we can write:

\[
[PX] = [P][X]/K_d
\]

(eqn. 1.5a)

\[
[P]_\text{tot} = [P] + [PX] = [P] \left(1 + [X]/K_d\right)
\]

From eqn. 1.5 we obtain:

\[
[P] = [P]_\text{tot} K_d/(K_d + [X])
\]

(eqn. 1.5b)

or

\[
[PX] = [P]_\text{tot} - [P] = [P]_\text{tot} [X]/(K_d + [X])
\]

(eqn. 1.5c)

Both equations correspond to rectangular hyperbolas, which asymptotically tend either to zero (1.5b) or to [P]_tot (eqn. 1.5c).

EIQ. 1.5c is most often employed and can be rearranged to represent the fractional saturation, defined as the fraction of bound over total ligand binding sites:

\[
\chi = [PX]/[P]_\text{tot} = [X]/(K_d + [X])
\]

(eqn. 1.6)

The fractional saturation is called in the ligand-binding literature \(\chi, \bar{\chi}, \theta, \) or \(\nu\). We prefer \(\chi\) since in reactions involving two or more ligands this allows us to call \(\chi\) the fractional saturation of the target for ligand X, \(\bar{\chi}\) for ligand Y and so on (see below).

EIQ. 1.6 describes a rectangular hyperbola with unitary asymptote (Figure 1.1A). For many biological systems this is the most meaningful representation of experimental data and it has been preferred traditionally by physiologists studying systems as different as oxygen carriers, hormone receptors, and enzymes. This representation has also the advantage of introducing minimal distortions in the experimental data and their errors. It is important to stress that the ligand concentration that appears in the law of
mass action, and hence in all the equations we developed thus far, except eqn. 1.4, refers to the free ligand, that is, \([X]_{\text{tot}} - [PX]\).

A variant of the hyperbolic plot \(X\) versus \([X]\) is obtained if one reports \(X\) as a function of the logarithm of the free ligand concentration. Chemists may prefer this representation over the hyperbolic one because the chemical potential of the ligand is proportional to the logarithm of its concentration (or activity), and thus the plot emphasizes some properties of the system that do not appear clearly in the hyperbolic plot. However, it is unusual for the physiological ligand concentrations to vary over several orders of magnitude; thus, this plot compresses the physiological range of ligand concentrations in a small region of the curve and physiologists seldom use this representation. The \(X\) versus log([X]) plot has the same overall accuracy as \(X\) versus \([X]\) plot, and does not distort the experimental error. Actually, the error on ligand concentration, which is usually assumed to be negligible, may in some cases be better represented on a logarithmic than a linear scale (see Chapter 3).

From eqn. 1.6 one may calculate the free ligand concentration (expressed as a multiple or sub-multiple of \(K_d\), as shown on Figure 1.1) required to achieve any value of \(X\). As an example, we may calculate that at \([X]=1/3 \ K_d\) we have \(X = 0.25\) and at \([X]=3 \ K_d\), \(X = 0.75\); or that to raise \(X\) from 0.1 to 0.9 an increase of the logarithm of \([X]\) of 1.91 units is required. Generalizing, if we consider ligand concentrations that are multiples or sub-multiples of the \(K_d\) by the same factor \(i\), we have that \(\bar{X}_{[X]/K_d/i} = 1 - \bar{X}_{[X]/K_d}\), which demonstrates that the \(X\) versus log([X]) plot is symmetric (at least in the cases of 1:1 and 1:2 stoichiometries).

An important property of the \(X\) versus log([X]) plot is that its shape is perfectly invariant with respect to \(K_d\): that is, if one explores a set of experimental conditions that cause \(K_d\) to vary, one obtains a series of symmetric sigmoidal curves of identical shape, shifted right or left according to their different \(K_d\)s. The shape of the plot can be quantified.

**Figure 1.1** Two common representations of ligand-binding isotherms. Panel A: the \(X\) versus \([X]\) plot; Panel B: same as A but \([X]\) is logarithmically scaled. In both panels the points are calculated from eqn. 1.6. The two lines above and below the points on each panel are obtained by adding and subtracting a constant error of 0.01 \(X\) units, and demonstrate that in these representations there is no distortion of the error.
as the approximate slope of the straight line passing through two points symmetric with respect to the center of the curve. For example, the points $\bar{X}_{[X]=K_d/3} = 0.25$ and $\bar{X}_{[X]=3K_d} = 0.75$ are symmetric in the sense defined above, and are joined by a straight line with slope $\sim 0.52$ (for a single-site macromolecule). The slope of the $\bar{X}$ versus log $([X])$ plot was called the binding capacity of the macromolecule by Di Cera and Gill (1988), in analogy with the physical concept of heat capacity. This terminology is potentially confusing because the term capacity is also used to indicate the total amount of some substance that the sample may contain (e.g., the maximum amount of oxygen that a given volume of blood can transport is commonly referred to as its oxygen capacity), which is not what these authors meant. Nevertheless, the concept conveyed by the slope is important because changes in slope from the canonical value given above provide important clues on the binding properties of the protein, and/or the composition of the experimental system. In particular, as we shall demonstrate further on, positive cooperativity increases the slope, whereas chemical heterogeneity of the protein or negative cooperativity decreases it (see Chapters 3 and 4).

In the present chapter we deal only with single-site targets, whose changes in ligand affinity shift the position of the $\bar{X}$ versus log $([X])$ curve along the X axis but do not change its shape, and in particular do not change its slope; this will be true for all types of linkage considered in this chapter (Sections 1.8–1.10) and for the effect of temperature (Section 1.7). However we call the attention of the reader to this point, because in later chapters we shall describe systems having steeper or shallower $\bar{X}$ versus log $[X]$ plots, and we shall develop an interpretation of the increased or decreased slope of these plots, which may constitute the first indication that the protein:ligand stoichiometry differs from 1:1.

The derivative of the $\bar{X}$ versus log $([X])$ plot, $\Delta \bar{X}$ versus log $([X])$, has been used by S.J. Gill to describe the ligand-binding isotherms of hemoglobin, as recorded using the thin layer dilution method (Figure 1.2) (Gill et al., 1987). This is a very specialized

![Figure 1.2](image)

**Figure 1.2** Plot of $\bar{X}$ increments versus $[X]$ (on a log scale). Same data set as in Figure 1.1; because the errors added to calculated $\bar{X}$ values are constant and independent of $\bar{X}$, in this plot the points and the lines exactly overlap. Notice that the values on the ordinate axis depend on the dimension of the intervals of log $[X]$ explored and that to obtain a readable plot, these should be kept constant.
plot justified by the experimental method used, and true to the experimental data in that it aims to minimize the error distortion implicit in other types of plots. However, this graphical representation is strongly linked to the experimental method, and greatly benefits from constant logarithmic steps of increase or decrease of the ligand concentration, which is why it never gained widespread use. A more general use of the derivative plot of the sigmoid-binding curve, applicable independently of the specific experimental setup, is to reveal the inflection point (i.e., the midpoint, $X_{1/2}$, whose significance is discussed in the next Section) even when the sigmoid is incomplete.

### 1.5 **The Important Concept of $X_{1/2}$**

An important point of the $X$ versus $[X]$ or the $X$ versus log $[X]$ plots is their midpoint. We may calculate that $X$ equals 0.5 (saturation of half the available binding sites) when $[X]=K_d$, consistent with the symmetry of the $X$ versus log $[X]$ plot for monomeric proteins.

The free ligand concentration required to achieve $X=0.5$ is called $X_{1/2}$ (or $X_{50}$) and is always a function of $K_d$. However, the simple equation $X_{1/2}=K_d$ applies only to the simple cases of monomeric, single-site proteins or multiple, identical, non-interacting sites; we shall consider below some cases in which this relationship is more complex and we shall devote Chapters 4, 5 and 7 to proteins with multiple interacting ligand binding sites.

In view of the widespread use and misuse of the term $X_{1/2}$, and its equivalence with $IC_{50}$ in enzymology (Section 8.6), it is important to provide a clear and unequivocal definition. $X_{1/2}$ is the free ligand concentration required to saturate half the available binding sites under the chosen experimental conditions, provided that chemical equilibrium has been reached. This definition is consistent with that of $IC_{50}$ (Cheng and Prusoff, 1973) and stresses the fact that $X_{1/2}$ is a thermodynamic parameter that may depend on the experimental conditions, but must be independent of the direction in which the equilibrium condition is approached (e.g., whether the experiment was carried out by successive additions of the ligand to the target, as in a titration, or by removing the ligand by dilution or dialysis). Moreover, $X_{1/2}$ cannot depend on the time required by the mixture to reach its equilibrium state.

A related, but perhaps less intuitive, concept is that of $X_m$, the ligand concentration required to express half the binding free energy change. If the $X$ versus log $[X]$ plot is symmetric, as always occurs in monomeric, single-site proteins, $X_m = X_{1/2}$ (Wyman 1963; Ackers et al., 1983).

Due to its necessary relationship with $K_d$, $X_{1/2}$ is commonly used as an overall empirical parameter to define the apparent affinity of the protein for its ligand. However, $X_{1/2}$ may depend not only on $K_d$, but also on the concentration of components of the mixture other than $X$. If this happens, the type of dependence, whether linear, hyperbolic, or other, is a clue to the type of thermodynamic linkage between $X$ and these components (Sections 1.8 and 1.9).

### 1.6 **Other Representations of the Ligand-Binding Isotherm**

Before the widespread use of electronic computers, the analysis of binding isotherms was carried out using graphical methods, and representations aimed at linearizing the $[PX]$ versus $[X]$ hyperbola were widely employed. Unfortunately, the transformations required to linearize the hyperbola entail significant distortion of the magnitude of the
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Experimental error, and are statistically unsound, thus they should not be used to obtain a quantitative estimate of the thermodynamic parameters (i.e., $K_d$ or $X_{1/2}$). They are nevertheless discussed here because a thorough understanding of these representations (and their weaknesses) is required to read and understand many classical papers on ligand binding; moreover, these representations may still have some limited usefulness because they may provide visual evidence of some property of the system that must be confirmed using statistically sounder methods.

A classical and widely employed plot was proposed by the Nobel laureate Archibald Vivian Hill to represent his hypothesis on hemoglobin cooperativity. The Hill plot is based on a simple re-elaboration of eqn. 1.6, which yields:

$$\frac{[PX]}{[P]} = \frac{X}{1 - X} = \frac{[X]}{K_d}$$

and in logarithmic form:

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

The Hill plot for a single site macromolecule is a straight line with unitary slope and intercept $-\log (K_d)$. Although this representation of experimental data lacks a sound thermodynamic basis and was based on a hypothesis for the function and structure of hemoglobin that was later proven wrong (Bellelli, 2010), it had considerable success due to its simplicity and apparent information content. Indeed, it allows the researcher to measure $\log (K_d)$ using a ruler, by drawing a straight line through the experimental points in the range $0.1 < Y < 0.9$ (outside this range the experimental error affects the data very significantly, as shown in Figure 1.3), an important

![Figure 1.3](image.png)

**Figure 1.3** The Hill (A) and Scatchard (B) plots.

Ligand concentration is expressed as multiples of $K_d$ (i.e., $[X]/K_d$).

These representations severely distort the experimental errors (continuous lines are calculated to represent an error of 0.01 on $X$, i.e., $X + 0.01$ and $X - 0.01$), and should never be used for quantitative analyses. However, they may be helpful to visualize some qualitative details of the ligand-binding curve (e.g., the presence of heterogeneity or cooperativity), and may maintain some value for figurative purposes.
advantage at a time when personal computers able to implement sounder statistical methods were not available. Moreover, deviations from the unitary slope may indicate either heterogeneity or cooperativity, consistent with Hill’s original objective (see Chapter 7).

Like several other graphical representations, the Hill plot severely distorts the experimental errors of the original data set. Thus it can be used, at most, as an instrument to depict a property of the system, but should not be used for quantitative analysis (Forsén and Linse, 1995). Some authors advocated a quantitative use of the Hill plot for the special cases of instruments with demonstrated greater precision (and smaller errors) at very low and very high values of ligand saturations (e.g., Imai et al., 1970), but this suggestion cannot be generalized.

Figure 1.3A reports the Hill plot of the data set of Figure 1.1, together with the lines representing the error range of +/−0.01 independent of $X$. The actual amplitude of the error of the experimental points cannot be defined a priori: it is a function of the experimental method used, of the value of the variable one measures, and often of $X$ itself (e.g., the error on absorbance measurements is usually a small fraction of the actual value recorded by the instrument, which is in turn a function of $X$, the protein concentration, and the extinction coefficients of the liganded and unliganded derivatives). Thus, the +/−0.01 confidence interval in Figure 1.3A is only representative of how severely a constant error is distorted in the Hill plot. The figure shows that the central region of the Hill plot, in the range $−1 < \log(\bar{X}/(1−\bar{X})) < 1$ (approximately equivalent to $0.1 < \bar{X} < 0.9$) is relatively free from error distortion, whereas, outside this range, the distortion becomes very pronounced.

The Hill plot has been widely used in the past to empirically evaluate the cooperativity of multimeric proteins (as described in Chapters 4 and 7); however, the advent of personal computers has made it obsolete. Moreover, its slope fails to provide a thermodynamically interpretable quantitative measure of the magnitude of cooperativity (Forsén and Linse, 1995) and remains an empirical parameter essentially devoid of any direct physical meaning. However, since the Hill plot has been widely employed in the past, interpretation of a host of literature data entails an understanding of its parameters ($\log(X_{1/2})$ and the slope, often called the Hill coefficient $n$).

The Scatchard plot is another type of graphical representation of great historical value that is unsuitable for quantitative data analysis, despite continuing to be in use. It aims to highlight different properties of a binding system than the Hill plot, but shares the same concerns for error distortion. This graphical representation was proposed by G. Scatchard (Scatchard, 1949) and is based on the following linear rearrangement of eqn. 1.6:

$$\frac{[PX]}{[X]} = \frac{[P]}{K_d} = \frac{[P]_{tot}}{K_d} - \frac{[PX]}{K_d}$$

or:

$$\bar{X}/[X] = 1/K_d - \bar{X}/K_d$$

Thus, a plot of $[PX]/[X]$ versus $[PX]$ (or $\bar{X}/[X]$ versus $\bar{X}$) yields a straight line with slope $−1/K_d$ and two intercepts: with the abscissa at $[P]_{tot}/K_d$ (or $1/K_d$) and with the ordinate at $[P]_{tot}$ (for the case of 1:1 binding) (Figure 1.3B).
The Scatchard plot may seem to have little to offer: (i) it severely distorts the experimental errors; (ii) it uses [PX], which is error-prone, in both the dependent and independent variables; and (iii) it is strongly counterintuitive, as can be seen from a comparison of Figure 1.3B with the same data plotted in Figure 1.1.

The Scatchard plot was used to graphically detect heterogeneity of the protein preparation (i.e., the presence of isoforms or non-equivalent binding sites; see Section 3.10); however, it is obsolete, because the same information can be obtained by sounder statistical methods at the expense of minimal computing power.

1.7 Effect of Temperature: Thermodynamic Relationships

The reaction scheme of eqn. 1.1 neglects any energy contribution to the formation or dissociation of the protein-ligand complex; a more reasonable scheme would be:

\[ P + X + Q \leftrightarrow PX \quad \text{or} \quad P + X \leftrightarrow PX + Q \]  
\[ \text{eqn. 1.7} \]

In which energy (\( Q \), which may indicate the free energy, the enthalpy or the entropy change) appears as a reactant or as a product of the reaction. Many ligand-binding reactions are exothermic, that is, they release heat as a consequence of the formation of the protein-ligand bond(s); however, there are also examples of endothermic reactions, in which the amount of heat released upon bond formation is exceeded by the heat absorbed because of changes within the protein or the ligand (eqn. 1.7).

The fundamental relationship between the reaction free energy and the equilibrium constant is:

\[ \Delta G^0 = -RT \ln K \]  
\[ \text{eqn. 1.8} \]

The \( \Delta G^0 \) (\( = G^0_{\text{products}} - G^0_{\text{reagents}} \)) is defined for standard thermodynamic conditions (1 M for every species in solution, 1 atm for gases), and measures the free energy that is absorbed or released during the process in which the system, starting from the standard conditions, reaches its chemical equilibrium state (at constant temperature). Biological systems rarely or never can be equilibrated under standard conditions, thus their free energy is corrected for their actual conditions and is called their \( \Delta G' \); the correction is such that a system under equilibrium conditions has \( \Delta G' = 0 \). \( \Delta G' \); if different from zero, defines the direction in which the system will evolve: if \( \Delta G' > 0 \) then evolution is in the direction of a net increase of products at the expense of the reactants; if \( \Delta G' < 0 \) then evolution is in the opposite direction. Progression of the reaction changes the concentrations of reactants and products, hence the \( \Delta G' \) that becomes zero when equilibrium is reached.

Eqn. 1.8 allows the researcher to calculate the binding energy of the protein-ligand pair, or, to be more precise, the free-energy difference between the PX complex and the dissociated P and X pair. Thus, one might completely replace the equilibrium constant with the binding free energy. Although we do not suggest the reader to take this step, it is useful to keep in mind the order of magnitude of the binding energies commonly encountered in biological systems; thus a \( K_d \) of 1 mM corresponds to a free-energy difference of approximately 4 kcal/mole of complex at 25 °C, a \( K_d \) of 1 uM to 8 kcal/mole,
and a $K_d$ of 1 nM to 12 kcal/mole. These values have a positive sign in the direction of dissociation, that is, free energy is released when the complex forms and is absorbed when the complex dissociates.

One should resist the simplistic idea of equating $\Delta G^0$ to the protein-ligand bond energy, since this parameter also includes the energy of any ligand-linked structural rearrangement of the protein and the ligand, and any solvation-desolvation processes of the ligand and the binding site.

The equivalence of equilibrium constants and energies clarifies that no chemical reaction can be truly irreversible, as this would imply an infinite $\Delta G^0$ and would violate the first principle of thermodynamics. What we usually call irreversible is a chemical reaction whose equilibrium constant is so large that the concentration of reagents at equilibrium escapes detection by any practical means. For example, the solubility product of mercuric sulfide is so low that the precipitate is in equilibrium with less than one of the constitutive ions in essentially any volume of water one may practically use, the equilibrium concentrations of mercuric and sulfide ions being of the order of $10^{-27}$ M. In cases like this one, thermodynamic relationships are used instead of direct measurements to infer the equilibrium constants, and we should imagine “concentrations” as the probability of at least one molecule of the reagent to be present in solution at any given time in the reaction mixture.

Protein-ligand interactions rarely approach these affinities, but may still reach levels that may make it difficult or impossible to detect the free ligand. For example, the $K_d$ of the avidin-biotin complex is in the order of $10^{-15}$ M and that of heme-hemopexin is $<10^{-12}$ M, as is that of the Mb-NO complex. In these and similar cases competition (see below and Chapter 3) or kinetic (Chapter 2) experiments may be required to determine equilibrium constants.

The study of the dependence of the equilibrium constant on the temperature allows the researchers to better quantify the components of its $\Delta G^0$. Indeed, if we combine eqn. 1.8 with the Gibbs’ free energy equation:

$$\Delta G = \Delta H - T \Delta S$$

we obtain:

$$\ln K = - (\Delta H^0 / RT) + (\Delta S^0 / R)$$

(eqn. 1.9)

In many simple chemical equilibria $\Delta H^0$ is independent of temperature (at least on small temperature intervals) and the plot $\ln K$ versus $1/T$ yields a straight line, with slope $-\Delta H^0 / R$ and intercept $\Delta S^0 / R$ (the van t’ Hoff plot; Figure 1.4). In such cases one may derive all three fundamental thermodynamic parameters for the reaction, $\Delta G$, $\Delta H$, and $\Delta S$ from determinations of the equilibrium constant at different temperatures. When $\Delta H$ is not temperature independent, the van t’ Hoff plot is curved, and the dependence of $\Delta H$ on temperature conveys information about the heat capacity change for the system.

We stress once more that when we say that a function is a straight line whose slope and intercept correspond to certain parameters, we do not imply that the statistically soundest method to determine those parameters is by linear regression: our aim is to offer to the reader the description of a function or a concept in a way that is easy to visualize and remember. Actually, the soundest method to determine the thermodynamic
parameters of a protein-ligand couple is to fit all the experimental points ($X$ values as functions of $[X]$ and $T$) globally with a function that includes the lowest possible number of relevant parameters. In the present case, this would mean $K_d$ at a given temperature, chosen as the reference state (usually 298 K, independently of the fact that this temperature has been actually explored), $\Delta H^0$, and $\Delta S^0$ (see eqn. 1.9). The $\Delta G^0$ will be derived from Gibbs’ free energy equation.

An interesting practical application of the van’t Hoff plot is to extrapolate the value of $\ln K_d$ to temperatures at which it is too large or too small to be determined directly. For example, if $K_d$ is too small to allow precise determination, for example, because the condition $[P]_{tot} << K_d$ cannot be achieved (see Section 1.3), but the reaction has a large $\Delta H$, the researcher can measure $\ln K_d$ in a range of temperatures where it is greater, calculate $\Delta H$ and extrapolate $\ln K_d$ value at the temperature of interest. Since this approach assumes that the $\Delta H$ is temperature independent, a condition that is not necessarily true, some caution in the use of the final value is required. For this use, the integrated form of eqn. 1.9 (van’t Hoff equation) may be useful:

$$\Delta H = R \frac{T_1 - T_2}{T_1 T_2} \ln \left( \frac{K_2}{K_1} \right)$$

(eqnn. 1.10)

where $K_1$ and $K_2$ are the equilibrium constant measured at temperatures $T_1$ and $T_2$, respectively. Needless to say, $\Delta H$ is lower than zero (negative) in the case of exothermic reactions (heat appearing as a product in eqn. 1.7) and greater than zero (positive) in the case of endothermic reactions (heat appearing as a reactant).

An important point to be considered when determining the apparent $\Delta H$ from van’t Hoff plots is that temperature changes may affect the ligand-binding equilibrium in more aspects than the one described by eqn. 1.9, and their effect on the equilibrium constant is the sum of a large number of contributions, only one of which is the reaction
heat. The heat of solubilization of the ligand, of its dilution, and so on, all contribute; the reaction heat of the buffer may cause pH variations (and in these measurements it is a good idea to use a buffer with as small as possible \( \Delta H \) of protonation, e.g., phosphate), and so on. If the contributions other than the reaction heat cannot be neglected, they should be measured by careful control experiments carried out in the absence of the protein. Moreover, the true reaction \( \Delta H \), after correction for the above contributions, is itself the sum of several components that include the heat of bond formation, the heat of desolvation of the ligand and of the binding site, the heat of eventual structural changes in the protein, and the reaction heat of titratable groups on the protein may cause changes in their protonation state. These components are to be considered because they represent important properties of the biological system under study.

A clear-cut example of the above-mentioned effects is the following. \( \Delta H \) for the reaction of oxygen binding to heme model compounds is \(-14\) kcal/mol (Traylor and Berzinis, 1980). Overall, \( \Delta H \) for the same reaction in hemoproteins from different sources varies over a significant range, from \(-14.9\) for sperm whale myoglobin (Antonini and Brunori, 1971), to \(-11\) kcal/mol for human hemoglobin, down to \(-2\) kcal/mol for musk ox and reindeer hemoglobins (Coletta et al., 1992). The chemical reaction in all the above cases is the same, namely the reversible binding of oxygen to the histidine/imidazole coordinated heme iron. However, when the heme is embedded in the protein moiety, oxygenation is coupled to other reactions (e.g., structural changes and proton release, see Chapter 7), whose \( \Delta H \) adds to that of heme oxygenation, thus explaining the variations.

### 1.8 Replacement Reactions: Competitive Ligands

A biologically important problem is that of the specificity of ligand binding, that is, the ability of a protein to discriminate between two ligands of similar molecular structure that may happen to be simultaneously present in the biological or experimental system. Common examples of this condition are steroid hormones and their receptors, substrates and competitive inhibitors in enzymology, and competition between CO and oxygen for the heme iron in hemoglobin and myoglobin, which leads to carbon monoxide poisoning. The set of relationships that describe a protein and two ligands competing for the same binding site has been called identical linkage by Wyman and Gill (1990). The same authors recognized an analogy with the partition of a solute between two phases and called this experiment the partition of the protein between two ligands (or two complexes), a term that we shall use as synonymous with ligand replacement.

When two similar molecules are present in solution and the protein can bind either but not both of them at the same binding site, the two ligands effectively compete with each other. The intrinsic affinity of each ligand is not affected by the presence of the other, but the apparent affinity is, depending on their relative affinities and concentrations. A detailed understanding of this feature is relevant because one may turn it to advantage. Let us describe the system as follows:

\[
Y + PX \rightleftharpoons Y + X + P \rightleftharpoons X + PY
\]

with:

\[
K_{d,X} = [P][X]/[PX]
\]
and

\[ K_{d,Y} = [P][Y]/[PY] \]

The binding polynomial, which we write using P as the reference species, results:

\[ [P]_{tot} = [P] + [PX] + [PY] = [P](1 + [X]/K_{d,X} + [Y]/K_{d,Y}) \]  \hspace{1cm} (eqn. 1.11)

From eqn. 1.11 we derive the fraction of saturation for both ligands:

\[ \bar{X} = [X]/K_{d,X} / (1 + [X]/K_{d,X} + [Y]/K_{d,Y}) \]  \hspace{1cm} (eqn. 1.12)
\[ \bar{Y} = [Y]/K_{d,Y} / (1 + [X]/K_{d,X} + [Y]/K_{d,Y}) \]  \hspace{1cm} (eqn. 1.13)

and

\[ X_{1/2} = K_{d,X} \left(1 + [Y]/K_{d,Y}\right) \]
\[ Y_{1/2} = K_{d,Y} \left(1 + [X]/K_{d,X}\right) \]  \hspace{1cm} (eqn. 1.14)

Eqn. 1.14 demonstrates that the apparent \( X_{1/2} \) is increased by the factor \([Y]K_{d,X}/K_{d,Y}\) in the presence of the competing ligand Y; that is, the competing ligand shifts the binding curves to the right, as shown in Figure 1.5. Conversely, the experimental finding that the \( X_{1/2} \) of a ligand is directly proportional to the concentration of another component of the solution strongly suggests identical linkage.

Unless both ligands are present at very low concentration relative to their \( K_d \)s, the fraction of the unliganded species P may be neglected, and the reaction scheme may be simplified.

\[ Y + PX \leftrightarrow X + PY \]

![Graph](image)

**Figure 1.5** A typical ligand replacement experiment. The dashed line represents the binding isotherm of ligand X in the absence of competing ligand Y; the three lines on the right represent the replacement of ligand Y by ligand X at constant fixed concentrations of ligand Y (from left to right: \([Y] = 10 K_{d,Y}, 25 K_{d,Y}, 50 K_{d,Y}\) ). Notice the right shift of each curve, that is, the decrease of the apparent affinity for ligand X, as a response to successive increases of the concentration of ligand Y, and their shape invariance, anticipated in Section 1.4.
with:

\[ X = \frac{[X]}{K_{d,X} + \left( \frac{[X]}{K_{d,Y}} + \frac{[Y]}{K_{d,Y}} \right)} = \frac{[X]}{[X] + \left( \frac{[Y]}{K_{d,X}} + \frac{[X]}{K_{d,Y}} \right)} \]  

(eq. 1.15)

Under experimental conditions where the total protein concentration is significantly lower than those of X and Y, eqn. 1.15 describes (and eqn. 1.12 approximates) a binding function analogous to eqn. 1.6, with the apparent constant:

\[ K_p = \frac{[PY][X]}{[PX][Y]} = \frac{K_{d,X}}{K_{d,Y}} \]

Kp, the ligand partition constant, is a measure of the relative specificity of the protein for the two ligands. The value of Kp is easily determined, provided that one can discriminate the two protein-ligand complexes PX and PY from each other (and from the unliganded protein P if present at all under the chosen experimental conditions).

If the experiment is carried out at constant concentration of either one of the ligands (under experimental conditions in which the amount of ligand bound to or released by the protein during the experiment causes negligible variations of its concentration), the above equation reduces to a simple hyperbola, fully analogous to Figure 1.1A, and described by the same equation except that in place of the unliganded protein concentration we find the complex of the protein with the constant ligand. For example, in the case of the concentration of ligand Y being constant:

\[ K_p = \frac{[PY][X]}{[PX][Y]} = \frac{K_{d,X}}{K_{d,Y}} \]  

(eqn. 1.16)

We finally remark that the interpretation of ligand replacement experiments in the case of proteins having more than a single ligand-binding site differs from the one presented above, thus the interested reader is encouraged to compare this section with Section 4.8.

Two practical problems may take great advantage of a ligand replacement experiment: that of ligands that provide poor or no signal, and that of high-affinity ligands, as we shall detail in Chapter 3.

The reason high-affinity ligands benefit from replacement experiments is evident from eqn. 1.15 and Figure 1.5. When K_{d,X} is small with respect to the protein or ligand concentration required to produce a measurable signal (a condition that we recognized above as undesirable), addition of a competing ligand will increase the apparent equilibrium constant by the factor \(1 + [Y]/K_{d,Y}\), which the experimenter controls at will (eqn. 1.14 and Figure 1.5). K_{d,X} is then calculated from K_p or, better, from a series of experiments carried out at different concentrations of ligand Y fitted globally to eqn. 1.12, or to eqn. 1.15 if the concentration of unliganded protein can be neglected, to find the statistically soundest estimates of K_{d,X} and K_{d,Y}. This experimental approach demands great attention to the incubation time required to reach the equilibrium condition after each addition of the variable ligand: indeed high-affinity ligands often have very low dissociation rate constants and replacement equilibration may require many minutes or hours (see Chapter 2).

Replacement reactions can also be used to determine ligand affinities when formation or dissociation of their complexes does not provide a detectable signal. For example, the complex of fatty acid binding protein (FABP) with its physiological ligands is indistinguishable from the mixture of the unbound protein and fatty acids. However, FABP binds the fluorescent probe anilinonaphthalene sulfonate (ANS) at the same site as the
fatty acid, and the reaction is associated with a large increase of its quantum yield. Thus, the affinity of FABP for ANS can be measured directly. In an experiment in which ANS-saturated FABP is titrated with the physiological ligand palmitic acid (or vice versa), the ligand replacement can be followed as a decrease in fluorescence, caused by the release of ANS, and the partition constant of the two ligands can be measured (see Angelucci et al., 2004). From $K_d$ of ANS and the partition constant for the ANS/fatty acid, $K_d$ of the fatty acid can be calculated.

### 1.9 Heterotropic Linkage: Non-Competitive Binding of Two Ligands

Proteins are complex macromolecules and the case of one and the same protein having two binding sites for two different ligands is by no means uncommon. The two different ligands may or may not affect each other’s affinity. When they do, one has the condition called heterotropic linkage by J. Wyman (Wyman, 1948; Wyman, 1964; Wyman and Gill, 1990). Examples of ligands involved in this type of linkage include uncompetitive enzyme inhibitors, allosteric effectors in hemoglobin, enzymes, and many other proteins, and regulatory ligands such as lactose for the repressor protein in the Lac operon.

The reaction scheme is as depicted in Figure 1.6.

The reaction scheme of Figure 1.6 is a thermodynamic cycle, and energy conservation dictates the important property:

\[
K_X^X K_Y^Y = K_Y^X K_X^Y = \frac{[P][X][Y]}{[PXY]} \\
(\text{eqn. 1.17})
\]

Thus any equilibrium constant can be determined from the other three.

In the absence of linkage, the equilibrium constants for each ligand are independent of the presence of the other ligand, that is, $K_X = K_Y^X$ and $K_Y = K_X^Y$. In the presence of linkage, the following relationships apply:

\[
X K_Y = K_Y^Y K_X^X / K_Y \\
Y K_X = K_X^X K_Y^Y / K_X
\]

These relationships may be summarized as follows: if $X$ increases the affinity of $P$ for $Y$ (i.e., $X K_Y < K_Y$) then $Y$ also increases the affinity of $P$ for $X$ (i.e., $Y K_X < K_X$), and we have a case of positive heterotropic interaction. Conversely, if $X$ decreases the affinity of $P$ for

![Figure 1.6](image-url) Reaction scheme for a protein binding two ligands at different binding sites. Notice that the ternary complex $PXY$ is possible in this case, whereas it was impossible in the case of identical linkage.
Y (i.e., $X_K > K_Y$) we have a case of negative heterotropic interaction (and $Y_K > K_X$). Negative heterotropic interactions are particularly common in biology and may play important regulatory roles, typically in negative feedback mechanisms.

The effect of ligand $Y$ on the apparent affinity of the protein for ligand $X$ may be derived from the above relationships (Wyman, 1948, 1964). The binding polynomial for the reaction scheme of Figure 1.6 is:

$$[P]_{tot} = [P] + [PX] + [PY] + [PXY]$$

$$= [P] \left(1 + \frac{[X]}{K_X} + \frac{[Y]}{K_Y} + \frac{[X][Y]}{K_X K_Y}\right)$$  \hspace{1cm} (eqn. 1.18)

If the experiment is carried out at constant concentration of one ligand, for example, $Y$, the fractional saturation of the other ligand results:

$$\tilde{X} = \left(\frac{[PX] + [PXY]}{[P] + [PX] + [PY] + [PXY]}\right)$$

$$= \left[\frac{K_X Y + \frac{Y}{K_X}}{K_Y + \frac{Y}{K_X}}\right] \left(\frac{[K_X + [Y] + [X][K_Y + [Y]/K_X]}{[K_X + [Y] + [X][K_Y + [Y]/K_X]}\right)$$  \hspace{1cm} (eqn. 1.19)

Note that if $K_X = Y_K$,  (absence of linkage between the two ligands) or $[Y]=0$, this case reduces to eqn. 1.6.

$X_{1/2}$ (i.e., the concentration of free ligand $X$ required to half saturate the protein in the presence of ligand $Y$) results:

$$X_{1/2} = K_X \left(\frac{Y + [Y]}{K_Y + \frac{Y}{K_X}}\right)$$  \hspace{1cm} (eqn. 1.20)

which, as expected, is limited between $X_{1/2} = K_X$ in the case $[Y] \ll \left(K_Y, X_K\right)$ and $X_{1/2} = Y_K$ in the case $[Y] \gg \left(K_Y, X_K\right)$. Contrary to the direct proportionality of $X_{1/2}$ to $[Y]$ observed in the ligand replacement experiment described in Section 1.8, in this case the dependence of $X_{1/2}$ on $[Y]$ is hyperbolic and essentially analogous to a binding curve; its midpoint has coordinates $[K_Y Y/K_X, 0.5(K_X + Y_K)]$ (Table 1.1 and Figure 1.7).

An important consequence of the relationships described above is that one can measure $K_Y$ from the effect of $[Y]$ on $X_{1/2}$. This feature may turn out very useful if either ligand yields a poor signal: for example, one can measure the affinity of Hb for its heterotropic effectors, whose binding is spectroscopically silent, from the changes they induce in its affinity for oxygen, which is associated with large changes in spectroscopic signal (Figure 1.8).

**Table 1.1** Relationships between $X_{1/2}$ and $K_d$ in monomeric single-site proteins.

<table>
<thead>
<tr>
<th>$X_{1/2}$ independent of effectors</th>
<th>absence of linkage; $X_{1/2} = K_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_{1/2}$ depends linearly on effector concentration</td>
<td>competitive binding; identical linkage. $X_{1/2}$ as in eqn. 1.14.</td>
</tr>
<tr>
<td>dependence of $X_{1/2}$ on effector concentration is hyperbolic</td>
<td>non-competitive binding; heterotropic linkage. $X_{1/2}$ as in eqn. 1.20.</td>
</tr>
</tbody>
</table>
Reversible Ligand Binding

Figure 1.7 A comparison of identical and heterotropic linkages.
Panel A: Identical linkage between ligands X and Y causes $X_{1/2}$ to be a linear function of [Y] (dashed line); heterotropic linkage causes $X_{1/2}$ to depend hyperbolically on [Y]. Panel B: dependence of $X_{1/2}$ on [Y] for the case of heterotropic linkage plotted on a logarithmic scale. $K_X = 10 K_Y$; $K_Y = 10 K_X$. The figure demonstrates that dependence of $X_{1/2}$ on the thermodynamic parameters of the system ($K_X$, $K_Y$ and $K_{XY}$) and the concentration of the other ligand unequivocally identifies the type of linkage between the two ligands X and Y. However, $X_{1/2}$ should not be used for determination of those parameters, since a sounder statistical procedure is to fit the original experimental data (fractional saturation $\bar{X}$ as a function of [X] and [Y]) directly with eqn. 1.14 or 1.19, and recover the three thermodynamic parameters from the fit.

Figure 1.8 Measurement of the affinity of human hemoglobin for glycerate 2,3 bis-phosphate from its effect on the affinity for oxygen. Source: Adapted from Antonini et al. (1982). Experimental conditions and symbols: 0.1 M Bistris or Tris HCl buffers, containing 0.1 M NaCl, pH 6.5 (squares), 7.5 (triangles), or 8.6 (circles); T = 20°C; Hb concentration 60 μM /tetramer.
1.10 Allostery and Allosteric Phenomena in Monomeric Proteins

The term allostery (a neologism from the Greek words for “other structure”) was created by J. Monod to describe a possible interpretation of the functional behavior of enzymes (or other macromolecules) presenting cooperativity and/or heterotropic linkage (Monod et al., 1963). Two years later Monod, Wyman, and Changeux (Monod et al., 1965] elaborated the underlying formal concepts in what became known as the two-state allosteric model (often nicknamed the MWC model). This model suggests that allosteric proteins can sample two (or more) spatial configurations, each characterized by its own set of equilibrium constants for binding to each of its ligands. Monod's original idea applied to proteins made up by multiple identical subunits, each having a ligand-binding site, and required the oligomer to be symmetric (Monod et al., 1965). However, it was successively demonstrated that also monomeric proteins may exist as equilibrium mixtures of conformational isomers (Austin et al., 1975). This idea, which when formulated was ahead of its time, has tremendous implications, first of all the thermodynamic necessity that all protein isomers bind all ligands, albeit with different affinities (Figure 1.9). The reaction scheme of a monomeric allosteric protein capable of binding two ligands is quite complex see Figure 1.9.

The important features of this reaction scheme are as follows:

i) The protein has access to (at least) two conformational isomers P and P*, independently of the presence of its ligands; the equilibrium constant of the isomerization reaction is $L = [P^*] / [P]$.

ii) The two isomers have different affinities for the protein's ligands, which bind to different sites (the case of two or more molecules of the same ligand binding to identical sites will be considered in Chapter 4).

iii) The binding of a ligand biases the equilibrium between the isomeric forms of the protein and favors the form for which it has higher affinity, increasing its relative population at the expense of the other(s).

The two-state model was meant to explain not only the heterotropic interactions between two ligands of a monomeric protein, but also the homotropic interactions of the same ligands binding to multiple identical sites in an oligomeric protein such as

\[ \text{Reaction scheme of an allosteric monomeric protein binding two ligands.} \]

Under the limiting condition where each ligand has significant affinity for only one of the different conformational isomers of the protein, some intermediates may be neglected.
Reversible Ligand Binding

hemoglobin. A full description of allostery in ligand binding is presented in Chapter 4. However, it is important to stress that allosteric phenomena have been described in monomeric proteins (Cui and Karplus, 2008), and actually some crucial experiments on this subject were carried out on myoglobin (Austin et al., 1975).

A fundamental, yet elusive, tenet of the concept of allostery is the presence of the two (or more) conformational isomers of the protein in the absence of the ligand. Although today the existence of protein conformational dynamics is widely accepted, there was little evidence supporting the MWC viewpoint in its day. Indirect evidence for protein dynamics generally, and for multiple conformational states in particular, became available for selected systems within the decade following publication of the MWC model. Among the earliest and best known of these two categories of evidence are the following. Lakowicz and Weber (1973) demonstrated that compounds as large and chemically diverse as molecular oxygen, acrylamide, or iodide ion could quench the native fluorescence of myoglobin during the lifetime of the excited state of its tryptophan residues (i.e., they showed that the quenching was dynamic rather than static). This result was unexpected because the crystal structure of myoglobin clearly showed the absence of any channels that could allow such compounds to reach the buried tryptophan residues from the protein exterior. Shortly thereafter, Frauenfelder and coworkers, working at the same institution as Lakowicz and Weber (the University of Illinois at Urbana), demonstrated that the photodissociation product of the myoglobin–CO complex at low temperature contains a mixture of conformational isomers (Austin et al., 1975). This result implied that the conformational isomers are present in the bound complex, before the photodissociating light pulse, and made it difficult to argue that unliganded Mb does not also sample multiple states.

We shall not discuss the two-state model further in this section since its formulation is clearly redundant if applied to a monomeric, single ligand-binding site protein, and shall defer its analysis to Chapter 4.

1.11 The Special Case of Cys Ligands (and Similar Reactions)

There are cases in which the protein-ligand reaction may not conform to the equations developed above, and great caution is in order before the researcher commits himself or herself to concepts developed for the standard case. Probably the most common of these is that of ligands of Cys residues. Thus in this section we shall devote our attention to these, and delay the analysis of other special cases to the next section. However, the reaction schemes considered in this section are of general relevance, Cys residues and their reagents being only the most typical examples of a class.

Eqns. 1.1 through 1.20 refer to reversible reactions in which the ligand and the protein have the same chemical formula in the bound and free states. This is the case for the majority of ligands forming weak bonds (e.g., steroid hormones and serum albumin) or coordination complexes with the protein (e.g., the ferrous heme iron of hemoglobin and its gaseous ligands). There are cases, however, in which the ligand has a different chemical formula in its bound and free state, and these cannot be treated like the preceding cases. It is important to firmly state this point because in these cases usage of the equations developed in this chapter may lead to errors.
This phenomenon commonly occurs with *covalent ligands*. An example is given by the reaction of Cys residues with alkylation agents, metal donors, or other reagents capable of transferring a reactive group. The reaction scheme may be described as follows:

\[
P - SH + RX \rightleftharpoons P - SX + HR (\text{or } H_2O^+ + R^-) \quad (\text{eqn. 1.21})
\]

where P is the protein, RX the free ligand used, and HR the leaving group. Some -SH reagents do not present a leaving group, and thus their reaction, if reversible, conforms to eqn. 1.1 instead of 1.21; these are not considered in this Section.

The reaction mechanism and leaving group(s) may vary. Examples of such ligands include gold organometallic complexes like aurothiomalate and auranofin, iodoacetamide, iodoacetate, and their derivatives, and so on (see Cai *et al.*, 2012 for a review). Cys residues are not the only sites where covalent ligand binding may occur, and in all cases where the leaving group is different from water a similar treatment of the experimental data is required.

The reaction described by eqn. 1.21 may be irreversible or reversible. The former case is probably more common, a typical example being provided by alkylation agents. However, the latter case cannot be excluded. An important example of a physiologically relevant reversible ligand of Cys is provided by nitrosothiols. The reaction of these compounds follows the scheme:

\[
P - SH + R - SNO \rightleftharpoons P - SNO + R - SH
\]

with R-SNO being nitroso-cysteine or nitroso-glutathione. This reaction is reversible and excess free Cys or GSH effectively removes the protein-bound NO (Meyer *et al.*, 1994).

If the reaction is reversible, a dimensionless equilibrium constant can be defined and determined.

\[
K = \frac{[P - SH][RX]}{[P - SX][HR]} \quad (\text{eqn. 1.22})
\]

The following considerations apply:

i) *Reversibility should be carefully assessed.* A covalent protein-ligand complex is often irreversible for all practical purposes, and its equilibrium constant is indistinguishable from zero. However, covalent binding does not necessarily imply irreversibility since the reaction's $\Delta G^0$ reflects the algebraic sum of the free energies of all the chemical bonds that break and form in the reaction, and breakage of the RX bond may require an amount of energy similar to that released by the formation of the P-SX bond. As a consequence, in spite of the fact that the protein-ligand complex entails covalent bond(s), we cannot take for granted that the complex is irreversible, even though this is often the case. We suggest that a system involving the formation of covalent bonds is considered irreversible until proven otherwise by extensive dialysis under the appropriate experimental conditions, but we also recommend that reversibility is carefully assessed in all cases. Because of the very high affinity of most Cys reagents and their slow reactivity true thermodynamic reversibility has been demonstrated in few cases, the best example being probably that of nitrosothiols.
ii) Diazalysis removes equally RX and HR and thus is ineffective in dissociating the complex, unless one takes the precaution of adding HR to the diazalysis buffer. As a consequence, if one does not take the necessary precautions these complexes may appear irreversible even when they are not.

iii) The system is insensitive to dilution and does not obey Ostwald’s law unless the concentration of HR (or H3O+ and R−) is made constant. In fact, if HR dissociates into H3O+ and R− in an unbuffered solution, and no external R− or HR is added, dilution would be expected to promote complex formation!

iv) If the reaction proves reversible and allows the researcher to define an equilibrium constant, the equilibrium constant is a pure number or has units of M−1 rather than M. X1/2 (in this case RX1/2), the concentration of free RX necessary to half-saturate the protein, depends on the protein concentration, unless one forces the system to behave in a way consistent with eqn. 1.1 by carrying out the measurement in the presence of added HR (or R− ions) at high and constant concentration (see below). (In the buffered solutions usually employed in biochemistry one need not worry about changes in hydrogen ion concentration.)

v) Binding may be slow because of the high activation energy for covalent bond breaking and formation, and reaching the true equilibrium condition may require long incubation times. If the incubation time is not long enough, one obtains an X versus [RX] plot that may superficially resemble a hyperbola, but is actually an exponential (see Section 3.4).

vi) A phenomenon that for lack of a better name we call pseudo-reversibility may be observed. Pseudo-reversibility occurs when the complex dissociates according to a reaction scheme different from that of association. As a consequence, only the native state of the protein is recovered, and not the native state of the ligand. An example of pseudo-reversibility is provided by the case of thioredoxin reductase covalently alkylated by nitrosoureas (Schallreuter et al., 1990; Saccoccia et al., 2014). In this case thioredoxin, the substrate of the enzyme, can extract the ligand from the Cys residues in the active site (and can also catalyze a redox reaction, which releases a reduced state of X).

The consequence pseudo-reversibility is recovery of the unmodified protein, via a reaction that is not the inverse of the binding reaction, and the compound RX is not recovered at the end of the process. We refer the reader to the review by Saccoccia and co-workers (Saccoccia et al., 2014) for a more detailed discussion of sulfhydryl ligands. The reason pseudo-reversibility is important is that, besides its possible biological relevance, it may simulate true thermodynamic reversibility, and may induce the researcher to assume that a reaction is reversible when in fact it is not.

Obviously, the correct identification of the reaction scheme and application of the appropriate formula for the equilibrium constant is necessary and sufficient to determine the equilibrium constant from the experimental data. Provided that the reaction has a signal, that reversibility has been carefully checked by diazalysis against excess HR, and that the time required for the complex to reach its equilibrium condition has been determined (see points 2 and 5, above), a binding isotherm may be recorded by successive additions of RX to the protein. The experiment may be carried out in two different ways.
In the presence of added HR at a concentration significantly higher than that of the protein, one may assume that the concentration of this component is nearly constant throughout the measurement, leading to a rearrangement of eqn. 1.22

\[ K' = K[HR] = [P-SH][RX]/[P-SX] \]

which is analogous to eqn. 1.3. Under these conditions all the equations developed in this Chapter may be applied, using \( K' \) instead of \( K \).

In the absence of added HR, equal amounts of P-SX and HR are produced (i.e., \([HR]=[P-SX]\)), which leads to the following rearrangement of eqn. 1.22

\[ K = [P-SH][RX]/[P-SX]^2 \]

Introducing \([P]_{tot} = [P-SH] + [P-SX]\) leads to:

\[ K[P-SX]^2 + [P-SX][RX] - [P]_{tot}[RX] = 0 \]

from which \([P-SX]\) (and \( \bar{X} = [P-SX]/[P]_{tot} \)) can be calculated.

The \( \bar{X} \) vs log[RX] plot has a symmetric sigmoidal shape, broader than that one would obtain from eqn. 1.6. The midpoint of the plot, that is, the concentration of RX required to half-saturate the protein, is defined by the condition: \([P-SX] = [HR] = [P-SH] = 0.5[P]_{tot}\), and results

\[ RX_{1/2} = 0.5K[P]_{tot} \]

We call the reader’s attention on the fact that the concept of \( X_{1/2} \) does not usually depend on the target’s concentration, and thus the above definition of the \( RX_{1/2} \) is atypical. It is common that enzymes having reactive Cys residues in their active site are inhibited by -SH reagents having reaction mechanisms similar to those described in this Section, for which \( IC_{50} \) values (fully analogous to \( RX_{1/2} \)) are of interest. In these cases one must pay attention to the necessary controls and considerations (points 1 to 6, above), and to the atypical form of \( RX_{1/2} (IC_{50}) \). This point is further discussed under Section 11.5.

### 1.12 Other Special Cases

Other special cases not fully conformant to eqns. 1.1–1.20 include reactions having semi-stable intermediates, and those cases that, unknown to the researcher, are replacement reactions rather than binding equilibria, and obey eqns. 1.11–1.16, even though the researcher expects them to obey eqns. 1.1–1.6.

Monomeric proteins having more than a single binding site for the same ligand will not be considered here and are deferred to Chapter 4. This case is often observed when the ligand is a low molecular weight ion: for example, the monomeric protein calmodulin binds four calcium ions. We only remark here that these cases highlight the importance of direct determination of binding stoichiometry and reaction mechanism, even in the case of monomeric proteins.
A ligand-binding reaction having a stable (equilibrium populated) intermediate is as follows:

\[ P + X \rightleftharpoons PX \rightleftharpoons PX^* \]

where PX and PX* are structural isomers, whose ratio is independent of ligand concentration. In this case the bound state of the protein is a mixture of two conformers in fixed proportions, that may or may not be recognized depending on whether the signal associated with PX and PX* is the same or not.

If the researcher assumes that the full signal change corresponds to the complete titration of the protein with the ligand, this case may be unnoticed. However, if the signal quantitatively measures the concentration of only one of the two bound states (e.g., PX*), while the other is not distinguished from the unbound state, incomplete binding may be erroneously suspected. Kinetics may often provide the clues to correctly identify this case (see Chapter 2).

Reactions that, unknown to the researcher, are replacements rather than ligand binding equilibria are not uncommon. A typical example is the reaction of several ferric hemoproteins their ionic ligands, for example, the reaction of ferric myoglobin with azide, cyanide or other ligands:

\[ \text{MbFe}^{+3} - \text{N}_3^- + \text{H}_2\text{O} \rightleftharpoons \text{MbFe}^{+3} - \text{OH}^- + \text{HN}_3 \]

The heme iron of several ferric hemoproteins, including vertebrate hemoglobins and myoglobins, is usually six-coordinated, either a water molecule or a hydroxyl ion occupying the sixth coordination position, depending on pH. When these proteins are titrated with their typical ligands (azide, cyanide, etc.), the water or hydroxyl ion must be displaced. Since the concentration of these species is constant (water because of its abundance and OH\(^-\) because of the buffer), the equilibrium experiments fully conform to a titration; but the kinetics does not (see Chapter 2). Moreover, the ionic ligands of ferric hemoproteins are Lewis bases and the reaction above may be read as a double ligand replacement: the azide ion is exchanged between two Lewis acids (iron and the hydrogen ion) at the same time as it is replaced at the iron by water.

A similar type of problem may occur in the case of the binding of metal ions to proteins, since upon dissociation these react with solution components, and may possibly precipitate. For example, copper(II), dissociated from a carrying protein, forms copper hydroxide Cu(OH)\(_2\), which has a solubility product of 4.8 x 10\(^{-20}\) M\(^3\).

As a general rule, the problem in these cases is to unravel the reaction mechanism and stoichiometry, in order to apply the analysis appropriate to the experimental data one collects.

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