# 17

# Ligand Binding Studies

## INTRODUCTION

Ligand binding studies play an important role in many aspects of protein chemistry and enzymology. As discussed in Chap. 1, many proteins must be assayed based on their specific ligand binding since they have no enzymatic activity. In Chap. 1 we briefly reviewed some of the experimental approaches used for determining specific ligand binding in the context of assaying nonenzymatically active proteins, and also referred to the use of the determination of the number of binding sites per molecular weight unit, and employing such information in establishing an extinction coefficient for a purified protein. The chapters on initial rate kinetic studies (Chaps. 13 to 15) have emphasized the importance of independently determined dissociation constants that can be used in conjunction with kinetically determined dissociation constants as a test of the validity of a proposed kinetic mechanism. In addition, as emphasized in Chap. 16, ligand binding studies are essential when examining systems that exhibit nonhyperbolic kinetics. In Chap. 18 we emphasize the importance of independently determined dissociation constants in studies of rapid kinetics and in the elucidation of mechanisms of enzyme regulation.

In any binding study, two parameters describe ligand binding: $B_{max}$, the maximum number of ligand molecules bound per mole of protein, and $K_d$, the dissociation constant of the reversible binding process. A variety of techniques have been developed to study ligand binding, although some cannot give a value for $B_{max}$ because of the nature of the assumptions made in analyzing the data. Such methods have great use, however, often in terms of ease and accuracy, and can be employed where knowledge of $K_d$ but not $B_{max}$ is important. In other instances, $B_{max}$ may be the

parameter whose value is primarily required, and of course techniques must be chosen that will yield such information.

Approaches for studying ligand binding can be divided into two categories—direct and indirect—and we examine these separately. In addition to experimental methods for determining ligand binding, a number of ways of presenting ligand binding have been developed, and depending on the information being sought, different types of data presentation are more appropriate. In the context of the graphical representation of ligand binding data we examine the effects of systems that do not follow hyperbolic saturation.

## METHODS TO STUDY LIGAND BINDING

### *Direct Methods*

The various direct methods for estimating the amount of ligand bound to a protein have all evolved from equilibrium dialysis and depend on physically separating bound ligand from free. Assuming that there are direct methods for quantitating the amount of ligand bound and free in solution, these techniques all give unequivocal (except for experimental error) estimates of $B_{max}$ and $K_d$. In equilibrium dialysis, a typical setup of which is shown in Fig. 17-1, the free ligand is allowed to reach an equilibrium across a semipermeable membrane that separates the protein from the bulk phase of the solution.

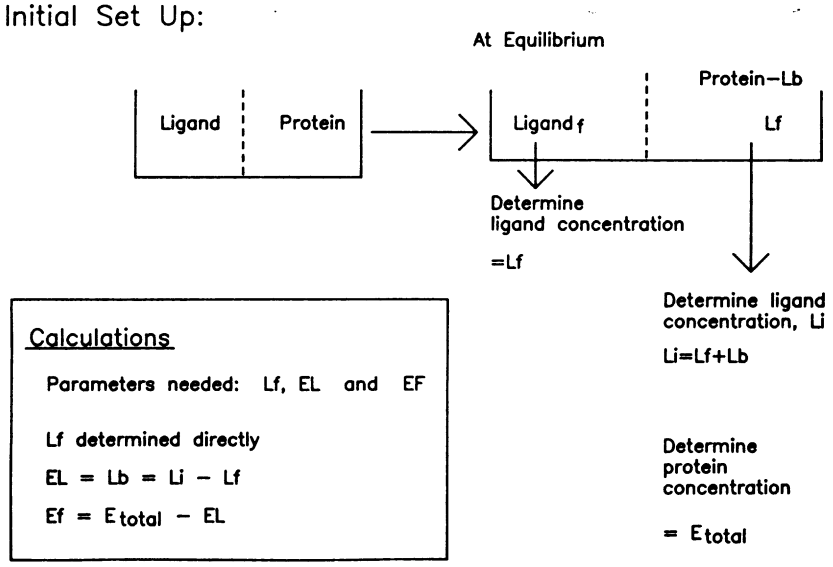


**Figure 17-1** Scheme for an equilibrium dialysis experiment.

At equilibrium the concentration of ligand in the non-protein-containing compartment equals the concentration of *free* ligand ($L_f$) in the protein-containing compartment. If the *total* ligand concentration ($L_i$) in the protein-containing compartment is known (from experimental determination or, if this is not possible, by subtraction of the amount of ligand in the non-protein-containing compartment from the total amount of ligand initially added), the amount bound to the protein ($L_b$) can be calculated by subtracting the contribution of free ligand. From a single experiment one can find $K_d$ by substitution into

$$K_d = \frac{[E_f] \cdot [L_f]}{[EL]} \tag{17-1}$$

where $L_f$ is experimentally determined, [EL] ($= L_b$) is calculated as described previously and $E_f$ obtained by subtraction of [EL] from $E_{total}$, which is presumably known. Application of this equation provides a value for $K_d$ but does not give a value for $B_{max}$. In the simple situation where hyperbolic saturation of a single class of sites occurs, $K_d$ is an accurate estimate. In a more complex situation, the dialysis experiment is repeated with a series of different total ligand or protein concentrations. Results are then plotted using one of the graphical methods described later.

Before moving on to some of the other direct methods for studying ligand binding, we examine in more detail the experimental procedures of equilibrium dialysis. A number of problems may be encountered:

1. True equilibrium of the free ligand may not be achieved. This is usually controlled for in two ways: (a) a duplicate experiment is set up with *no* protein in either compartment—if equilibrium is reached in the time period used in the experiment, the ligand concentrations in each compartment will be equal, and (b) duplicate determinations in the presence of protein are made, but in one the ligand is initially placed in the compartment containing the protein, while in the other the ligand is initially placed in the other compartment. If an effective equilibrium is established during the time course of the experiment, identical results will be obtained.

2. During the time required to reach equilibrium (often 10 to 15 hours), either the ligand or the protein may decay to inactive forms that interfere with the concentration determination. A more serious problem exists if the ligand decays to a form that competes with the true ligand at the protein binding site. The former artifacts can be controlled for by assaying the protein activity and the effective total ligand concentration before and after dialysis.

3. In many cases the free ligand concentration is conveniently assayed by spectrophotometric measurements. If the bound ligand has different spectral properties from the free ligand, the total ligand concentration in the protein-containing compartment will be incorrectly estimated. This can be overcome by denaturing the protein after equilibrium has been achieved and determining the concentration of the total ligand in that compartment when it is all free in solution. As will be discussed later, it is often possible to determine an extinction coefficient for the bound ligand

via spectral titrations, and if it is known, the concentration can be determined directly from the total absorbance of the protein-containing compartment after subtraction of the absorbance of the free ligand (which is known from the compartment lacking protein). These problems are overcome if a radioactive ligand is used.

4. In situations where the total ligand concentration is varied (usually the case), one reaches a situation as $B_{max}$ is approached where the amount bound is calculated by subtraction of one large number (for free ligand concentration) from a slightly larger number (for [free] + [bound])—a situation that can lead to large experimental error.

Having considered these potential pitfalls, we now examine some of the other "direct" methods for following ligand binding together with some of their problems.

*Forced Dialysis Methods.* In a number of variants of equilibrium dialysis, there is physical separation of free from bound ligand via a semipermeable membrane. The ligand and protein are initially in the same compartment, separated from a collection vessel by the semipermeable membrane (see Fig. 17-2).

At the start of the experiment, pressure is applied to the protein-containing compartment to cause a "forced" dialysis: solvent, containing free ligand solute but *not* the protein solute, is forced through the membrane, collected, and the concentration of free ligand determined. In the early versions of such schemes the forced dialysis was continued until the protein (and its bound ligand) remained associated with the inside of the semipermeable membrane. The assumption was made that the solvent, containing free solute ligand, was in equilibrium with the protein-containing compartment, and hence the concentration of ligand in the forced dialysate was equal to the concentration of free ligand in the protein-containing compartment. To avoid problems that might arise from concentrating the protein, the experiment is terminated at a fixed point so that a constant protein concentration can be used. To

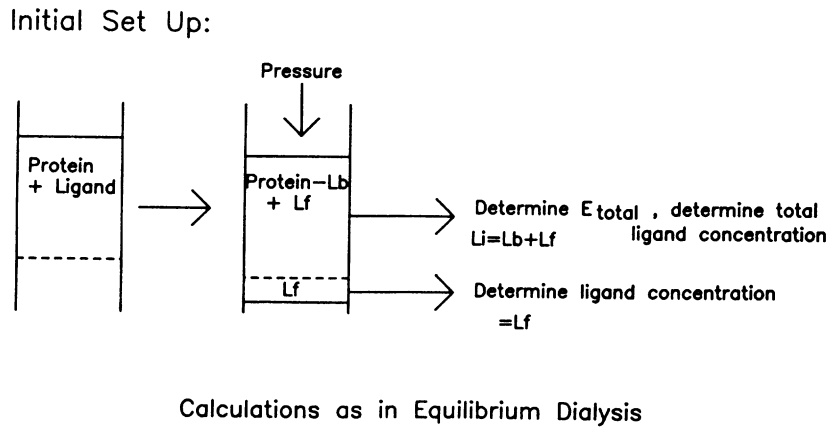


**Figure 17-2** Scheme for forced-dialysis method of determining equilibrium binding.

achieve multiple determinations, buffer containing ligand is added to the protein-containing compartment at the termination of one run and the process repeated, allowing either increasing or decreasing total ligand concentrations to be used.

*Protein Transport Methods.* Several methods have been developed where protein is transported through a solution containing ligand, and the amount of ligand associated with the region containing protein is compared to regions lacking protein to give the amount of ligand bound to the protein. In particular, gel filtration and sedimentation have been successful in this approach.

The simplest way of using gel filtration chromatography to study ligand binding is exemplified by the *Hummel–Dreyer method*, the general principles of which are given in Fig. 17-3A; a gel filtration column is equilibrated with buffer containing a particular ligand at a defined concentration. Once the column is equilibrated (as determined by the eluent containing the same ligand concentration as the starting buffer), the protein sample (which is preequilibrated with the same ligand concentration as the equilibrating buffer) is introduced to the column and the column developed with the equilibrating buffer. Under circumstances where the protein binds the ligand, the sample solvent is depleted with respect to free ligand. As elution proceeds,

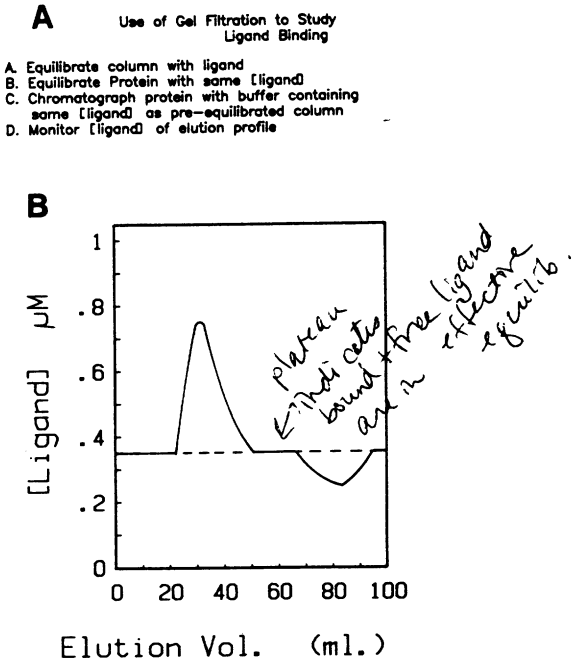


**Figure 17-3** (A) Outline of steps in the use of gel filtration to study ligand binding; (B) typical experimental data obtained in Hummel–Dreyer method.

the protein is separated from ligand-depleted solvent and elutes as a peak with its bound ligand such that the *total* ligand concentration in the protein peak is higher than the concentration of the equilibration buffer: as a result, a corresponding trough follows this peak. When the *ligand* concentration is monitored as the column develops, the profile obtained looks like that in Fig. 17-3B. If the ligand does not bind to the protein, the measured concentration of *ligand* during elution does not deviate from the concentration of the equilibration buffer (the dashed line in Fig. 17-3B).

To achieve the best results, it is important to choose a gel matrix that gives maximum separation between the ligand and the protein. This is usually one that totally excludes the protein. It is also essential that the *ligand* elution profile has a plateau of constant ligand concentration between the peak and the trough. This indicates that for the flow rate used to elute the column, the bound and free ligand are in effective equilibrium. If this plateau is not observed, the binding equilibria are too slow relative to the flow rate of the column and the experiment must be repeated at a lower flow rate.

The simplest way to calculate the amount of bound ligand is to collect fractions and determine the ligand concentration per fraction. Although in theory the calculation of the amount of bound ligand can be made using either the peak or the trough, in practice it is easier to use the trough, as interference of the ligand concentration determination by the protein is minimized. Assuming that the ligand concentration is determined by absorbance measurements, the concentration of bound ligand is given by

$$\mu\text{mol bound} = \frac{\sum_i (\Delta A_i)(\text{ml}_i)}{\varepsilon_{\text{m}M}} \tag{17-2}$$

where $\Delta A_i$ is the difference in the absorbance of the fraction, $i$, and the baseline absorbance (determined by the equilibrating ligand concentration), $\text{ml}_i$ is the volume of fraction $i$ and $\varepsilon_{\text{m}M}$ is the millimolar extinction coefficient of the ligand. The summation is carried out for each fraction in the trough region of the chromatogram, giving the amount of ligand bound *by the amount of protein* in the initial sample.

Although the dissociation constant for the ligand binding process can be calculated from a single experiment, it is necessary to obtain additional data for more detailed analysis of the binding isotherm. Therefore, the experiment must be repeated at either different protein concentrations of the loaded sample or, preferably, at different concentrations of the equilibrating ligand. Essentially the same approach is used when bound and free ligand are separated by centrifugation. This method is outlined in Fig. 17-4.

*Protein Precipitation Approaches.* In a number of instances, especially where large molecule ligands are involved, it is convenient to study binding by precipitating the protein–ligand complex away from the free ligand and then determining the amount of protein and ligand in the complex; the free ligand concentration is then

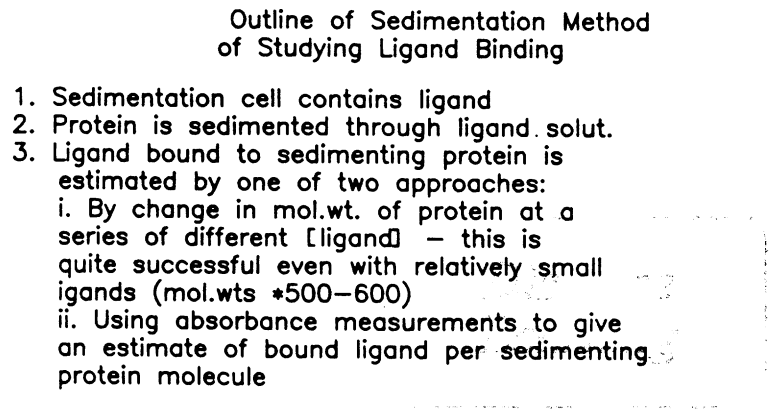


**Figure 17-4** Outline of approach used to determine ligand binding by sedimentation methods.

determined in the supernatant. This method is outlined in Fig. 17-5. Although frequently used (out of necessity), it is subject to the criticism that the binding of ligand in the presence of the appropriate precipitant may not accurately reflect that found under normal circumstances. In addition, it is necessary to control for nonbound ligand that is included in the precipitated material. When a radioactive ligand is employed, this is often achieved by repeating the precipitation in the presence of a large excess of unlabeled ligand. In this case precipitated radioactive material is used as a control blank to be subtracted from the specifically bound ligand.

### *Indirect Methods*

*Spectroscopic Methods.* These depend on either a change in the ligand's or the protein's spectral properties on complex formation, and are probably the most widely employed techniques for studying ligand binding. They have the advantages of being rapid, reproducible, and quite accurate. *However*, there are some potential sources of deception in what is an otherwise simple approach, and also (in general), these methods do not give a value for $B_{max}$, only for $K_d$.

Consider two cases, both involving fluorescence measurements (although the same arguments that we will use could be made for any other spectral parameter).

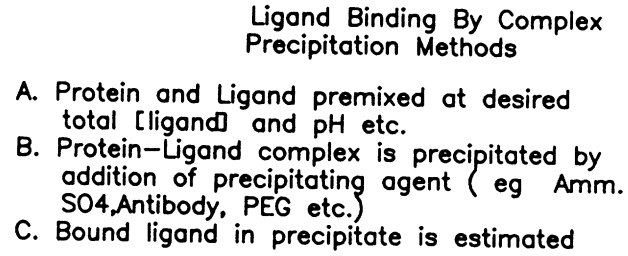


**Figure 17-5** Scheme outlining the determination of ligand binding by complex precipitation.

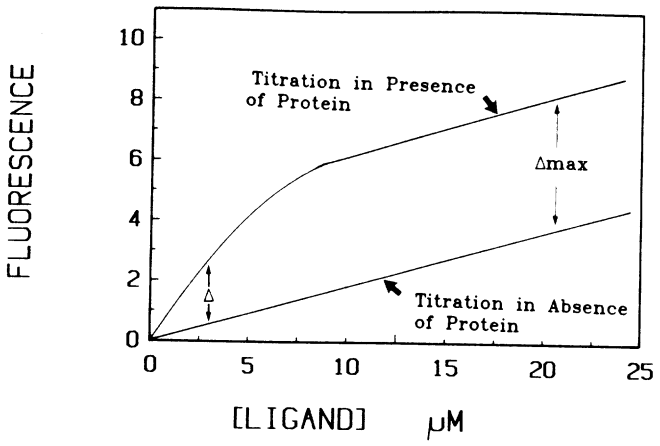


**Figure 17-6** Fluorescence titrations in the presence and absence of protein.

In the first, we assume that the fluorescence of a ligand is enhanced upon binding to the protein. A typical experiment is shown in Fig. 17-6.

In the absence of protein, fluorescence intensity is linearly related to the ligand concentration. In the presence of protein the fluorescence intensity rapidly increases, but eventually becomes parallel to that obtained in the absence of protein. The maximum change in fluorescence is defined as $\Delta_{max}$, as shown in Fig. 17-6. If experimentally the two titrations do not become parallel, a value for $\Delta_{max}$ can be obtained from a double reciprocal plot of $1/\Delta$ versus 1/[ligand], as in Fig. 17-7.

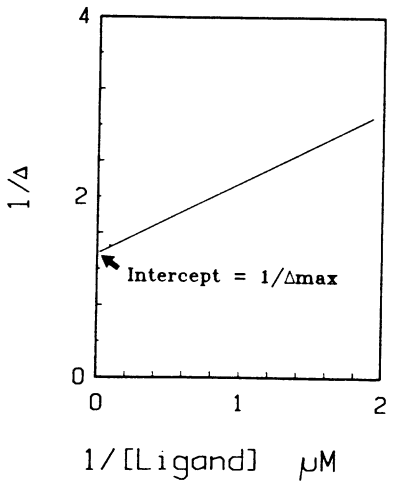


**Figure 17-7** Experimental determination of $\Delta_{max}$ from double-reciprocal plots.

If it is assumed that $\Delta_{max}$ occurs when all the protein sites for the ligand are occupied, one can calculate the amount of ligand bound ($L_b$) at any point in the titration curve, if the initial concentration of binding sites (which is $B_{max}$) is known, from

$$L_b = \frac{\Delta}{\Delta_{max}} B_{max} \tag{17-3}$$

Knowing the total ligand concentration at any point in the titration allows calculation of $L_f$ and subsequently a value for $K_d$. *This approach depends on prior knowledge of* $B_{max}$.

An alternative calculation is represented by the following: Let $F_m$ be the experimentally determined fluorescence *in the presence* of protein at a given total ligand concentration $T$. $L_b$ is the concentration of *bound* ligand at any given total ligand concentration. If we define $F_b$ as the *specific molar fluorescence* of bound ligand and $F_f$ as the specific molar fluorescence of free ligand, then at any given point in the titration,

$$F_m = F_b L_b + F_f(T - B) \tag{17-4}$$

$$= F_b L_b + F_f T - F_f B \tag{17-5}$$

However, since the experimentally measured fluorescence in the *absence* of protein $F_t = F_f T$, we get

$$F_m = F_b L_b + F_t - F_f L_b \tag{17-6}$$

Dividing both sides of Eq. (17-6) by $F_t$ gives

$$\frac{F_m}{F_t} = \frac{F_b L_b}{F_t} + 1 - \frac{F_f L_b}{F_t} \tag{17-7}$$

Therefore,

$$\frac{F_m}{F_t} - 1 = \left(\frac{F_b}{F_t} - \frac{F_f}{F_t}\right) L_b \tag{17-8}$$

and we obtain an expression for $L_b$:

$$L_b = \frac{(F_m/F_t) - 1}{(F_b/F_t) - (F_f/F_t)} \tag{17-9}$$

Since $F_f T = F_t$,

$$L_b = \frac{(F_m/F_t) - 1}{(F_b/F_f)T - \dfrac{F_f}{F_f T}} \tag{17-10}$$

Multiplying top and bottom by $T$ gives

$$L_b = T\frac{(F_m/F_t) - 1}{(F_b/F_f) - 1} \tag{17-11}$$

Defining a new constant, the *fluorescence enhancement*, FE, as $FE = F_b/F_f$, yields

$$L_b = T\frac{(F_m/F_t) - 1}{FE - 1} \tag{17-12}$$

which allows calculation of the amount of bound ligand, $L_b$, at any point in the titration, provided that the total ligand concentration ($T$) and the fluorescence enhancement (FE) are known. $F_m$ and $F_t$ are the experimentally determined fluorescences at any given ligand concentration in the presence and absence of protein, respectively. Provided that FE is experimentally determined, these titrations permit calculation of the amount of ligand bound without prior knowledge of $B_{max}$.

These two ways of approaching essentially the same type of experimental data illustrate an important point: $\Delta_{max}$ is a *protein-dependent parameter*, while FE is a *ligand-dependent parameter*. The experimental determination of ligand-dependent parameters allows $B_{max}$ to be determined from indirect methods; however, if only a protein-dependent parameter can be followed, then only information about $K_d$ can be obtained since $B_{max}$ must be assumed.

Ligand-dependent parameters such as FE must be independently determined from an experiment of the type illustrated in Fig. 17-8. FE is determined by fluorescence titrations *at a fixed ligand concentration* with varied protein concentrations. Control titrations omit the ligand to allow the contribution (if any) of the protein to the fluorescence. $\sigma_0$ is the fluorescence of the chosen ligand concentration in the absence of protein. $\sigma_i$ is the measured fluorescence at various protein concentrations (minus the protein fluorescence) and $\sigma_{max}$ is obtained when the experimental and control titrations become parallel. At $\sigma_{max}$ *all* the ligand is bound by protein and

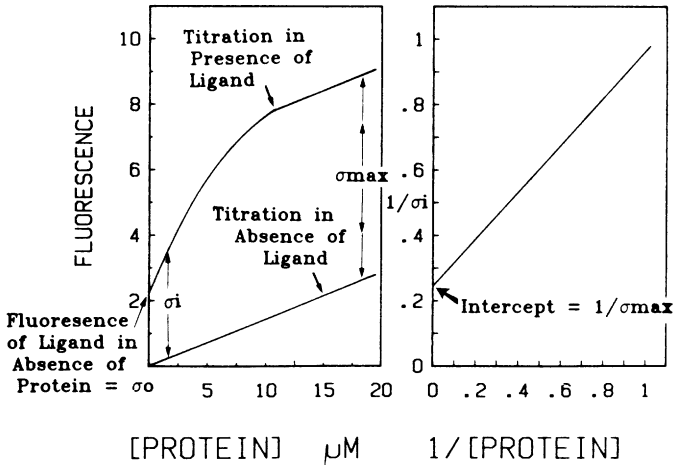


**Figure 17-8** Outline of the determination of the ligand-dependent parameter of FE.

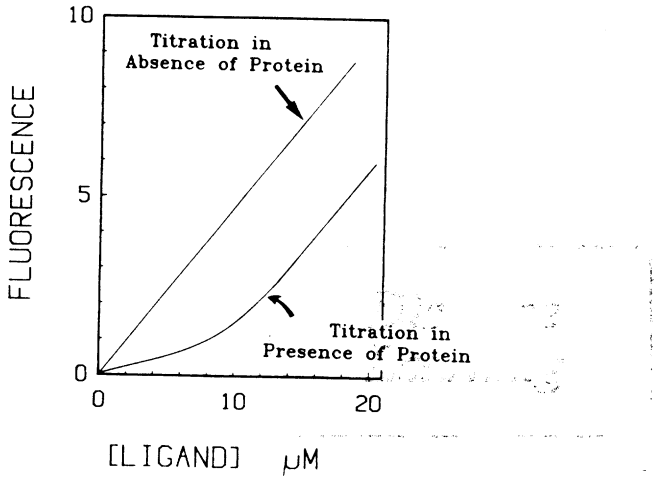


**Figure 17-9** Fluorescence titration data obtained when the ligand fluorescence is quenched upon binding to protein.

hence FE is simply given by

$$\mathrm{FE} = \frac{\sigma_{\max}}{\sigma_0} \tag{17-13}$$

If $\sigma_{\max}$ is not experimentally obtained from the titration, a double reciprocal plot of $1/\sigma_i$ versus 1/[protein] gives an intercept of $1/\sigma_{\max}$ as in Fig. 17-8.

This example involves a fluorescent ligand that undergoes a fluorescence enhancement on binding to a protein. In some instances the ligand fluorescence may be quenched, and a titration of the type shown in Fig. 17-9 is obtained.

As with the case of enhanced fluorescence, equations for use in the calculation of the bound ligand concentrations at any point in the titration can be derived using either the protein-dependent parameter, $\Delta_{\max}$, or a ligand-dependent parameter, $Q_c$, defined by

$$Q_c = \frac{F_b}{F_f} \tag{17-14}$$

where $F_b$ and $F_f$ are defined as previously. The equation for the protein-dependent parameter is as before, while the equation using $Q_c$ is

$$\mathrm{L}_b = T\frac{1 - (F_m/F_t)}{1 - Q_c} \tag{17-15}$$

Although this discussion has centered on fluorescent ligands, any spectral property of the system (i.e., protein or ligand) that changes upon complex formation can be used to study ligand binding. Some of these are summarized in Table 17-1.

**TABLE 17-1** Spectral properties used to study ligand binding

| Parameter[a] | Dependence[b] | Comment |
|---|---|---|
| Protein fluorescence | Usually P | Usually quenching of protein fluorescence observed |
| Polarization | L | Bound ligand has higher polarization than free ligand |
| Absorbance | P or L | Can present problems if the ligand absorbs in the region 260–280 nm since protein absorbance changes may interfere with detection of ligand absorbance changes |
| ESR | Usually P | Useful with paramagnetic metal binding (e.g., Mn); otherwise, must introduce spin label to ligand |
| NMR | Usually P | In theory almost universally applicable; in practice is limited by experimental considerations such as protein concentration needed |
| NOE | L | Especially useful with weakly bound ligands |

[a] ESR, Electron spin resonance; NMR, nuclear magnetic resonance; NOE, nuclear Overhausser effect.
[b] P, Protein dependent; L, ligand dependent.

In addition to the question of whether a protein-dependent or a ligand-dependent parameter can be determined, spectral methods of following ligand binding suffer from a fundamental limitation that can be resolved only through independently determining $B_{max}$ using a direct method. Implicit throughout all of this is the assumption that in a multisite-per-molecule system (either two or more sites for the same ligand per polypeptide chain *or* a multi-subunit situation) *all* molecules of bound ligand contribute equally to the followed parameter. If one or more sites in a multisite system are spectrally unobservable, only binding of ligand molecules that do contribute to the signal are observed, and the true $B_{max}$ cannot be determined, even using a ligand-dependent parameter.

*Other Indirect Methods.* Any property of a protein that changes upon the binding of a ligand can be used to study that ligand binding process. All such approaches depend on the experimental determination of a parameter that is equivalent to the $\Delta_{max}$ discussed previously. As a result, these methods must assume a value for $B_{max}$, and can only allow determination of $K_d$, but they are, however, quite useful, as they are often experimentally easy.

Some of the experimental properties that have been used to study ligand binding are given in Table 17-2.

**TABLE 17-2** Protein properties that have been used to follow ligand binding

| Property | Comment |
|---|---|
| Susceptibility to proteolysis<br>Denaturation by solvents<br>Heat stability<br>Chemical modification | In all instances the presence of ligand in a protein–ligand complex may either increase or decrease susceptibility to approach; in either case the dependence of the change on ligand concentration can give binding information. |

### *Affinity Chromatography*

The general principles were described in some detail in Chap. 3. Because the retardation of passage of a specific protein through an affinity column is directly due to a specific ligand–protein interaction, it is natural that affinity chromatography can be used to study ligand binding.

If we consider a situation where a solution containing a protein, P, ligand, L, and complex, PL, are in equilibrium, we can write the equilibrium constant, $K_{pl}$:

$$K_{pl} = \frac{[PL]}{[P_f][L_f]} \tag{17-16}$$

If this mixture is now chromatographed on an affinity column with immobilized X, which is capable of interacting reversibly with *any* component of the equilibrium, we can write a series of equations for the appropriate equilibrium constants and calculate the product concentration,

$$P + X \longleftrightarrow PX \qquad [PX] = K_{px}[P_f][X_f] \tag{17-17}$$

$$L + X \longleftrightarrow LX \qquad [LX] = K_{lx}[L_f][X_f] \tag{17-18}$$

$$L + PX \longleftrightarrow LPX \qquad [LPX] = K_1K_{px}[P_f][L_f][X_f] \tag{17-19}$$

$$P + LX \longleftrightarrow LPX \qquad [LPX] = K_2K_{lx}[P_f][L_f][X_f] \tag{17-20}$$

$$X + PL \longleftrightarrow LPX \qquad [LPX] = K_3K_{pl}[P_f][L_f][X_f] \tag{17-21}$$

where the designation $f$ indicates the free species.

The volume ($V_a$) of the affinity column that is accessible to the protein is given by $t$:

$$V_a = V_0 + K^*_{av}V_s \tag{17-22}$$

where $V_0$ is the void volume, $V_s$ the volume of the stationary phase, and $K^*_{av}$ that fraction of the stationary phase accessible to the protein.

Affinity chromatography usually involves a gel matrix as the stationary phase, and retardation of the protein involves a gel filtering effect as well as specific interaction. The concentration of "immobilized" protein, $[P]_i$, at any point is given by

$$[P]_i = K_{px}[P_f][X_f] + Y[P_f][L_f][X_f] + [P_f](1 + K_{pl}[L_f])K^*_{av} \tag{17-23}$$

where $Y = K_1K_{px} + K_2K_{lx} + K_3K_{pl}$.

The concentration of protein in the mobile phase, $[P]_m$, is given by

$$[P]_m = [P_f](1 + K_{pl}[L_f]) \tag{17-24}$$

and the ratio of the concentration of "immobilized" to "mobile" protein is given by

$$K_{av} = \frac{[P]_i}{[P]_m} \tag{17-25}$$

From Eqs. (17-23) to (17-25) it is apparent that

$$K_{av} = \frac{K^*_{av} + [X_f](K_{px} + Y[L_f])}{1 + K_{pl}[L_f]} \tag{17-26}$$

$K_{av}$ and $K^*_{av}$ are converted into elution volumes by

$$V^*_p = V_0 + K^*_{av}V_s \tag{17-27}$$

$$V_p = V_0 + K_{av}V_s \tag{17-28}$$

where $V_p$ and $V^*_p$ are the elution volumes of the protein in the presence or absence, respectively, of interaction with the immobilized ligand, X.

From Eqs. (17-26) and (17-27), the equation

$$V_p - V^*_p = \frac{V_s[X_f](K_{px} + Y[L_f])}{1 + K_{pl}[L_f]} \tag{17-29}$$

is obtained. $[X_f]$ may be written in terms of the *total* concentration of X, $[X]_t$, as shown by

$$[X_f] = \frac{[X]_t}{1 + K_{px}[P_f] + K_{lx}[L_f] + Y[P_f][L_f]} \tag{17-30}$$

Using Eqs. (17-29) and (17-30), we get the general equation for $(V_p - V^*_p)$,

$$V_p - V^*_p = \frac{V_s[X]t(K_{px} + Y[L_f])}{(1 + K_{pl}[L_f])(1 + K_{lx}[L_f] + [P]t)(K_{px} + Y[L_f])} \tag{17-31}$$

where $[P]_t$ is the total protein concentration.

Clearly, this is completely general in that it contains all six of the equilibrium constants defined by Eqs. (17-16) to (17-21). Specific equations for use in particular experimental systems are obtained from this general equation by setting certain equilibrium constants equal to 0. We consider here the two cases most likely to be encountered. They are as follows: (1) protein binds to either immobilized ligand or to free ligand B, but not to both, and (2) only the protein–ligand complex binds to the immobilized ligand. These two cases yield the following equations (obtained by setting other constants = 0):

Case 1:

$$\frac{1}{V_p - V^*_p} = \frac{K^l_p[L_f]}{V_s[X][K_{px}]} + \frac{1 + K_{px}[P]}{V_s[X]K_{px}} \tag{17-32}$$

Case 2:

$$\frac{1}{V_p - V^*_p} = \frac{1}{(V_s[X]K_3K_{pl}[L_f]} + \frac{1 + K_3[P]}{V_s[X]K_3} \tag{17-33}$$

In case 2, $V^*_p = V_p$ and $V_p$ can be substituted directly. In case 1, however, $V^*_p$ is not equal to $V_p$, but it is possible to use $V_p$ by subtracting the expression for $V_p - V^*_p$

[obtained by setting [L] = 0], in which case $V_p = V_p^*$, giving

$$(V_p - V_p^*) = \frac{V_s[\mathrm{X}]K_{px}}{1 + K_{px}[\mathrm{P}]}$$

and we get, for case 1,

$$\frac{1}{V_p - V_p^*} = -\left\{\frac{(1 + K_{px}[\mathrm{P}])^2}{V_s K_{px} K_{pl}[\mathrm{X}][\mathrm{L}_f]} + \frac{1 + K_{px}[\mathrm{P}]}{V_s K_{px}[\mathrm{X}]}\right\} \tag{17-34}$$

Now we have expressions in terms of $V_p$ and $V_p^*$ and where $V_p$ is the elution volume of the protein under conditions where it interacts with the matrix and $V_p^*$ is the elution volume where no interaction occurs.

These equations also involve the equilibrium concentration of the *free* ligand $[\mathrm{L}_f]$, which is related to the total ligand concentration [L] by

$$[\mathrm{L}_f] = \frac{[\mathrm{L}]}{1 + K_{pl}[\mathrm{P}]} \tag{17-35}$$

which upon substitution into Eqs. (17-32) and (17-33), gives, for cases 1 and 2, respectively,

$$\frac{1}{V_p - V_p^*} = -\left\{\frac{(1 + K_{px}[\mathrm{P}])^2(1 + K_{pl}[\mathrm{P}])}{V_s K_{px}[\mathrm{X}] K_{pl}[\mathrm{L}]} + \frac{1 + K_{px}[\mathrm{P}]}{V_s K_{px}[\mathrm{X}]}\right\} \tag{17-36}$$

and

$$\frac{1}{V_p - V_p^*} = \frac{1 + K_{pl}[\mathrm{P}]}{V_s[\mathrm{X}]K_3 K_{pl}[\mathrm{L}]} + \frac{1 + K_3[\mathrm{P}]}{V_s[\mathrm{X}]K_3} \tag{17-37}$$

In *either* case a plot of $1/(V_p - V_p^*)$ versus $1/[\mathrm{L}]$ is curvilinear, and the limiting slopes and intercepts (as $1/[\mathrm{L}] \rightarrow 0$) are given in Table 17-3.

From these it is obvious that in either case, a single set of experiments at a fixed protein concentration yields two expressions in three unknowns. A third expression is required. Although several approaches can be used, the simplest is to perform a second set of experiments at a different protein concentration with a range of ligand concentrations, including 0, to allow determination of the new value of $V_p$. The dissociation constant $K_{pl}$ for the protein-free ligand complex can now be determined.

**TABLE 17-3** Values of slopes and intercepts from plots of $1/(V_p - V_p^*)$ versus $1/[\mathrm{L}]$

| Case | Slope | Intercept |
|---|---|---|
| 1 | $-\left\{\frac{(1 + K_{px}[\mathrm{P}])^2}{V_s K_{pl} K_{px}[\mathrm{X}]}\right\}$ | $-\left\{\frac{1 + K_{px}[\mathrm{P}]}{V_s K_{px}[\mathrm{X}]}\right\}$ |
| 2 | $\frac{1}{V_s[\mathrm{X}]K_3 K_{pl}}$ | $\frac{1 + K_3[\mathrm{P}]}{V_s[\mathrm{X}]K_3}$ |

## REPRESENTATION OF LIGAND BINDING DATA

For the simple equilibrium $P + L \rightleftharpoons PL$, the dissociation constant, $K_d$, is given by

$$K_d = \frac{[P_f][L_f]}{[PL]} \tag{17-38}$$

where $f$ denotes the concentration of free species at equilibrium. The total concentration of protein, $P_t = [PL] + [P_f]$, and the concentration of PL, may be expressed as a function of $[L_f]$ if $[P_f]$ is eliminated from Eq. (17-38), as shown in

$$[PL] = \frac{[P_t]}{1 + K_d/[L_f]} \tag{17-39}$$

If $P_t$ is constant, as is usually the case, a plot of $[PL]$ versus $[L_f]$ is a rectangular hyperbola and, analogous to the Michaelis–Menten equation, the concentration of $[L_f]$ at which $[PL] = \frac{1}{2}[P_t]$ is equal to $K_d$. This is summarized in Fig. 17-10.

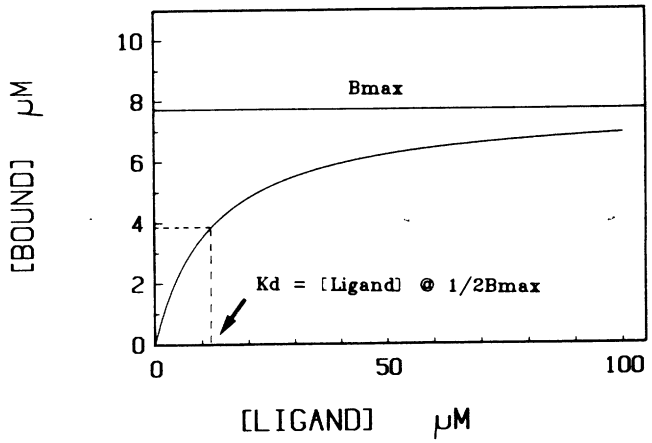


**Figure 17-10** Saturation curves for ligand binding to protein.

As with enzyme kinetics, several linearized versions of this equation are used, most notably the Klotz equation,

$$\frac{1}{[PL]} = \frac{1}{[P_t]} + \frac{K_d}{[P_t]}\frac{1}{[L_f]} \tag{17-40}$$

and the Scatchard equation,

$$[PL] = [P_t] - \frac{K_d[PL]}{[L_f]} \tag{17-41}$$

The equivalent linear plots, the Klotz plot and the Scatchard plot, are illustrated in Figs. 17-11 and 17-12, respectively. All of these equations refer to a case where a single protein molecule has a single binding site for ligand.

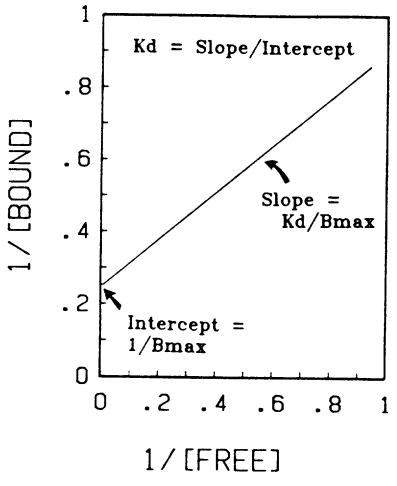


**Figure 17-11** Klotz plot of equilibrium binding data.

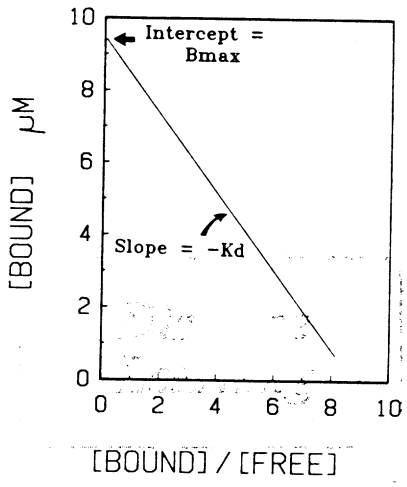


**Figure 17-12** Scatchard plot of equilibrium binding data.

### *Identical, Independent Binding Sites*

Many oligomeric proteins contain more than one binding site per molecule for a particular ligand, and in cases where the sites are independent *and* have the same microscopic dissociation constant, the interactions of the protein (P) with the ligand (L) can be characterized by the following equilibria:

$$P_0 + L \longleftrightarrow P_1$$
$$P_1 + L \longleftrightarrow P_2$$
$$P_{n-1} + L \longleftrightarrow P_n$$

where the number indicates the number of ligand molecules that are bound to the protein. Each site has the same microscopic dissociation constant, designated by $mK$. The *macroscopic dissociation constant* $K_d$, however, depends on the level of occupancy of the molecule, as indicated by

$$K_{d_1} = \frac{[P_0][L_f]}{[PL_1]} \tag{17-42}$$

$$K_{d_2} = \frac{[PL_1][L_f]}{[PL_2]} \tag{17-43}$$

$$K_{d_n} = \frac{[PL_{n-1}][L_f]}{[PL_n]} \tag{17-44}$$

The macroscopic dissociation constant $K_{d_i}$ is related to the microscopic dissociation constant $mK$ in such a system by the relationship given in

$$K_{d_i} = \left(\frac{\sum n, i-1}{\sum n, i}\right) mK \tag{17-45}$$

where $\sum n, i$ is the number of microscopic forms that make up $PL_i$. From Eqs. (17-42) to (17-45) we can write

$$[PL_i] = \frac{[PL_{i-1}][L_f]}{K_{d_i}} \tag{17-46}$$

and since $\sum n, i = n!/(n-1)!i!$, we can write

$$[PL_i] = [PL_{i-1}] \frac{n-i+1}{i} \frac{[L_f]}{mK} \tag{17-47}$$

Similarly, from expressions for $PL(i-1)$, $PL(i-2)$, and so on, we get

$$PL_i = PL_0 \left(\prod_{j=1}^{i} \frac{n-j+1}{j}\right) \left(\frac{[L_f]}{mK}\right)^i \tag{17-48}$$

The moles of ligand bound per mole of protein, designated as $L_b$, is given by

$$L_b = \frac{\sum_{i=0}^{n} i[PL_i]}{\sum_{i=0}^{n} [PL_i]} \tag{17-49}$$

and using Eq. (17-48) for $[PL_i]$, we get

$$L_b = \frac{\sum_{i=1}^{n} i\left\{\prod_{j=1}^{1} [(n-j+1)/j]\right\}([L_f]/mK)^i}{1 + \sum_{i=1}^{n} \left\{\prod_{j=1}^{i} [(n-j+1)/j]\right\}([L_f]/mK)^i} \tag{17-50}$$

which, using the expression for $\sum n, i$, simplifies as follows:

$$\prod_{j=1}^{i} [(n-j+1)j] = \frac{n!}{(n-i)!\, i!} \tag{17-51}$$

Therefore,

$$L_b = \frac{\sum_{i=1}^{n} i[n!/(n-i)!\, i!]([L_f]/mK)^i}{1 + \sum_{i=1}^{n} [n!/(n-i)!\, i!]([L_f]/mK)^i} \tag{17-52}$$

The denominator of this equation is the binomial expansion of $(1 + [L_f]/mK)^n$:

$$\left(1 + \frac{[L_f]}{mK}\right)^n = 1 + \sum_{i=1}^{n} [n!(n-i)!\, i!] \left(\frac{[L_f]}{mK}\right)^i \tag{17-53}$$

which, upon differentiation with respect to $[L_f]/mK$ and multiplication by $[L_f]/mK$, yields

$$n\left(\frac{[L_f]}{mK}\right)\left(1+\frac{[L_f]}{mK}\right)^{n-1}=\sum_{i=1}^{n} i\left[\frac{n!}{(n-i)!\,i!}\right]\left(\frac{[L_f]}{mK}\right)^{i} \tag{17-54}$$

Substitution into the expression for $[L_b]$ gives

$$[L_b]=\frac{n[L_f]/mK}{1+[L_f]/mK} \tag{17-55}$$

Since by definition $L_b = [PL]/[P_t]$, we get, after multiplication of both sides by $[P_t]$,

$$[PL]=\frac{n[P_t]}{mK/[L_f]+1} \tag{17-56}$$

giving

$$[PL]+[PL]\frac{mK}{[L_f]}=n[P_t] \tag{17-57}$$

which rearranges to

$$[PL]=n[P_t]-mK\frac{[PL]}{[L_f]} \tag{17-58}$$

which is the Scatchard equation derived earlier for a single site per molecule, where $n$ is the number of sites per molecule. As before, a plot of [PL] versus $[PL]/[L_f]$ is linear with a slope of $-mK$ and an intercept of $n[P_t]$.

### *Multiple Classes of Independent Sites*

A frequently encountered situation is the case where a Scatchard plot is nonlinear, as illustrated in Fig. 17-13. Where there are $n_i$ independent sites with intrinsic microscopic dissociation constants $mK_i$, we can write

$$[L_b]=\sum_i \frac{n_i[L_f]/mK_i}{1+[L_f]/mK_i} \tag{17-59}$$

which gives (following the same process as previously)

$$[PL]=\sum_i n[P_t]-\sum_i mK\frac{[PL]}{[L_f]} \tag{17-60}$$

In the Scatchard plot of Fig. 17-13, the intercept on the [PL] axis is, for the case shown (which involves *two* classes of sites) $n_1 + n_2$, and the intercept on the $[PL]/[L_f]$ axis is $n_1/mK_1 + n_2/mK_2$. The most realistic values of $n_1$, $n_2$, $mK_1$, and $mK_2$ are obtained by an iterative process.

Assuming initially that the $x$-axis intercept is dominated by the smaller $mK$ value (i.e., $mK_1$), a tangent to the curve at regions approaching [PL] = 0 gives an intercept

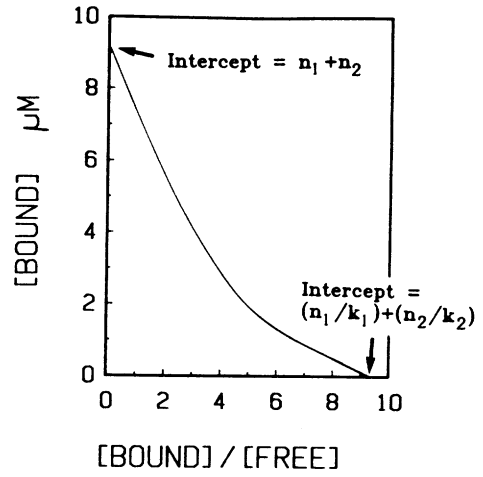


**Figure 17-13** Nonlinear Scatchard plot as might arise from two independent but nonidentical binding sites.

of $n_1/mK_1$, and on the $y$-axis an initial value for $n_1$. With initial estimates of $n_1$ and $mK_1$, we can subtract the contribution of the high-affinity sites from the data obtained at higher degrees of saturation, which can then be plotted to give initial estimates of $n_2$ and $mK_2$. Once these estimates of $n_2$ and $mK_2$ have been obtained, the contribution of the *low*-affinity sites to the data at low degrees of saturation can be subtracted and new estimates of $n_1$ and $mK_1$ obtained. Throughout the procedure $n_1 + n_2$ must equal the observed [PL] intercept, and the iterations are continued until $\sum_i (n_i/mK_i)$ equals the $x$-axis intercept.

### *Dependent Binding Sites*

In a situation where two identical ligand molecules bind to a protein molecule (either one subunit with two sites or two subunits, each with a binding site), it may be that binding the ligand to the first binding site alters the affinity of ligand binding to the second site. Two cases are possible: In the first, ligand binding at the first site *increases* the affinity of the second site, while in the second case, ligand binding at the first site decreases the affinity of the second site. In either instance nonlinear Scatchard (Fig. 17-14) or Klotz (Fig. 17-15) plots result.

As discussed in the chapters on nonlinear kinetics and allosteric models (Chaps. 16 and 21, respectively) it is not possible to distinguish cases of independent nonidentical sites from cases where the first ligand decreases the affinity of the second ligand. The Scatchard plot in Fig. 17-13 resembles that of Fig. 17-14, curve B. Mechanistically, such binding can result from allosteric interactions giving negative cooperativity, or from direct steric interaction of the bound ligands. In contrast to this situation, Scatchard or Klotz plots indicating that the first ligand molecule to bind increases the affinity of the second ligand molecule can be explained only by allosteric models.

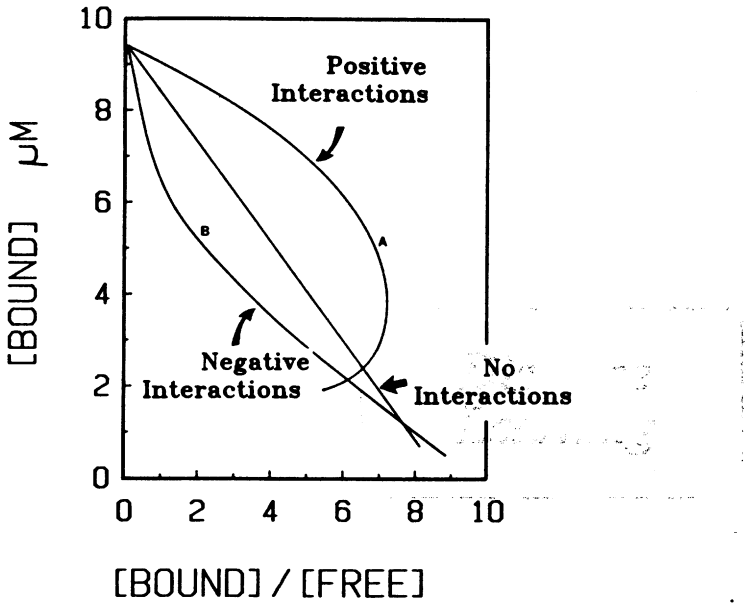


**Figure 17-14** Scatchard plots for dependent binding sites: (A) increased affinity of second ligand; (B) decreased affinity of second ligand.

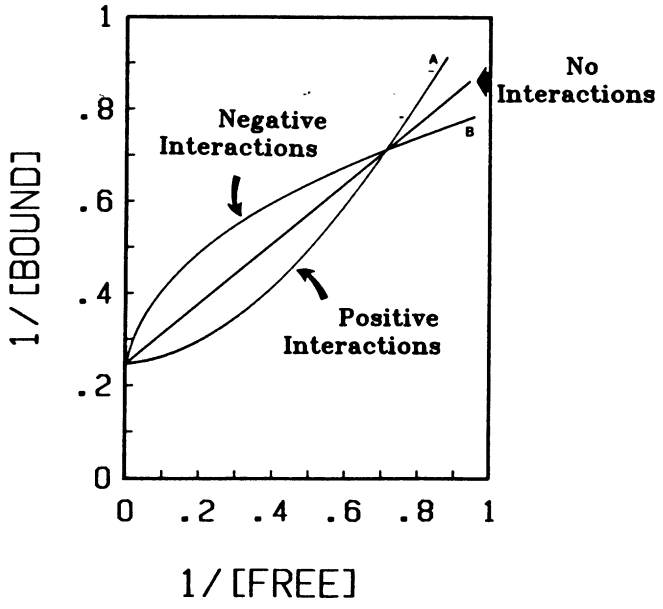


**Figure 17-15** Klotz plots for dependent binding sites: (A) increased affinity of second site; (B) decreased affinity.

This chapter examined a variety of experimental approaches for determining the dissociation constant and maximum binding capacity of a ligand binding to a protein. It must be emphasized that in the treatments developed here the binding is assumed to be in a free equilibrium on the time scale at which the experiment is performed. As discussed in Chaps. 14 to 16, such equilibrium binding studies are of extreme importance in elucidating kinetic mechanisms of enzymes and establishing the basis of nonlinear kinetics in systems that show non-Michaelis–Menton behavior. Similarly, as developed in Chap. 18, equilibrium binding studies complement the information obtained in rapid kinetic studies on the rates of ligand binding and release to and from protein complexes.