[25] Measurement and Analysis of Ligand-Binding Isotherms Linked to Protein Self-Associations

In the preceding chapter [24], we examined the method of studying ligand-induced protein self-associations using the velocity sedimentation technique.[1] Toward the end of the chapter, we discussed how to utilize the apparent self-association constants derived from the velocity sedimentation study and analyze them as a function of the ligand activity to probe the multiple equilibria of the system. In a ligand-induced protein subunit association system, the equilibria of the ligand-binding reaction and the self-association reaction are linked. Consequently, the apparent self-association constant and the apparent ligand-binding constant should be mutually dependent. A number of theoretical studies have shown that in linked self-association systems the shape of the ligand-binding isotherm depends on the macromolecular concentration, the intrinsic (microscopic) equilibrium constants, and the mechanism of the equilibrium linkage. These isotherms obtained at different protein concentrations and plotted in the Scatchard format could be concave either upward or downward and their Y intercepts could either converge or diverge.[2-5] Therefore, by analyzing the ligand-binding data and fitting it with theoretically calculated isotherms, one can also probe the multiple equilibria of the system. These two different approaches, although probing the same multiple equilibria, utilize different experimental methods. One measures the macromolecule self-association while the other determines the ligand binding. They are, therefore, independent of each other with regard to the nonidealities present in the experimental methods and the assumptions invoked in the data analyses. Consequently, by taking both approaches to examine a linked system and obtaining consistent results, one can gain much stronger confidence in the conclusions derived. Furthermore, as will become evident later in this chapter, these two approaches frequently shed light on different aspects of a linkage and complement each other well. On the phenomenological level, it is important to recognize that the cooperativity between a ligand-binding reaction and a self-association

[1] G. C. Na and S. N. Timasheff, this volume [24].
[2] J. R. Cann and N. D. Hinman, *Biochemistry* **15**, 4614 (1976).
[3] L. W. Nichol and D. J. Winzor, *Biochemistry* **15**, 3015 (1976).
[4] J. R. Cann, this series, Vol. 48, p. 299.
[5] R. F. Stein, *Mol. Cell. Biochem.* **31**, 5 (1980).

reaction can lead to curvilinear ligand-binding isotherms that are similar to those found in either allosteric or heterogeneous ligand-binding systems. Such similarities have led to frequent misinterpretations of ligand-binding data in the literature. In this chapter, we discuss the methods of diagnosing ligand-induced protein self-associations and probing the multiple equilibria of such systems through the measurement and analysis of their ligand-binding isotherms.

Measurement of Ligand–Protein-Binding Isotherms

There are countless numbers of methods available for the measurement of ligand–protein bindings. Many of them do not meet the criteria to be used in linked self-association systems. We shall examine some of the reasons why they do not, and then describe the experimental protocol of a batch gel filtration method which we have used successfully that can be adapted easily to other linked systems.

One can classify the methods of measuring ligand–protein-binding isotherms into two major categories, indirect measurement and direct measurement. In an indirect measurement, one monitors a specific change in a physical or chemical property of either the protein or the ligand that ensues from the ligand–protein interaction. This can be, for instance, a shift in the absorption spectrum, quenching of the fluorescence spectrum, or a change in NMR relaxation time. Such methods have the advantage that the measurements can be carried out without prior separation of the ligand from the macromolecule. However, they are subject to the serious drawback that, in order to calculate the amount of the bound ligand, one usually has to make the assumption that the characteristic property change is linearly proportional to the quantity of the ligand bound to the macromolecule. The validity of this assumption is frequently difficult to verify independently. It is very likely an oversimplification when dealing with proteins with multiple ligand-binding sites or those with the ligand-binding reaction linked to the subunit association reaction. This difficulty essentially rules out the use of indirect measurements in the rigorous and unambiguous determination of ligand binding in linked systems.

It is frequently easier to measure the total concentration of a ligand in a solution through monitoring a physical or chemical property of the ligand such as radioactivity or light absorption. With this type of approach, the property of the ligand to be monitored should be independent of the interaction of the ligand with the macromolecule, a condition that is usually not difficult to establish. However, in order to obtain directly the concentration of both the bound and free ligand, one usually has to separate the macromolecule from the free ligand. Thus, the method of separat-

ing the unbound ligand from the macromolecule, instead of the actual measurement of the ligand activity itself, often becomes the issue. The selection of the method of separation is dictated by two requirements. First, the separation must be carried out without perturbing the chemical equilibrium of the system, i.e., the results must reflect the true equilibrium of the reaction. Second, it must allow precise control of the protein concentration so that a ligand-binding isotherm can be determined under a constant protein concentration. Molecular transport methods, such as ultracentrifugation, size exclusion chromatography, and electrophoresis, have been used frequently to separate the unbound ligand from macromolecules. For instance, one can precipitate macromolecules by centrifugation, leaving only the free ligand in the supernatant. Such an approach has been demonstrated by Steinberg and Schachman, using an analytical ultracentrifuge equipped with an optical scanner to monitor the distribution of the ligand across the centrifuge cell.[6] However, as described in the preceding chapter,[1] in fast reversible ligand-induced protein associations, due to the differences in the sedimentation coefficient and ligand affinity between the monomer and the polymers, the ligand concentration found in the supernatant could differ substantially from the equilibrium concentration. This renders the method unacceptable for the present purpose. This difficulty with velocity sedimentation can be circumvented by using instead equilibrium ultracentrifugation. That method, however, is not suitable for protein concentration-dependent studies. Furthermore, the low sensitivity of the UV absorption optics often places a limit on the magnitude of the binding constant that can be measured by the instrument. In zonal methods such as the Hummel–Dreyer column gel filtration, the results are usually expressed as ligand binding across the complete protein peak instead of at a given protein concentration.[7] It is, therefore, not suitable for protein concentration-dependent studies. Paper discs impregnated with ion-exchange resins also have been used to separate the unbound ligand from the protein. This method requires extensive washing of the disc after the adsorption of the protein to the ionic resin to remove the unbound ligand. Since the bound ligand can be released and the protein concentration can be changed during the washing, the method is unacceptable unless one can demonstrate that the rates of the ligand and the protein association/dissociation are both very slow.

The methods that seem most suitable for the determination of the ligand binding of linked systems are those that use the principle of size exclusion to separate, yet maintain an equilibrium between the macromolecule and the free ligand. For instance, with certain ions, one can use

specific electrodes which employ semipermeable glass or membranes that allow the determination of the free ion activity in the presence of the macromolecule. Two other methods that use the same principle are equilibrium dialysis and batch gel filtration. The technique of equilibrium dialysis has been discussed in detail in earlier volumes of this series and will not be discussed here.[8,9] The batch gel filtration method is, in principle, quite similar to equilibrium dialysis. It uses a porous gel instead of a semipermeable membrane to maintain chemical and diffusional equilibria between two separate phases. The external phase corresponds to the mobile phase in column gel filtration and contains both the macromolecule and the ligand. The internal phase corresponds to the stationary phase in column gel filtration and contains only the free ligand.[9-11] Compared with equilibrium dialysis, the batch gel filtration method usually requires less material and the diffusional equilibrium can be reached within a shorter period of time. These could be significant advantages when dealing with precious and/or unstable proteins. Furthermore, the batch gel filtration method allows more precise control of the protein concentration than does equilibrium dialysis. However, as in the case of equilibrium dialysis, the method is sometimes complicated by the adsorption of ligand, usually aromatic ones, to the gel matrix which requires corrections in data analysis.[10-13] Nevertheless, parallel studies have shown that the ligand-binding results obtained from the batch gel filtration method are consistent with those obtained from other techniques.[10,14] The following is an experimental protocol of the batch gel filtration method that we have used successfully in the determination of the binding of the anticancer drug vinblastine to tubulin.[16] One should be able to adopt it easily for other linked systems, and it is of course applicable to systems which do not exhibit linkage.

Determination of Ligand–Protein Binding by Batch Gel Filtration

Preliminary Considerations. Before embarking on the measurement of ligand–protein bindings, one should first experiment with different types of gels. They frequently show different degrees of ligand adsorption due

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[9] P. McPhie, this series, Vol. 22, p. 23.
[10] P. Fasella, G. G. Hammes, and P. R. Schimmel, *Biochim. Biophys. Acta* **103,** 708 (1965).
[11] W. H. Pearlman and O. Crépy, *J. Biol. Chem.* **242,** 182 (1967).
[12] J.-C. Janson, *J. Chromatogr.* **28,** 12 (1967).
[13] B. Gellote, *J. Chromatogr.* **3,** 330 (1960).
[14] M. Hirose and Y. Kano, *Biochim. Biophys. Acta* **251,** 376 (1971).
[15] G. C. Wood and P. F. Cooper, *Chromatogr. Rev.* **12,** 88 (1970).
[16] G. C. Na and S. N. Timasheff, in preparation.

to their different chemical characteristics. In addition to the commonly used gels, such as the polyacrylamide-based BioGel (Bio-Rad Chemical Co.)[17] and Dextran-based Sephadex (Pharmacia Chemical Co.), the silicate-based porous glass beads (Pierce Chemical Co.) often display less ligand adsorption and deserve consideration. It should be cautioned, however, that glass beads in the simple form often absorb proteins, particularly those with higher charge densities. This difficulty can be partially alleviated by choosing the carbohydrate-coated glass beads and using a higher ionic strength buffer. With a given type of gel, the ligand adsorption usually decreases with increasing pore size of the gel.[12-14] Consequently, it is best to select the largest pore size gel that still excludes the protein. The adsorption of the ligand to the gel is often time dependent. To minimize the adsorption, the gel can be swollen in the absence of the ligand so that the ligand of interest is allowed to be in contact with the gel for a short period of time, just sufficient to achieve diffusional and chemical equilibria. Adsorption is not the only factor that makes the partition coefficient of the ligand, defined as the ratio of the ligand concentration in the internal phase of the gel to that in the external phase, deviate from the ideal value of one. If the size of the ligand molecule is substantially larger than that of water, the partition coefficient of the ligand could be less than one. This is apparently due to the exclusion of the ligand from a portion of the internal volume of the gel that is accessible to water molecules. At low ligand concentrations, the adsorption of the ligand to the gel, if it occurs, is usually the dominant factor and the partition coefficient becomes higher than one. At high ligand concentrations, the amount of the adsorbed ligand plateaus and becomes insignificant relative to the total ligand. The size exclusion effect, if any, may emerge to reduce the partition coefficient of the ligand to less than one. Again, to account for the nonideality, due either to the adsorption of the ligand to the gel matrix or to the exclusion of the ligand from a portion of the internal volume of the gel, the data have to be corrected against the partition coefficient of the ligand determined in the absence of the protein.

Experimental Protocol. Borosilicate culture tubes (12 × 75 mm) are soaked in Nochromix solution (Godax Lab., Inc.) and then rinsed thoroughly before use. The washed glass tubes should show little solution adherence to the test tube wall which may affect the accuracy of the measurements. Our experience has shown that plasticwares, both test tubes and disposable pipettes, frequently absorb aromatic ligands and should be avoided. In the method described below, the volumes of the

aliquots are based on BioGel P-100. Both the weight of the gel and the volumes of the aliquots can be modified, scaled either up or down depending on the particular gel used and the availability of the material under investigation.

Forty milligrams of the dried gel is weighed in each tube using a semimicroanalytical balance. Of the experimental buffer 0.5–0.6 ml is then added to each tube. The buffer contains all the ingredients except the protein and the ligand of interest. The volume of the buffer is approximately 20% higher than the internal volume of the gel to ensure complete swelling of the gel. The tubes are tightly sealed with parafilm and the gel is allowed to swell for 20 hr at room temperature. At the end of the swelling, 0.2 ml of the experimental buffer containing a known concentration of the ligand is added and mixed gently. This is followed immediately by 0.2 ml of the protein stock solution, which had been preequilibrated with the same experimental buffer without the ligand through either column or batch gel filtration or equilibrium dialysis. The complete mixture is incubated at a constant temperature with gentle shaking to avoid splashing the solution to the test tube wall. The time interval of incubation is predetermined to be sufficient for achieving both chemical and diffusional equilibria, usually within 10 min. After the incubation, the tubes are left to stand still to allow the gel to settle by gravity to the bottom. Aliquots are then carefully removed from the supernatant for measurements of the ligand and protein concentrations. Controls are treated identically as the samples. They contain the same mixture except either the protein or the ligand solution is replaced by additional experimental buffer. The former controls give the partition coefficient of the ligand, while the latter ones give the excluded volume of the protein, both with respect to the particular gel used. Higher degrees of precision and accuracy can be achieved if all of the aliquots are measured gravimetrically with a semimicroanalytical balance. Weights of the aliquots can be converted to volumes using the density of the buffer solution which can be determined accurately with a precision densimeter.[18] The presence of the protein and the ligand at low concentrations should have little effect on the solution density and can be ignored in the calculation. The internal or excluded volume of the gel is also determined by using Blue Dextran 2000 (Pharmacia Chemical Co.). Three milliliters of Blue Dextran 2000 at concentrations ranging from 0.02 to 0.2% is added to each tube containing 100 mg of the dried gel. The gel is incubated for 20 hr at room temperature. Aliquots are then taken from the supernatant for measurement of the Blue Dextran concentration using its UV absorbance at 260 nm. If the gel is properly chosen, the excluded

volume obtained using Blue Dextran 2000 should not differ by more than 5% from the value obtained with the protein.

Data Calculation. The excluded volume per unit weight of the gel, $\bar{v}_i$, can be calculated from

$$A_e/A = v_t/v_e \quad (1)$$
$$v_t = v_e + v_i \quad (2)$$
$$\bar{v}_i = v_i/g \quad (3)$$

where A and A_e are the absorbances at 260 nm of Blue Dextran 2000 in the stock solution and the external phase, respectively, v_t is the total volume of the solution, v_e is the external volume, v_i is the internal volume, and g is the weight of the gel. Once $\bar{v}_i$ is determined, for a given weight of the gel, the internal and external volumes can be calculated from Eq. (3). Following the law of conservation of mass, $[X_i]$, the concentration of the ligand in the internal volume of the gel, can be calculated from

$$[X_i] = \frac{(X) - [X_e]v_e}{v_i} \quad (4)$$

where (X) is the total amount of the ligand added and $[X_e]$ is the concentration of the ligand in the external volume. In an ideal case, the partition coefficient of the free ligand should be equal to one. However, because of

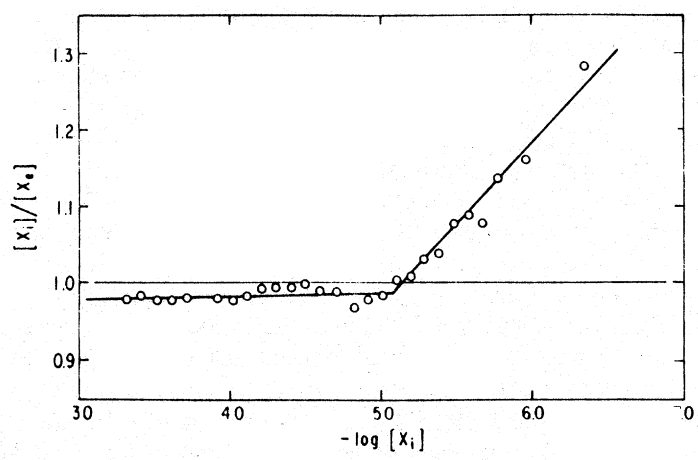

FIG. 1. The partition of [^{3}H]vinblastine in BioGel P-100. BioGel P-100 was swollen for 24 hr. [^{3}H]Vinblastine was then added and the mixture was incubated at 20° for 1 hr before aliquots were removed for radioactive scientillation counting. Data were least-squares fitted to two straight lines, from 10^{-7} to 10^{-5} M vinblastine and from 10^{-5} to 10^{-3} M vinblastine.

the factors described above, for many ligands it may deviate from one. The nonideality can be corrected using the partition coefficient of the free ligand determined in the absence of the protein as a function of the ligand concentration. Figure 1 shows the partition coefficient of vinblastine, a plant alkaloid and anticancer drug, in the presence of BioGel P-100. Above 1×10^{-5} *M* of ligand, the partition coefficient has a rather constant value of 0.98 ± 0.01. Below 1×10^{-5} *M*, the adsorption of the ligand to the gel caused a linear increase of the partition coefficient as a function of the logarithm of the ligand concentration within the gel. By assuming that these partition coefficients are not affected by the presence of the protein, one can obtain $[X_f]$, the concentration of the free ligand in the external volume, from $[X_i]$ by interpolating from the predetermined partition coefficients of the free ligand. Once $[X_f]$ is known, one can calculate the concentration of the ligand bound to the protein in the external volumes, $[X_b]$:

$$[X_b] = [X_e] - [X_f]$$

Analyses of Ligand-Binding Isotherms

Data Acquisition

Protein self-association, being an intermolecular event, is dependent on protein concentration. Consequently, a ligand-binding reaction linked to a protein self-association reaction must also be dependent on the protein concentration. This contrasts with the cases of heterogeneous, but independent ligand-binding sites on a protein and allosteric ligand binding in which free energy coupling takes place only intramolecularly between different ligand-binding sites. Although both of these types of ligand binding can display curvilinear binding isotherms, neither should be dependent on the macromolecular concentration. Thus, the first step toward establishing a ligand-induced self-association system is to demonstrate that the ligand-binding isotherms obtained are dependent on the total protein concentration. Subsequent to this, a more rigorous and quantitative linkage analysis can be carried out, if one has on hand several ligand-binding isotherms that span one order of magnitude or more of the protein concentration. The range of protein concentration used should depend on the self-association constant and the linkage mechanism. For quantitative analysis, the binding isotherm obtained at the lowest protein concentration should be close to that of the intrinsic binding, whereas that obtained at the highest protein concentration should manifest substantial deviation from the intrinsic binding.

Data Plotting

The ligand binding data obtained are best analyzed by the Scatchard plot[19] ($\bar{X}/[X_f]$ vs $\bar{X}$, where $\bar{X} = [X_b]/[A_t]$; $[X_f]$ and $[X_b]$ are the concentration of the free and bound ligand, respectively, and $[A_t]$ is the molar concentration of the macromolecule in terms of the monomer) rather than the double-reciprocal plot ($\bar{X}^{-1}$ vs $[X_f]^{-1}$) or the saturation plot ($\bar{X}$ vs $[X_f]$). When dealing with ligand bindings that are linked thermodynamically to self-associations, the Scatchard plot is superior to the other two plots in its ability to diagnose different equilibrium linkage mechanisms and to allow easy derivation of the intrinsic association constants.[2] The reason lies in the intrinsic differences between these plotting methods. In the Scatchard plot, the ligand binding constant can usually be extracted from either the slope or the Y intercept of the data.[20] Since ligand binding isotherms of linked self-association systems are usually curvilinear, the ligand binding constants can be more easily and accurately derived from the Y intercept rather than the slope of the curve. By using the Y intercept of the Scatchard plot, the data analysis is focused at the region where the ligand concentration approaches zero. At diminishing ligand concentrations, the Y intercept of the Scatchard plot can be expressed in simpler terms of the intrinsic equilibrium constants and the macromolecular concentration and is, therefore, more amenable to analysis. On the other hand, with a double-reciprocal plot, the binding constant is derived from either the slope of the data or the extrapolated value on the X axis. The extrapolation can be carried out only when the isotherm is linear. Consequently, for curvilinear isotherms which prevail in the ligand-induced association systems, one has to resort to the slope of the plot to derive the intrinsic equilibrium constants. The latter process requires additional manipulation of the data and is less accurate. Furthermore, as shown in the latter part of this chapter, different linkage mechanisms of ligand-induced self-association manifest most pronounced differences in their apparent ligand bindings in the region where the association-linked binding site is less than half saturated. Such differences appear more distinctly and dramatically in the Scatchard plot than in the other two plots.[2]

Although one can use the same Scatchard plot and extrapolate the data to the X axis to obtain the ligand-binding stoichiometry, the validity of the latter value depends on the linearity of the data. Since the Scatchard plots of self-association-linked ligand bindings are almost never linear, one should be cautioned against this practice. In most cases, it is better to plot the same data in the form of a saturation plot ($\bar{X}$ vs log [X])

which allows direct visualization of the degree of saturation of a given binding site or class of binding sites and is therefore more reliable than the Scatchard plot in deriving the ligand binding stoichiometry.[21]

No matter how the data are plotted, there is no way of assuring complete saturation of a protein by a given ligand. Consequently, the range of the ligand concentration used in a binding study must be decided through other means. The best guideline obviously can be found in a complementary study of the macromolecular self-association reaction, i.e., determination of the range of ligand concentrations that exerts a clearly observable effect on the equilibrium of the protein self-association reaction and measurement of the ligand–protein binding in that range, plus maybe one order of magnitude at each end. Ligand bindings that take place outside this range are independent of and irrelevant to the self-association reaction.

Model Fitting

Ligand-binding isotherms in the form of Scatchard plots can be analyzed quantitatively by fitting them with theoretically calculated isotherms. The characteristic shape of the ligand-binding isotherm is usually more uniquely dependent on the linkage mechanism and is generally insensitive to the macromolecular association stoichiometry, i.e., if one varies the macromolecular stoichiometry but maintains the same linkage mechanism, the resulting calculated isotherms usually change only quantitatively without losing their characteristic shape. Consequently, one can frequently deduce the linkage mechanism simply from the shape of the binding isotherm and without any knowledge of the stoichiometry of the macromolecular association. However, for a rigorous quantitative analysis, it is imperative to determine the stoichiometry of the macromolecular association through other means, such as analytical ultracentrifugation or light scattering, so that the exact theoretical ligand-binding isotherms can be calculated and used to fit the data. To aid the process of model selection and isotherm calculation, we present below the calculation of ligand-binding isotherms for the four different ligand-induced self-associations examined in the preceding chapter while discussing the linked function analysis of the apparent macromolecular self-association constant.[1] Each of these systems manifests a ligand-binding isotherm with a characteristic shape. They can be used by the readers, when confronted with an unknown system, as an initial guideline in selecting linkage mechanisms to fit the data.

In the following sections, we will use lower-case k's for the intrinsic (microscopic) equilibrium constants and upper-case K's for the apparent (macroscopic) equilibrium constants. Although only dimerization reactions are shown, each linkage mechanism can be extended to any macromolecular association stoichiometry. For a ligand-induced self-association, the binding of the ligand to the macromolecule, irrespective of the reaction mechanism, can be expressed in the Scatchard format as

$$\frac{\bar{X}}{[X_f]} = \frac{[X_b]}{[A_t][X_f]} = \frac{\sum_{i=1}^{n}\sum_{j=1}^{m} j[A_iX_j]}{[A_t][X_f]}$$

where $[X_b]$ and $[X_f]$ refer to the concentration of the bound and free ligand, respectively. $[A_t]$ is the total molar concentration of the macromolecule in terms of the monomer and $[A_iX_j]$ is the molar concentration of the i-mer containing j bound ligand. Two somewhat different approaches are available for the calculation of the theoretical ligand-binding isotherms. In the first approach, the concentration of each i-mer containing j bound ligand is expressed in terms of $[A_f]$, the free monomer concentration, $[X_f]$, the free ligand concentration, and the intrinsic equilibrium constants. Given the total protein concentration, the free ligand concentration, and the intrinsic association constants, one can calculate $[A_f]$, the free monomer concentration. For a dimerization reaction and an isodesmic indefinite self-association, the calculation is simple because it only requires the solving of a binomial equation. For associations with other stoichiometries, the values of $[A_f]$ can be obtained by solving the equation numerically using the Newton–Raphson rule.[22] Given $[A_t]$, $[A_f]$, $[X_f]$, and all the intrinsic equilibrium constants, the summation of Eq. (6) gives the Scatchard binding isotherm. In the case of an indefinite self-association, the summation of Eq. (6) at each free ligand concentration proceeds with increments of i until a major portion of the total protein, e.g., 99.9% is accounted for.

An alternative approach to obtain the theoretical isotherms is to calculate the total concentration of each i-mer containing all the liganded species with the use of the apparent self-association constant. From the total concentration of each i-mer, the amount of the ligand bound to the i-mer usually can be calculated. Both of these methods of calculating the ligand binding isotherms are demonstrated below.

Ligand-Mediated Self-Association. This association mechanism was defined as one in which the binding of the ligand to monomers precedes the macromolecular self-association. The original definition permits the

polymerization between a liganded and an unliganded monomer.[2,3] We further stipulate here that each and every monomer must first bind the ligand before it can participate in the self-association reaction. A one-ligand-mediated dimerization is depicted by the following equation:

$$\begin{array}{ll} A + X \rightleftharpoons AX & k_1 \\ 2\,AX \rightleftharpoons A_2X_2 & 2k_2 \end{array} \tag{7}$$

If the linkage mechanism is extended to an isodesmic indefinite one, then the apparent self-association constant for each step of the polymerization is identical. For a one-ligand-mediated isodesmic self-association, i.e., self-association induced by the binding of one ligand per monomer,[1] the apparent association constant for each step of the reaction can be expressed as

$$K_2 = \frac{[A_2X_2]}{([A] + [AX])^2} = \frac{2k_1^2k_2[X_f]^2}{(1 + k_1[X_f])^2} \tag{8}$$

where $[X_f]$ is the concentration of the free ligand and k_1 and k_2 have the same definitions as in Eq. (7). Since all polymers must have their association-linked ligand binding sites occupied, one can set $j = i$ and $m = n$ in Eq. (6) and obtain

$$\frac{\bar{X}}{[X_f]} = \frac{\sum_{i=1}^{n} ik_1^i(2k_2)^{i-1}[A_f]^i[X_f]^{i-1}}{[A_t]} \tag{9}$$

where $[A_f]$ is the concentration of unliganded monomers and $[A_t]$ is as defined above in Eq. (6). Consequently,

$$\lim_{[X_f]\to 0} \frac{\bar{X}}{[X_f]} = k_1 \tag{10}$$

which means that the Y intercept of a Scatchard plot of this type of linked system will have the value of k_1 regardless of the total protein concentration used.

Figure 2 depicts the Scatchard binding isotherms of a one-ligand-mediated isodesmic indefinite self-association at different protein concentrations. Similar characteristic ligand-binding isotherms were found in the ligand-mediated self-associations with lower macromolecular stoichiometries or with higher ligand stoichiometries.[2,3] There are several salient features in the ligand-binding isotherms of such systems which allow their easy identification. They approach a straight line near zero protein concentration but become increasingly concave downward as the protein

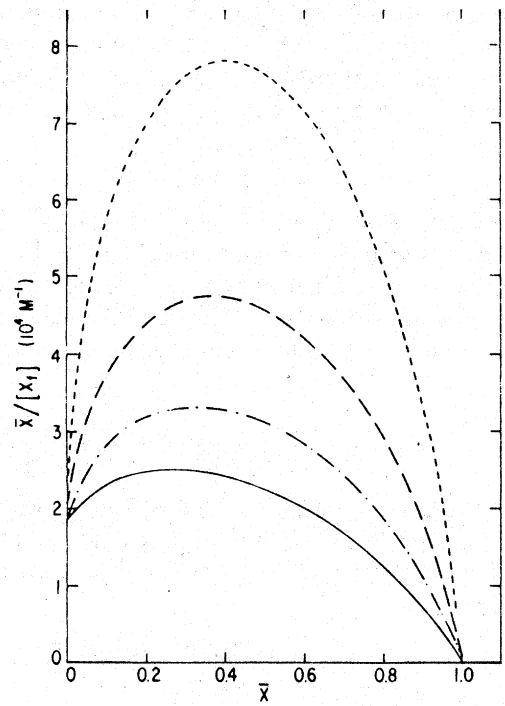

FIG. 2. Scatchard plots of the ligand binding of a one-ligand-mediated isodesmic indefinite self-association. The protein concentrations are 1.6×10^{-5} M (——), 3.4×10^{-5} M (- - -), 7.8×10^{-5} M (— —), and 2.3×10^{-4} M (– –). The intrinsic association constants used are $k_1 = 1.8 \times 10^4$ M^{-1} and $k_2 = 9 \times 10^4$ M^{-1}.

concentration increases. This is because the ligand binding is enhanced by the self-association reaction and, thus, increased at higher protein concentration. As the protein concentration approaches zero, the self-association diminishes and the apparent ligand binding approaches the intrinsic ligand binding. This is mathematically equivalent to setting the self-association constant k_2 to zero. At higher protein concentrations, a maximum can be found in the curvilinear isotherms between ligand stoichiometries of 0.3 and 0.5 for the one-ligand-mediated system. As predicted by Eq. (10), all binding isotherms show the same Y intercept k_1, the intrinsic ligand-binding constant for the ligand-binding site.

The ligand-binding isotherms of ligand-mediated self-association systems can be analyzed by first determining k_1, the ligand-binding constant of the monomer, from the Y intercept of the isotherms, and subsequent

fitting of the isotherms to obtain k_2, the association constant between two liganded monomers.

Ligand-Facilitated Self-Association. A ligand-facilitated self-association is defined as one in which the ligand binds only to the polymers. A one-ligand-facilitated dimerization is depicted by the following equations:

$$\begin{array}{rcll} 2A & \rightleftharpoons & A_2 & 2k_4 \\ A_2 + X & \rightleftharpoons & A_2X & k_3 \\ A_2X + X & \rightleftharpoons & A_2X_2 & k_3 \end{array} \tag{11}$$

The model assumes an identical ligand-binding constant, k_3, for the two association-linked sites on the dimer. For such a self-association scheme, the apparent self-association constant can be expressed by

$$K_2 = \frac{[A_2] + [A_2X] + [A_2X_2]}{[A]^2} = 2k_4 + 2k_3k_4[X_f] + 2k_3^2k_4[X_f]^2 \tag{12}$$

Let us consider a similar ligand-facilitated isodesmic indefinite self-association having the same intrinsic association constants k_3 for each ligand binding site and k_4 for each step of the self-association. The weight concentration of each i-mer, c_i, can be calculated by using the equation

$$c_t = \frac{c_1}{[1 - (K_2c_1/\mathrm{MW})]^2}, \qquad c_i = i\left(\frac{K_2}{\mathrm{MW}}\right)^{i-1} c_1^i \tag{13}$$

where MW is the molecular weight of the monomer. The concentration of the ligand bound to the macromolecule can be obtained by

$$[X_b] = \sum_{i=2}^{n} \frac{c_i}{\mathrm{MW}} \frac{k_3[X_f]}{(1 + k_3[X_f])} \tag{14}$$

Figure 3 shows the Scatchard binding isotherms for a ligand-facilitated isodesmic indefinite self-association obtained by using Eqs. (12), (13), and (14). The model assumes $k_4 = 9 \times 10^2\ M^{-1}$, a weak self-association of the monomer in the absence of the ligand. Similarly to the ligand-mediated self-association, the binding isotherms for the ligand-facilitated self-association are also concave downward. However, their Y intercepts increase with increasing protein concentration, instead of converging to a single point as do those of the ligand-mediated association systems. Consequently, at high protein concentrations, the isotherm loses its downward curvature. The amount of the ligand bound to the macromolecule can also be expressed as

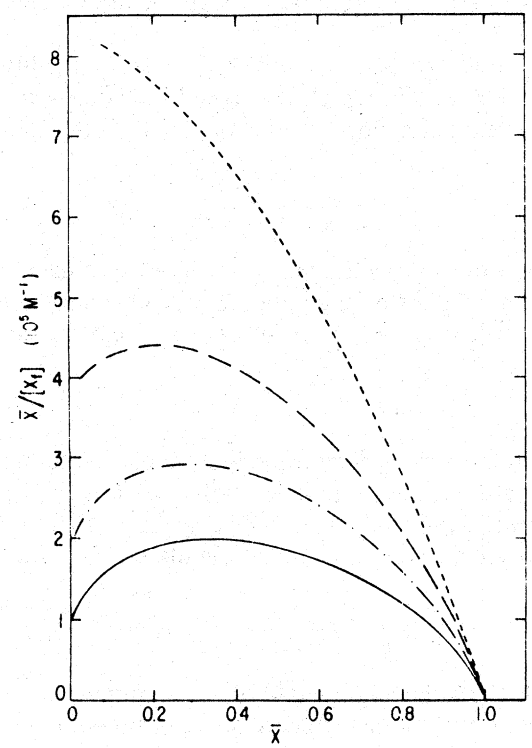

FIG. 3. Scatchard plots of ligand binding of a one-ligand-facilitated isodesmic indefinite self-association. The macromolecular concentrations increase from the bottom to the top and are the same as in Fig. 2. The intrinsic association constants used are $k_3 = 1.8 \times 10^6\ M^{-1}$ and $k_4 = 9 \times 10^2\ M^{-1}$.

$$[X_b] = \sum_{i=2}^{n} \sum_{j=1}^{i} j[A_iX_j]$$
$$= \sum_{i=2}^{n} \sum_{j=1}^{i} \frac{i!}{(i-j)!j!} j\, k_3{}^j(2k_4)^{i-1}[A_f]^i[X_f]^j \tag{15}$$

and the Y intercept of the Scatchard plot for this self-association scheme should be

$$\lim_{[X_f]\to 0} \frac{\bar{X}}{[X_f]} = \frac{\sum_{i=2}^{n} ik_3(2k_4)^{i-1}[A_f]^i}{[A_t]} \tag{16}$$

At low protein concentrations, if k_4 is small, $[A_f] \cong [A_t]$ and the Y intercept can be expressed by

$$\lim_{[X_f]\to 0} \frac{\bar{X}}{[X_f]} = \sum_{i=2}^{n} ik_3(2k_4)^{i-1}[A_t]^{i-1}$$

Figure 4a shows the Y intercepts of the Scatchard plots of the above linkage mechanism calculated from Eq. (16) and plotted against the total protein concentration. They show a curvilinear dependence on the total protein concentration and the curvature increases with increasing values of the intrinsic association constants. It is evident that the Y intercept approaches zero at low protein concentration. This result is a consequence of the assumption that the monomers which prevail at low protein concentration do not bind ligand.

A quantitative analysis of the ligand binding isotherms of ligand-facilitated self-association systems can be carried out by first analyzing the Y intercepts of the Scatchard isotherms as a function of the total protein concentration to obtain estimates of k_3 and k_4, followed by point-by-point fitting of the binding isotherms to determine quantitatively the intrinsic association constants.

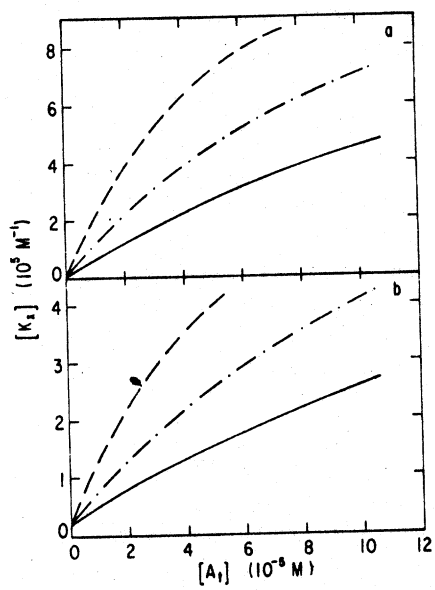

FIG. 4. Y intercept of the Scatchard plots of the ligand-binding isotherms of linked self-association systems as a function of total protein concentration. (a) Ligand-facilitated isodesmic indefinite self-association. The intrinsic association constants are $k_3 = 1.8 \times 10^6$, and $k_4 = 9 \times 10^2$ (——), 1.8×10^3 (–·–·), and 3.6×10^3 (– – –). (b) Ligand-mediated and facilitated self-association. The intrinsic association constants are $k_1 = 1.8 \times 10^4$, $k_3 = 1.8 \times 10^6$, and k_2 and k_4 are 9×10^4, 9×10^2 (——); 1.8×10^5, 1.8×10^3 (–·–·); and 3.6×10^5, 3.6×10^3 (– – –).

A system that behaves like a ligand-facilitated dimerization can be found in the interaction of the peptide hormone oxytocin with bovine neurophysin II.[25] Equilibrium ultracentrifugational studies showed that the protein undergoes a weak dimerization even in the absence of the ligand. The introduction of oxytocin shifted the existing equilibrium toward the dimeric state. The ligand-facilitated mechanism of association is also indicated by the downward curvature of the Scatchard ligand binding isotherms and the increase of the Y intercept of the binding isotherm with increasing protein concentration.

Ligand-Mediated and Facilitated Self-Association. This mechanism is defined as a combination of the above two mechanisms, i.e., ligand can bind to both monomers and polymers and both liganded and unliganded monomers can participate in the self-association reaction. A one-ligand-mediated and facilitated dimerization reaction is shown by

$$\begin{array}{ccccc} A_2 + 2X & \overset{k_1}{\rightleftharpoons} & XA_2 + X & \overset{k_3}{\rightleftharpoons} & A_2X_2 \\ \rotatebox{90}{$\rightleftharpoons$} & & \rotatebox{90}{$\rightleftharpoons$} & & \rotatebox{90}{$\rightleftharpoons$} \\ 2A + 2X & \overset{k_1}{\rightleftharpoons} & AX + A + X & \overset{k_1}{\rightleftharpoons} & 2AX \\ \rotatebox{90}{$\rightleftharpoons$} & & \rotatebox{90}{$\rightleftharpoons$} & & \rotatebox{90}{$\rightleftharpoons$} \\ A_2 + 2X & \overset{k_1}{\rightleftharpoons} & A_2X + X & \overset{k_1}{\rightleftharpoons} & A_2X_2 \end{array} \tag{18}$$

In addition to the self-associations defined by Eqs. (7) and (11), the association between a liganded and an unliganded monomer is permitted. The model assumes an asymmetry in the self-association, i.e., when two monomers self-associate, the association constant is dependent on the liganding state of only one of the two monomers. The association constant is assumed to be k_2 if that monomer is liganded and k_4 if it is not liganded. This model can simulate a system where the monomer, upon binding a ligand, undergoes a conformational change and acquires a stronger propensity to associate. For such a system, the apparent dimerization constant can be expressed as

$$\begin{aligned} k_2 &= \frac{[A_2] + [A_2X] + [A_2X_2]}{([A] + [AX])^2} \\ &= \frac{2k_4 + k_1k_2[X_f] + k_1k_4[X_f] + 2k_1^2k_2[X_f]^2}{(1 + k_1[X_f])^2} \end{aligned} \tag{19}$$

If the reaction scheme is extended to a stepwise isodesmic indefinite self-association, the ligand bound to the macromolecule can be expressed as

$$[X_b] = \sum_{i=1}^{n} k_1^i(2k_2)^{i-1}[A_f]^i[X_f]^i + \sum_{i=2}^{n}\sum_{j=1}^{i} \frac{(i-1)!}{(i-j)!(j-1)!} k_1^j(2k_2)^{j-1}(2k_4)^{i-j}$$

$$[A_f]^i[X_f]^j + \sum_{i=2}^{n}\sum_{j=1}^{i} \frac{(i-1)!}{(i-j-1)!j!} k_1^j(2k_2)^j(2k_4)^{i-j-1}[A_f]^i[X_f]^j \quad (20)$$

The first term of Eq. (20) represents those macromolecules with their self-association linked sites fully occupied. This is contributed by the ligand-mediated self-association. The second and third terms consist of those macromolecules with their self-association-linked sites unsaturated. They are contributed by the rest of the association mechanisms. The second term covers those polymers with a liganded monomer at the starting terminal of the polymer whereas the third term contains those polymers with an unliganded monomer at that position.

One can also take the alternate approach to calculate the concentration of the bound ligand, i.e., first calculate the total concentration of each i-mer using the apparent self-association constant. There are two classes of ligand binding sites on the i-mer, those with a ligand affinity k_1 and those with a ligand affinity k_3. The ligand affinity of a given site depends on whether it has been coupled to the self-association reaction. For the model described above, with the exception of one terminal site, all ligand-binding sites on the polymer are linked to the self-association reaction and have a ligand-binding constant of k_3. The terminal site not linked to the self-association should have a ligand binding constant of k_1. Consequently, the total ligand bound to the macromolecule can be expressed as

$$[X_b] = \sum_{i=1}^{n} \frac{C_i}{i\mathrm{MW}} \left[\frac{(i-1)k_3[X_f]}{(1+k_3[X_f])} + \frac{k_1[X_f]}{(1+k_1[X_f])}\right] \quad (21)$$

Figure 5 shows the theoretical binding isotherms of a one-ligand-mediated and facilitated isodesmic indefinite self-association system. They are concave upward and their Y intercepts increase with increasing protein concentration. In a manner similar to the ligand-facilitated self-association, the analysis of the ligand-binding isotherm of the ligand-mediated and facilitated self-association can be started by first obtaining estimates of the intrinsic equilibrium constant through analyzing the protein concentration dependence of the Y intercept of the Scatchard plot. According to Eq. (20)

$$\lim_{[X_f]\to 0} \frac{\bar{X}}{[X_f]} = \frac{k_1[A_f] + \sum_{i=2}^{n} k_1(2k_4)^{i-1}[A_f]^i + \sum_{i=2}^{n} (i-1)k_1(2k_2)(2k_4)^{i-2}[A_f]^i}{[A_t]}$$

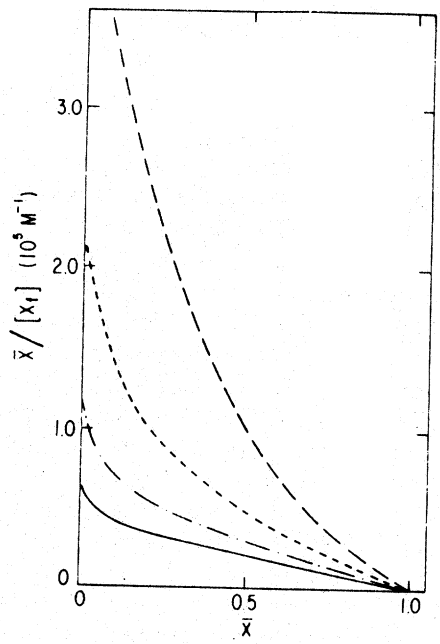

FIG. 5. Scatchard plots of the ligand binding of a one-ligand-mediated and facilitated isodesmic indefinite self-association. The macromolecular concentrations increase from the bottom to the top and are the same as Fig. 2. The intrinsic association constants used are $k_1 = 1.8 \times 10^4\ M^{-1}$, $k_2 = 9 \times 10^4\ M^{-1}$, $k_3 = 1.8 \times 10^6\ M^{-1}$, and $k_4 = 9 \times 10^2\ M^{-1}$

Figure 4b shows the Y intercept of the Scatchard plot for such a linked system calculated according to Eq. (22) as a function of the total protein concentration. If $[A_t]$ is small, all terms in Eq. (22) higher than first order in $[A_f]$ can be ignored, and Eq. (22) becomes

$$\lim_{[X_t]\to 0} \frac{\bar{X}}{[X_f]} = k_1\{1 + (2k_2 + 2k_4)[A_t]\} \tag{23}$$

Thus one can obtain estimates of k_1, k_2, and k_4 from the Y intercept and the slope of Fig. 4b. As the values of the intrinsic association constants k_2 and k_4 increase, the dependence of the Y intercept of the Scatchard plot on the total protein concentration becomes increasingly curvilinear and the data must be analyzed through nonlinear least-squares fitting technique. The validity of these estimated values of the intrinsic association constants can be tested subsequently through least-squares fittings of the experimental binding isotherms with the theoretical ones.

An example of the ligand-mediated and facilitated self-association can

be found in the interaction of the anticancer drug vinblastine with the microtubule protein, tubulin. Velocity sedimentation studies of the self-association showed that the apparent self-association constant plateaued at high ligand concentrations, suggesting the presence of the ligand-mediated association.[26,27] At lower ligand concentrations, the Wyman plot showed a slope of approximately one, suggesting that the binding of one ligand is required for the association between two monomers. Studies of the vinblastine binding isotherms have shown that they are concave upward which rules out the ligand-mediated association as the only association mechanism.[28] In fact, the experimental isotherms can be fitted well by a theoretical model where association between a liganded monomer and an unliganded monomer is permitted.[16,29] Further proof of the linkage mechanism was derived from the complete consistency of both the stoichiometry and the intrinsic association constants obtained from ligand-binding and self-association studies.

Ligand-Induced Cross-Linking Self-Association. A protein self-association induced by ligand cross-linking is defined by Eq. (24) where the ligand serves as a bridge connecting two macromonomers[3,27]:

$$\begin{array}{lll} A + X \rightleftharpoons AX & k_1 \\ AX + A \rightleftharpoons AXA & k_1 \\ AXA + X \rightleftharpoons AXAX & k_1 \\ \vdots \quad \vdots \quad \vdots & \end{array} \tag{24}$$

Each monomer has two ligand-binding sites. During its self-association, each macromonomer binds only the ligand and not another monomer. For such a ligand-induced association, the apparent self-association constant can be expressed as[27]

$$K_2 = \frac{[A_2X] + [A_2X_2] + [A_2X_3]}{([A] + [AX] + [AX_2])^2} = \frac{k_1^2[X_f]}{(1 + k_1[X_f])^2} \tag{25}$$

If the association proceeds indefinitely, the concentration of ligand bound to the macromolecule can be calculated from

$$[X_b] = \sum_{i=1}^{n} \left[\frac{i-1}{i}\frac{c_i}{MW} + \frac{2}{i}\frac{c_i}{MW}\frac{k_1[X_f]}{(1 + k_1[X_f]}\right] \tag{26}$$

where the first term represents the ligands that bridge two macromono-

[26] G. C. Na and S. N. Timasheff, *Biochemistry* **19**, 1347 (1980).
[27] G. C. Na and S. N. Timasheff, *Biochemistry* **19**, 1355 (1980).
[28] J. C. Lee, D. Harrison, and S. N. Timasheff, *J. Biol. Chem.* **250**, 9276 (1975).
[29] G. C. Na and S. N. Timasheff, *Abstr. Pap., 180th Meet., Am. Chem. Soc. Abstr. Biol.* p. 170 (1980).

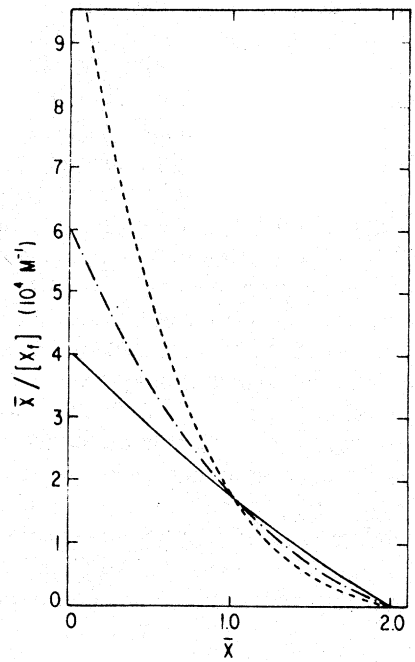

FIG. 6. Scatchard binding isotherms of an indefinite ligand-induced cross-linking of macromolecules. The details of the association mechanism are described by Eq. (24). The intrinsic ligand-binding constant to the monomer is 1.8×10^4. The protein concentrations are 1.6×10^{-5} M (——), 7.8×10^{-5} M (- · -), and 2.3×10^{-4} M (– – –).

mers and the second term represents the ligand bound to the two terminal monomeric units not involved in bridging two monomers. Figure 6 shows the binding isotherms of such a linkage system at different protein concentrations. They are concave upward and intersect with each other at the stoichiometry of one. In fact, this is where the apparent self-association constant reaches its maximum when plotted against the free ligand concentration. Since the polymers must dissociate in order to have two ligands bound to each monomer, the apparent self-association constant peaks at this ligand concentration and then drops off at higher ligand concentrations as shown in the preceding chapter.[1] Such a behavior has been observed in the mercuric chloride-induced association of human mercaptalbumin, suggesting that this protein is cross-linked by the divalent ligand.[30]

The dotted curve of Fig. 6 can be used as a good example of the danger in determining ligand stoichiometry from a Scatchard plot alone. In this curve the Y coordinate descended more than 80% from the Y intercept over the region in which the ligand binding increased from zero to one ligand per monomer. Consequently, without looking at the saturation plot, it is very tempting to stop the experiment at a stoichiometry near one, linearly extrapolate the data, and reach the erroneous conclusion that there is only one ligand binding site per monomer.

Concluding Remarks

The multiple equilibria in a ligand-induced self-association can be studied by measuring and analyzing either the apparent macromolecular association or the apparent ligand binding. These two approaches, one discussed in the preceding chapter of this volume[1] and the other in this chapter, each have its own advantages and drawbacks, and they complement each other well. For instance, probing the ligand binding through analysis of the apparent self-association constant as described in the preceding chapter gives information only on the self-association-linked ligand binding site(s) and not the self-association-independent site(s). Information regarding the latter ligand-binding site(s) can be obtained only from direct ligand-binding measurements as described in this chapter. Conversely, in a ligand-binding study of the linkage mechanism, the shape of the ligand-binding isotherm is usually not sensitive toward the stoichiometry of the macromolecular association and thus cannot be used to obtain that information. For a thorough and detailed characterization of a linked system, it is essential to follow both approaches, i.e., first examine the mechanism of the macromolecular association reaction using a method such as analytical centrifugation and then analyze the ligand-binding isotherms as described in the present chapter. Since these two approaches measure two entirely different molecular events, they should employ different physical techniques. Consequently, both the encountered nonidealities and the invoked assumptions are different. By taking both approaches in examining a given system and obtaining consistent results, one can gain much stronger confidence in the conclusions derived. Another advantage which is gained sometimes from the complementary use of different physical techniques is that they focus frequently at different regions of the binding isotherm. For a given system, the sensitivity of one approach may pick up where that of the other starts to drop off. For instance, in the linked function analysis of the apparent macromolecular self-association shown in the preceding chapter, self-association is stronger at higher ligand concentrations. Consequently, the

results obtained in the region where the association-linked ligand binding site is half to fully saturated are usually more reliable. If the apparent self-association constant plateaus at saturating ligand concentrations, it suggests the presence of a ligand-mediated association mechanism. The presence of a ligand-facilitated self-association should cause the apparent association constant to level off at low ligand concentration. However, if the apparent self-association constant does not level off at the lowest ligand concentrations examined, one still cannot rule out the presence of a weak ligand-facilitated pathway accompanying the ligand-mediated pathway that is beyond the detection by the available technique. Contrary to the self-association study, probing the linkage mechanism through analysis of the ligand binding as shown in this chapter focuses on the region where the association-linked ligand binding site is less than half saturated. It is in this region that different linkage mechanisms manifest most pronounced differences in the shape of their binding isotherms, and good quality data of ligand binding often can be obtained to allow differentiation of these linkage mechanisms. For instance, the question whether the ligand-mediated self-association is the sole association mechanism can be most easily tested by examining if the Y intercepts of the Scatchard binding isotherms converge to a single point.

There are instances where the self-association approach, rather than the ligand binding one, allows a more unambiguous differentiation of the linkage mechanisms. For example, the ligand-binding isotherms of the ligand-induced cross-linking of macromolecules exhibit certain similarities to those of the ligand-mediated and facilitated self-association. Given a certain amount of experimental uncertainties, it becomes difficult to differentiate these two linkage mechanisms on the basis of binding isotherms. However, the apparent self-association constants of the two systems show completely different dependences on the ligand concentration, one reaching a maximum and then dropping off, while the other plateaus at high ligand concentrations.

It should be noted that in the self-association study of a ligand-mediated and facilitated system, the value of the intrinsic self-association constant k_2 was obtained at saturating ligand concentration.[1] It should, therefore, correspond to the self-association constant between two fully liganded monomers. On the other hand, in the ligand-binding study of the same system, described above, the value of the intrinsic self-association constant is obtained at ligand concentrations approaching zero. Thus, in Eq. (23), k_2 corresponds to the self-association constant between a liganded monomer and an unliganded monomer. Although in the linkage mechanism defined by Eq. (18), the values of these two constants differ

only by a factor of two, due to a hypothetical statistical factor, in a real case these two constants can be totally independent of each other.

The linkage mechanisms discussed in this chapter assume that there is no self-association-independent ligand-binding site on the macromolecule. If self-association-independent ligand-binding sites do exist on the macromolecule, their contribution to the total ligand bound can be easily accounted for and added to the above equations. Assuming that there are n_0 such association-independent sites with an equivalent association constant of k_0, their contribution to the total ligand bound can be expressed as

$$[X_b] = \frac{n_0 k_0 [X_f]}{(1 + k_0 [X_f])} \tag{27}$$

In the actual data analysis, if the ligand affinity of these sites is weaker than that of the self-association-linked site, the binding of the ligand to the association-independent site will follow the association-dependent site and appear at the right hand side of the Scatchard plot. This will interfere very little with the analysis of the linked ligand-binding site. This is the case in the vinblastine-induced tubulin self-association system.[16,29] However, if the self-association-independent site has an affinity that is much stronger than that of the self-association-linked site, the ligand binding to the former site will precede the latter one and appear at the left-hand side of the Scatchard plot. This could complicate the data analysis.

Last, it is important to emphasize that the methods described in this chapter are intended only for equilibrium systems. It is prudent to test the attainment of equilibrium through either a kinetic measurement or by ascertaining that the same results can be obtained from either the binding of the free ligand or the dissociation of the bound ligand.