[21] Measurement of ligand-protein interaction by electrophoretic and spectroscopic techniques

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[21] Measurement of Ligand–Protein Interaction by Electrophoretic and Spectroscopic Techniques

By ROBERT W. OBERFELDER and JAMES C. LEE

Introduction

Determination of the equilibrium binding constant and the stoichiometry for a protein–ligand interaction requires an appropriate choice of methodology. Each technique has advantages and disadvantages, as well as practical and theoretical limitations, and these must be considered when choosing a procedure. It is important to have a number of approaches available from which to choose, so that techniques can be used which will be compatible with the constraints placed on the system due to the nature of the protein and ligand of interest. Several procedures have been discussed in this series including equilibrium dialysis,[1] Hummel–Dryer chromatography,[2] Womack–Colowick rapid dialysis,[3] and countercurrent distribution.[4] In these methods the formation of a ligand–protein complex is detected by directly determining the difference in ligand concentrations. Indirect approaches utilizing absorbance spectroscopy, fluorescence spectroscopy, or electrophoresis may also be employed to perform binding studies. These studies use changes in the spectral properties of the protein or ligand to measure equilibrium binding constants. The procedures used to perform binding studies utilizing spectroscopy, electrophoresis,[5] and isoelectric focusing[6] will be discussed and the advantages and disadvantages intrinsic to these techniques will also be described.

Spectroscopic Methods

Any spectroscopic technique can be employed to monitor ligand–protein interaction. Utilization of spectroscopic change as a measure of the extent of interaction is based on the explicit assumption that the fractional

[1] U. Westphal, this series, Vol. 15, p. 762.
[2] G. K. Ackers, this series, Vol. 27, p. 441.
[3] F. C. Womack and S. P. Colowick, this series, Vol. 27, p. 464.
[4] G. Kegeles, this series, Vol. 27, p. 456.
[5] E. M. Ritzén, F. S. French, S. C. Weddington, S. N. Nayfeh, and U. Hansson, *J. Biol. Chem.* **249,** 6597 (1974).
[6] J. R. Cann and K. J. Gardiner, *Biophys. Chem.* **10,** 211 (1979).

saturation of binding sites by ligand is linearly proportional to the fraction change in the spectroscopic signal. Such an assumption is the weakest link in these indirect methods of monitoring ligand–protein interaction to obtain binding constants and stoichiometry.

Difference Spectroscopy

Theory. Binding of a ligand to a protein may result in a change in the environment surrounding either the ligand or amino acid residues in the protein. Such a change may perturb the electronic interactions of the ligand or the residues in the protein producing a shift in the absorbance spectrum.[7,8] The difference between the unperturbed and the perturbed spectrum is termed the difference spectrum which can be calculated according to the following equation.

$$A_x\,(\mathrm{P} + \mathrm{L}) - A_x\,(\mathrm{PL}) = \Delta A_x \qquad (1)$$

where A_x is the absorbance at wavelength x. (P + L) indicates that the protein and ligand are separate, while (PL) indicates that mixing and presumably binding has taken place. ΔA_x is the difference in the absorbance of the two solutions at wavelength x. The difference spectrum is simply the expression of the ΔA_x values over the wavelength range studied.

Perturbation of the protein can most readily be detected in the 250- to 300-nm range for phenylalanine, tyrosine, and tryptophan. These residues when perturbed have difference spectra which are easily recognized. The spectral shifts may be a result of a direct interaction of the ligand in or around the binding site, or it may be a result of a conformational change distant from the binding site. Association and dissociation of the protein might also produce a spectral shift, but the binding of the ligand would have to be linked to the association or dissociation to affect a change that is ligand concentrations dependent.

The spectrum of the ligand may also be perturbed since it may be shifted from an aqueous environment to a hydrophobic one upon binding to the protein. Interaction of the ligand might also change the degree of ionization and thereby shift the spectrum. Thus, an observed spectral change can be caused by a wide variety of perturbations.

Examination of the observed difference spectrum should reveal a wavelength or a range at which ΔA_x is maximal. This wavelength will provide the maximum sensitivity in the observation of the change; hence, it should be employed in the studies to determine the effect of ligand

[7] P. Bennouyal and C. G. Trowbridge, *Arch. Biochem. Biophys.* **115,** 67 (1966).
[8] P. Cuatrecasas, S. Fachs, and C. B. Antinsen, *J. Biol. Chem.* **242,** 4759 (1967).

concentration on the spectral change. The maximum change in absorbance can then be determined by plotting $1/\Delta A_x$ versus $1/[L]$ and extrapolating to infinite ligand concentration. The y intercept will yield the maximum absorbance change, ΔA_{xm}. The fractional change in the absorbance $\Delta A_x/\Delta A_{xm}$ is assumed to be linearly proportional to the fractional saturation of binding sites, thus,

$$\frac{\Delta A_x}{\Delta A_{xm}} = \frac{\bar{\nu}}{n} \tag{2}$$

where $\bar{\nu}$ and n are the moles of ligand bound and the number of binding sites, respectively.

$\bar{\nu}$ may be determined by conducting another series of experiments. The increment in the ligand molar extinction coefficient due to binding, $\Delta\varepsilon = \varepsilon(\text{bound}) - \varepsilon(\text{free})$, can be determined from extrapolation of a titration of ligand solution of finite concentration with excess protein. Then aliquots of a protein solution are titrated with known total concentrations of ligands, the bound ligand concentration, $\bar{\nu}$, determined as $\Delta A_x/\Delta\varepsilon$ and the free ligand concentration estimated from the difference.

The binding isotherm can be expressed as

$$\bar{\nu} = \frac{n[L]_{\text{free}}}{K_d + [L]_{\text{free}}} \tag{3}$$

where $[L]_{\text{free}}$ and K_d are the free ligand concentration and the dissociation constant, respectively. The binding isotherm can also be expressed in the familiar Scatchard plot format as follows[9]

$$\frac{\bar{\nu}}{[L]_{\text{free}}} = \frac{n - \bar{\nu}}{K_d} \tag{4}$$

The slope of a plot of $\bar{\nu}/[L]_{\text{free}}$ versus $\bar{\nu}$ will yield a slope equal to $-1/K_d$.

An allosteric enzyme may be analyzed using difference spectroscopy as long as the relationship described by Eq. (2) is adhered to. The degree of cooperativity can be estimated since

$$\bar{\nu} = \frac{n\,[L]^h_{\text{free}}}{K_d + [L]^h_{\text{free}}} \tag{5}$$

where h is the Hill coefficient,[10] an indication of cooperativity in ligand binding.

Experimental Procedure. 1. The spectral data required for this procedure can be most readily obtained utilizing a double-beam spectrophotom-

[9] G. Scatchard, *Ann. N.Y. Acad. Sci.* **51,** 660 (1949).
[10] A. V. Hill, *J. Physiol. (London)* **40,** 190 (1910).

eter. Two tandem cuvettes will also be necessary to perform these experiments. The UV range is essential for the detection of absorbance changes in the protein and may be useful for detection of absorbance changes in the ligand as well. The visible range is useful in the detection of absorbance changes in the ligand or prosthetic groups associated with the protein. It is also useful to have the capacity to scan as a function of wavelength.

2. A stock protein solution should be prepared so that all of the solutions used in the experiments contain the same protein concentrations. Stock solutions of the ligand should be prepared using the ligand concentrations to be tested. All of these solutions should be prepared in the same buffer to avoid potential complications due to differing buffer compositions.

3. Of the protein stock 1.00 ml is added to one side of the two cuvettes and 1.0 ml of the ligand stock is added to the other side of both cuvettes. One cuvette will be employed as the sample and the other as the reference.

4. Scan the two unmixed cuvettes over a wavelength range encompassing both the range in which the protein and ligand absorb. This scan should be essentially flat, if the two cuvettes are well matched.

5. Mix the sample cuvette thoroughly, then scan again in order to detect spectral changes in the absorbance of the protein or the ligand. The spectral change may be either an increase or a decrease in the absorbance relative to the reference spectra.

6. Steps 3, 4, and 5 should be repeated for each ligand concentration to be tested.

7. Examination of the scans should reveal the wavelength at which the spectral change is maximal.

8. Utilizing the wavelength at which the perturbation was maximal for wavelength "x," the data should be plotted as $\bar{\nu}/[L]_{free}$ versus $\bar{\nu}$ so that the slope can be used to determine K_d in accordance to Eq. (4).

An illustration of the application of difference spectroscopy in monitoring ligand–protein interactions is shown in Fig. 1.[10a] The binding of 2-methoxy-5(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC) to calf brain tubulin induces a difference spectrum. The magnitude of absorbance increment is proportional to the amount of MTC added. The binding isotherm is shown in Fig. 2. It is evident that the binding isotherm determined by difference spectroscopy resembles that determined by the equilibrium method, although the absolute values of $\bar{\nu}$ are lower.

[10a] J. M. Andreu, M. J. Gorbunoff, J. C. Lee, and S. N. Timasheff, *Biochemistry* **23**, 1742 (1984).

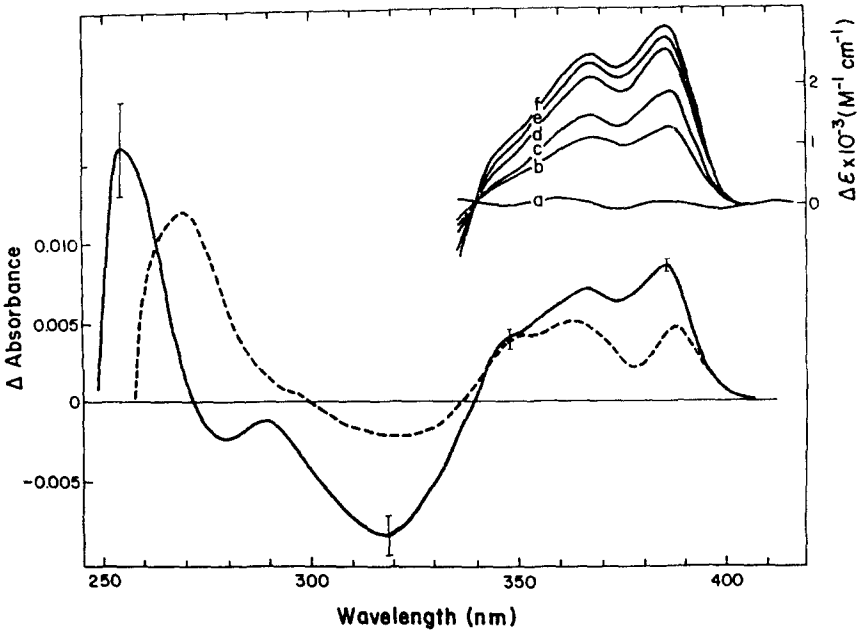

FIG. 1. Difference absorption spectra generated by the interaction of tubulin with MTC in 10^{-2} *M* phosphate, 10^{-4} *M* GTP (PG buffer) at pH 7.0 and 25°. (——) Difference spectrum of 3.1×10^{-5} *M* MTC and 4.0×10^{-6} *M* tubulin vs ligand and protein in separate solutions. The difference spectrum (– – –) generated by 1.6×10^{-5} *M* tubulin is shown for comparison. The 350 to 400 nm spectra in the upper right hand corner were generated by 9.5×10^{-6} *M* MTC with (a) no tubulin, (b) 6.9×10^{-6} *M* tubulin, (c) 1.37×10^{-5} *M* tubulin, (d) 2.72×10^{-5} *M* tubulin, (e) 4.02×10^{-5} *M* tubulin, and (f) 6.55×10^{-5} *M* tubulin. (Reprinted with permission from Andreu *et al.*[10a] Copyright 1984 American Chemical Society.)

General Comments. The validity of the results of the difference spectroscopy experiments is entirely dependent upon the relationship described by Eq. (2). One should check the results by performing direct binding experiments to establish that the assumed relationship between the fractional spectral perturbation and the fractional saturation of the protein is correct. The apparent dissociation constant should be determined utilizing several different protein concentrations so that affects due to association–dissociation phenomena may be detected.

Fluorescence Spectroscopy

Theory. Formation of a ligand–protein complex may result in alteration in the fluorescence intensity of the aromatic amino acids of the protein, extrinsic probes or the ligand. Monitoring ligand–protein interaction by fluorescence has one complication in addition to those cited for differ-

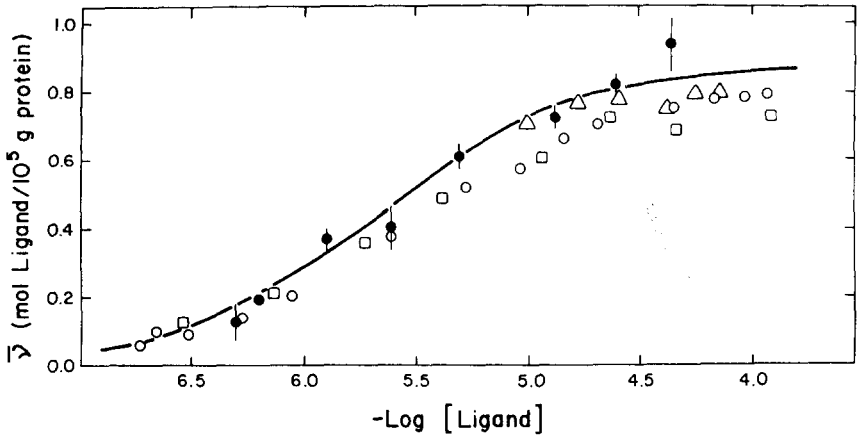

FIG. 2. Binding isotherm of MTC to tubulin; PG buffer pH 7.0, 25°. Solid circles (●) are column measurements at a protein concentration of (0.5–1.2) × 10^{-5} *M*. Open symbols are binding measurements from ligand fluorescence at (□) 1.8 × 10^{-6} *M*, (○) 5.3 × 10^{-6} *M*, and (△) 3.6 × 10^{-5} *M* protein. The solid line is a fit to the column measurements (K_b = 4.8 × 10^{-5} M^{-1}; n = 0.88) obtained from a Scatchard plot of these data. (Reprinted with permission from Andreu *et al.*[10a] Copyright 1984 American Chemical Society.)

ence spectroscopy, the inner filter effect. Fluorescence intensities are proportional to concentrations over a narrow range of optical densities. If the titrant in the measurement of ligand–protein interaction absorbs light at the excitation wavelength, then the intensity of the exciting light will decrease with each addition of the titrant, thus, an artifactual decrease in emission light intensity will be observed. Various methods have been proposed to correct for the inner filter effect and one of the simplest procedures is[11]

$$F_{corr} \cong F_{obs} \text{ antilog } \frac{OD_{ex} + OD_{em}}{2} \tag{6}$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence intensity, respectively and OD_{ex} and OD_{em} are the optical density of the sample at the excitation and emission wavelengths, respectively. The best solution to inner filter effect is, however, to employ samples of low optical density, i.e., less than 0.05.

Having corrected for the inner filter effect, quenching of the intrinsic protein fluorescence by ligand can be employed to estimate the binding affinity. The maximal fluorescence quenching by excess ligand is mea-

[11] J. R. Lakowicz, "Principles of Fluorescence Spectroscopy." Plenum, New York, 1983.

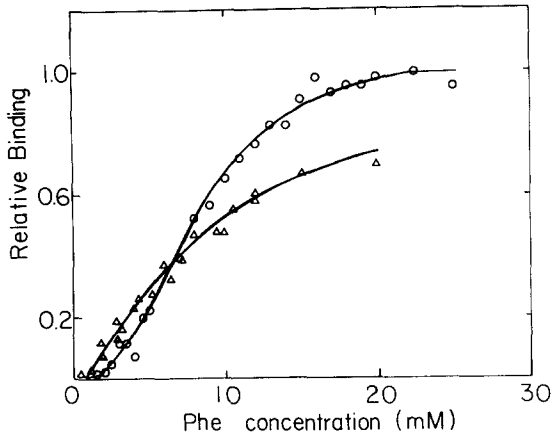

FIG. 3. The binding of Phe to rabbit muscle pyruvate kinase at pH 7.5 and 23°. The symbols and source of data are (△) equilibrium dialysis data (reproduced with permission from Kayne and Price[12a]) and (○) fluorescence data. (T. G. Consler and J. C. Lee, unpublished data.)

sured, then the fraction of sites occupied, θ, must be assumed to be equal to the fraction of the maximal quenching effect at a given total ligand concentration. The binding equilibrium constant can be determined employing the relationship[12] $\theta/(1 - \theta) = K_b$ [A], where [A] is the free ligand concentration calculated from the total ligand and protein concentrations and θ is the fraction of the protein in bound form, assuming a binding stoichiometry, if it has not been determined independently by other means.

Experimental Procedure. 1. To further limit the complication caused by inner filter effects small fluorescence cells of 0.5 × 0.5 cm or smaller can be used.

2. A stock ligand solution should be prepared. The concentration of the stock solution should ideally be about 100- to 500-fold that of the final ligand concentration to be tested. This will ensure minimal dilution of the protein solution, which should not be diluted by more than 5%.

An illustration of the application of fluorescence spectroscopy in monitoring ligand–protein interaction is shown in Fig. 3.[12a] The binding of L-phenylalanine to rabbit muscle pyruvate kinase induces a change in the intensity of the intrinsic protein fluorescence. Assuming the change in fluorescence intensity is directly proportional to ligand–protein complex formation, a binding isotherm can be defined. The sigmoidal nature of the

[12] S. S. Lehrer and G. D. Fasman, *Biochem. Biophys. Res. Commun.* **23,** 133 (1966).
[12a] F. J. Kayne and N. C. Price, *Arch. Biochem. Biophys.* **159,** 292 (1973).

binding isotherm indicates a significant degree of cooperativity in the binding of phenylalanine to the enzyme. Comparison of the fluorescence data with data obtained using equilibrium analysis (Fig. 3) shows that the two methods indicate different degrees of cooperativity. Clearly the isotherms are dependent upon the methods used to obtain them. Thus additional experiments must be performed in order to explain the discrepancy. It has been shown that pyruvate kinase undergoes a cooperative global structural change upon binding of phenylalanine, so the change in the fluorescence intensity may reflect both the structural change and ligand binding. These results illustrate the major limitation in using indirect methods to monitor ligand–protein interaction.

Electrophoretic Methods

A majority of methods available to monitor ligand–protein interaction rely on separation of the ligand–protein complex from free ligand. Thus, transport methods are involved, such as the chromatographic procedure of Hummel and Dreyer,[13,14] the gel exclusion procedure of Hirose and Kano,[15] and the sedimentation procedure of Steinberg and Schachman.[16] In this section, methods utilizing electrophoresis will be discussed.

Steady-State Polyacrylamide Gel Electrophoresis

Theory. Polyacrylamide gel electrophoresis at steady-state conditions can be employed to obtain reliable information on the equilibrium constant and stoichiometric relationship in ligand–protein interaction, as demonstrated by Ritzén *et al.*[5]

The principle utilized in this procedure is essentially the same as that of the chromatographic procedure of Hummel and Dreyer. The limitation is, however, that the ligand of interest should be noncharged so that the electric field does not affect the distribution of the ligand. The ligand is introduced into the polyacrylamide gel matrix by including it in the solution during the polymerization process, which should result in uniform distribution throughout the gel. As the protein migrates through the gel, it encounters a constant concentration of ligand and it will bind an amount of ligand which will be dependent upon the free ligand concentration stoichiometry and the binding constant. The amount of ligand bound to the protein will continue to increase until the free concentration of ligand

[13] J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta* **63,** 530 (1962).
[14] G. F. Fairclough and J. S. Fruton, *Biochemistry* **5,** 673 (1966).
[15] M. Hirose and Y. Kano, *Biochim. Biophys. Acta* **251,** 376 (1971).
[16] I. Z. Steinberg and H. K. Schachman, *Biochemistry* **5,** 3728 (1966).

in the region of the protein is equivalent to the initial free ligand concentration. Once this state has been achieved, there will no longer be any net exchange of ligand between the gel and the protein. This should lead to the observation of a peak in the ligand concentration for ligand associated with the protein, while a trough should be observed in the early portion of the gel. Addition of a large amount of protein may deplete the gel of ligand, so that net binding of the ligand occurs during the entire period of electrophoresis of the protein, thus making determination of bound and free ligand difficult. The attainment of equilibrium may be checked by determination of the concentration of ligand both in front of and behind the protein peak. The concentrations should be the same if equilibrium has been attained.

In order to determine the equilibrium binding constant, the concentration of the free ligand in the gel is required. The amount of ligand added to the solution prior to polymerization is known, and the total volume of solution is also known. The concentration of the ligand in the gel should be equivalent to the initial concentrations in the solution prior to polymerization. The free ligand concentration, C_L, should then be

$$C_L = L/V \tag{7}$$

where L is the amount of ligand added, and V is the total volume of liquid added. The calculated value can be verified experimentally. The density of the solution used in polymerizing the gel, excluding acrylamide should be measured. A slice of gel is then blotted free of excess moisture and weighed. The amount of ligand present in the slice (L) is then quantitated. The ligand concentration is

$$[\mathrm{L}] = (L)\left[\frac{(S)}{G - AG}\right] \tag{8}$$

where [L] is the concentration of the ligand, G is the total weight of the gel, A is the fraction of acrylamide in the gel by weight, and (S) is the density of the polymerization solution excluding acrylamide in grams/liter. It is assumed in this calculation that the ligand concentration is not appreciably affected by acrylamide, i.e., the ligand is neither bound by acrylamide nor preferentially excluded.

The amount of bound ligand must also be determined. The amount of ligand in each gel slice is measured so that one can assess the magnitude of the peak in the ligand concentration in the region of the protein. The free ligand concentration for each slice of gel can be determined from the region immediately in front of or behind the protein peak. The amount of ligand bound per mole of protein, $\bar{\nu}$, can be calculated using the following equation

$$\bar{\nu} = \frac{\Sigma L_B - \Sigma L_F}{p} \tag{9}$$

where L_B is the total amount of ligand in the gel slices in the region of the protein, L_F is the amount of free ligand expected over the gel slices in which L_B was determined, and p is the amount of protein in the gel.

A range of free ligand concentrations must be tested and the values for $\bar{\nu}$ and $[L_F]$ determined. The data can then be plotted in the form of a Scatchard plot[9] to determine the stoichiometry and the equilibrium binding constant.

Experimental Procedure. 1. Preparation of the polyacrylamide tube gels is carried out according to the procedure described in detail by Davis,[17] omitting the stacking gel. Elimination of the stacking gel abolishes potential complication which might arise from the passage of the protein through zones containing two different hydrogen ion concentrations. A 1-cm space is left above the running gel so that the protein can be layered on top. The weight percent of the acrylamide can be varied to suit the protein, but 7% is generally satisfactory. The density of the running gel solution without the acrylamide should be determined so that the free ligand concentration can be calculated based upon Eq. (8). The ligand is included in the solution during polymerization of the running gel to ensure uniform distribution. The ligand must not be modified during the polymerization process, and this should be checked by extracting it from the gel followed by characterization by analytical procedures such as NMR, mass spectrometry, or high-pressure liquid chromatography.

The ligand used should be readily detectable in small quantities. Radioactive ligand provides such sensitivity.

2. The protein should be dissolved in buffer with 10% glycerol. The solution may also include the ligand at a concentration equivalent to that employed in the gel. Preequilibration of the protein with the ligand facilitates formation of the complex. The protein containing solution should be layered through the reservoir buffer and on top of the polymerized gel.

3. The electrophoresis apparatus should be water jacketed and the temperature of the system carefully controlled using a circulating constant temperature bath. Regulation of the temperature is important since the equilibrium constant may be significantly affected by temperature changes.

4. The time and current may be varied to match the time required for equilibrium to be achieved and to allow for the stability of the protein.

5. Once electrophoresis is concluded, the gel is removed from the tube as discussed by Davis[17] and sliced into small sections, approximately

[17] B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).

2 mm in height, using a gel slicer. The gel slices can then be placed in one of the commercially available solubilizers such as Soluene 350 from Packard, then added to Insta-Fluor (Packard) and the amount of radioactive ligand determined using a liquid scintillation counter. The observed counts per minute are corrected for quenching and the amount of ligand in each slice determined from the specific radioactivity of the ligand. The gels should also be stained according to any of the procedures described by Wilson[18] to determine the location of the protein peak.

6. A plot of the moles of ligand versus the slice number should show a peak in the amount of ligand at the position of the protein. The peak should be flanked by regions in which the amount of ligand present in the slices is equivalent to the amount expected, based upon the original concentration of ligand in the gel. The electrophoresis time should be extended or the amount of protein loaded on the gel should be decreased until the flanking regions are clearly detectable.

7. A range of ligand concentrations should be tested utilizing a single protein concentration so that enough data can be amassed to determine the bound and free ligand concentrations. The free ligand concentration can be determined using Eq. (7) based on the initial ligand concentration in the solution used to prepare the running gel. Equation (8) should also be applied after determination of G, S, and L to check the free ligand concentration. The amount of bound ligand is then determined utilizing Eq. (9) after determination of ΣL_B in the region of the protein, ΣL_F and the total amount of protein added to the gel. Once the free and bound ligand concentrations have been determined, the data can be plotted in the form of a Scatchard plot.

General Comments. Results obtained using the steady-state polyacrylamide gel electrophoresis technique show good agreement with those determined by equilibrium methods.[5] The precision of the technique is estimated to be ±4%.

A potential advantage of this technique is that it is not necessary to employ a homogeneous protein sample. As long as the target protein can be resolved from the other proteins, a binding isotherm is estimated. Furthermore, simultaneous demonstration of several binding components is feasible in one sample under identical experimental conditions. Ritzén *et al.*[5] have demonstrated the simultaneous binding of dihydrotestosterone to albumin, corticosteroid-binding globulin and testosterone-binding globulin, as shown in Fig. 4. By this technique it is possible to measure binding to specific components over a wide range of binding affinities and to quantitate each binding component for which the binding constant is known.

[18] C. M. Wilson, this series, Vol. 91, p. 236.

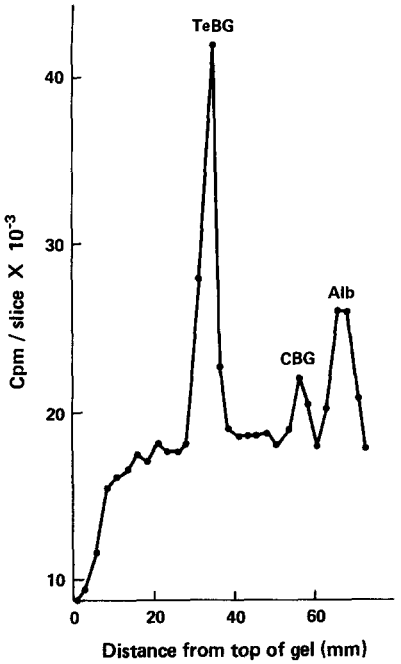

FIG. 4. Fractionation of prepubertal monkey serum by steady-state polyacrylamide gel electrophoresis. Serum diluted 1 : 10 was incubated for 16 hr at 0° with 10^{-8} *M* [^{3}H]dihydrotestosterone. One hundred microliters (corresponding to 0.7 mg of protein) was applied on a 6.5% polyacrylamide gel (10 × 70 mm) containing 2 n*M* [^{3}H]dihydrotestosterone. Steady state between association and dissociation after 3.5 hr of electrophoresis is shown by the equal levels of free radioactivity in front and behind the peaks. TaBG is testosterone-binding globulin. (Reprinted with permission from Ritzén *et al.*[5] Copyright 1974. The American Society of Biological Chemists, Inc.)

The technique can be successfully applied for quantitative measurements when the interaction between ligand and protein is in rapid equilibrium relative to the migration rate of the protein. To ascertain that this critical condition is fulfilled, it is necessary to conduct the experiment as a function of voltage across the gels. If the system is in rapid equilibrium, identical results should be obtained regardless of voltage.

Counterion Electrophoresis

Theory. The method of steady-state counterion electrophoresis was developed by Ueng and Bronner[19] to monitor the binding of ionic ligands to proteins. The basic theory is very similar to that for steady-state electrophoresis.

Experimental Procedure. 1. The ionic ligand is added to the lower

[19] T.-H. Ueng and F. Bronner, *Arch. Biochem. Biophys.* **197**, 205 (1979).

buffer chamber of a conventional disc electrophoresis apparatus,[17] while the protein sample is applied to the gel in the upper buffer chamber.

2. Under the influence of the applied electric field, the ionic ligand and protein will migrate in opposite directions, leading to complex formation.

3. A steady state is reached when the concentrations of ligand in front and behind the protein zone are identical.

4. The gel is sliced and the amount of ligand in each slice quantitated. Since in most cases the ligand employed is radioactive, it can be quantitated by counting.

5. Repeating the experiment as a function of ligand concentration, a binding isotherm can be established and the data analyzed to obtain the stoichiometry and apparent binding constant.

General Comment. This method has been applied to the study of calcium binding to rat intestinal calcium-binding protein,[19] as shown in Fig. 5. It is evident that there are two calcium binding components, band 1 and 2. A binding isotherm can be established for these two components by varying the amount of calcium in the buffer chamber. Figure 6A shows the linear relation between the amount of calcium added and the amount of calcium detected in the baseline. Figure 6B shows the binding isotherm for band 1 and band 2. The data were further analyzed by double reciprocal plots to yield apparent binding constants (Fig. 6C).

Cann and Fink[20] presented a theoretical analysis of this method and show that the apparent binding constant is highly dependent on protein concentrations. Thus, the apparent binding constant should be determined as a function of protein concentration. The value of the apparent binding constant at infinite dilution of protein is actually the product of a kinetic factor and the intrinsic binding data.

Isoelectric Focusing

Intrinsic ligand binding constants may be obtained for some protein–ligand interactions utilizing isoelectric focusing. This procedure is useful for interactions in which the binding of ligand results in a change in the isoelectric point of the protein, the theory of which has been discussed by Cann and co-worker.[6,21] Since the ligand free species can be distinguished electrophoretically from the liganded protein, the relative proportion of each can be determined. Utilizing a range of ligand concentrations, one should observe a ligand concentration-dependent shift in the position of the protein from the position of the unliganded species to that of the saturated protein. The position of the center of mass of the protein is assumed to be linearly proportional to the amount of ligand bound. Quan-

[20] J. R. Cann and N. H. Fink, *Biophys. Chem.* **21,** 81 (1985).
[21] J. R. Cann, *Biophys. Chem.* **11,** 249 (1980).

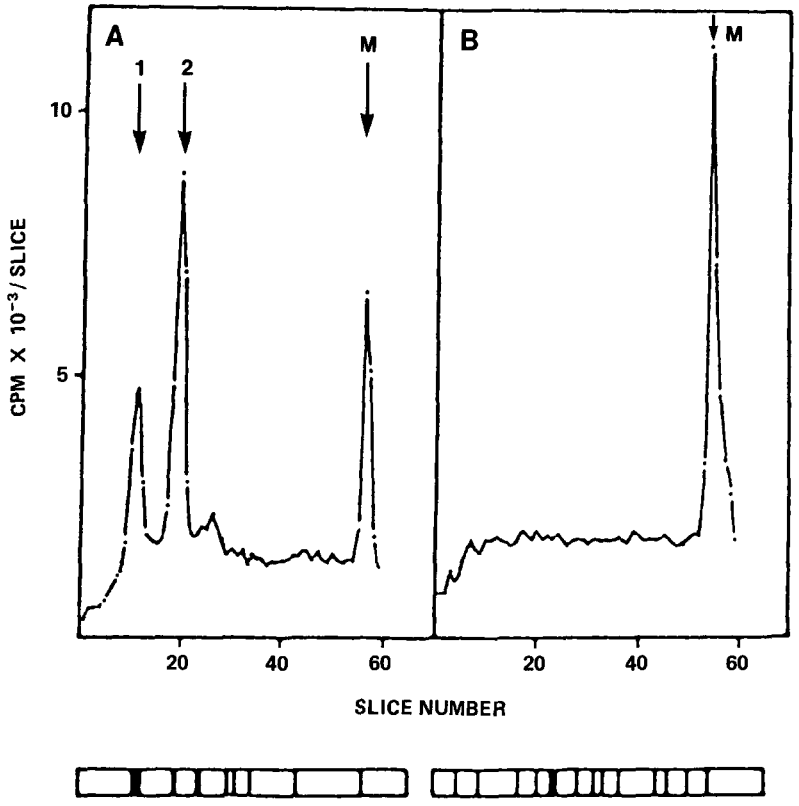

FIG. 5. Binding activity profiles and protein staining patterns of partially purified calcium-binding protein obtained from mucosal scrapings from animals on a low calcium (I) or a low calcium, vitamin D-deficient (I minus D) regimen. The specific activity was 33 nmol Ca_{bound}/mg protein from diet I animals (A) and 8 in material from animals on diet I minus D (B). Protein samples of 150 μg were electrophoresed with the anode buffer (700 ml) containing 0.4 μM $CaCl_2$; 13,400 cpm ^{45}Ca/nmol; 0.25 ml of 0.014% bromphenol blue had been added to the cathode buffer (300 ml). Following electrophoresis gels were either stained or sliced (1 mm/slice) and the slices assayed for radioactivity. The protein patterns have been sketched from the destained gels. Protein migration was from left to right, toward the anode (increasing slice number); Ca^{3+} migrated from right to left toward the cathode. Arrows designate calcium-binding protein in band 1 and band 2 and the position of the marker dye (M, bromphenol blue). The baseline radioactivity of slices 36 to 50 (A) averaged 1480 cpm/slice; the binding activity in band 1 or band 3 was estimated to be 0.16 or 0.37 nmol Ca_{bound}, respectively, by dividing the radioactivity in the peak area above the baseline value by the specific radioactivity. Note the absence of bands 1 and 2 in the material from the D-deficient animals (B). (Reprinted with permission from Ueng and Bonner.[19] Copyright 1979, Academic Press.)

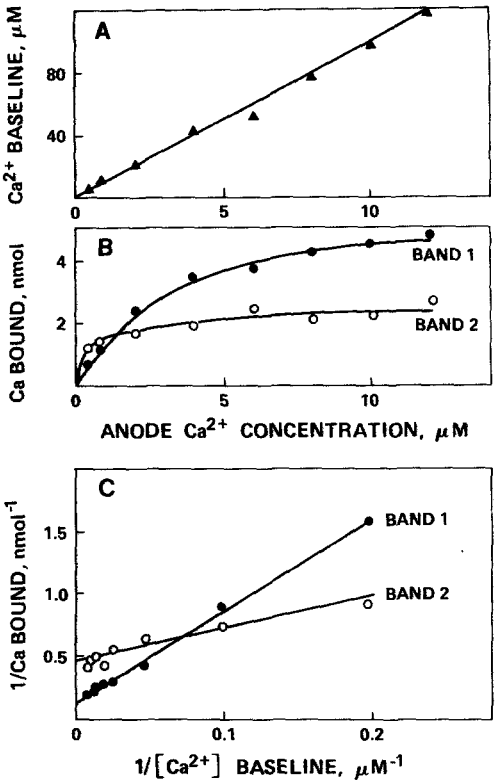

FIG. 6. Calcium binding of calcium-binding protein in counterion electrophoresis. Protein (200 μg), prepared from mucosal scrapings of animals on diet I, with a specific activity of 47 nmol Ca_{bound}/mg protein, was applied to the gel, with the anode buffer calcium concentration varied from 0.4 to 12 μ*M*, while the radiospecific activity varied from 11,200 to 42,500 cpm ^{45}Ca/nmol. The cathode buffer contained 250 μl of 0.015% bromphenol blue. Following electrophoresis the gels were stained or sliced (0.8 mm/slice) and assayed. (A) Baseline calcium as a function of calcium concentration at the anode. Baseline calcium levels were estimated from the mean baseline counts divided by the buffer specific radioactivity. (B) Calcium content of bands 1 and 2 as a function of calcium at anode. The calcium content was estimated from the difference between baseline and peak area of bands 1 and 2. (C) Estimation of apparent dissociation constants. The graph was constructed from the values for baseline calcium (ordinate, A) and band calcium content (ordinate, B). (Reprinted with permission from Ueng and Bonner.[19] Copyright 1979, Academic Press.)

titative evaluation of the ligand concentration dependence of the position of the protein can be used to determine the equilibrium binding constant for the ligand.

Theory. The equilibrium between the protein, P, and the ligand, L, to form the complex, PL, is illustrated below:

$$\begin{aligned} P + L &\rightleftharpoons PL \\ PL + L &\rightleftharpoons PL_2 \\ PL_{i-1} + L &\rightleftharpoons PL_i \\ PL_{n-1} + L &\rightleftharpoons PL_n \end{aligned}$$

where n is the maximum number of ligand molecules bound. If binding of the ligand affects the electrophoretic mobility and isoelectric point of the protein, then the binding of each ligand should shift the isoelectric point of the complex by a constant increment in the velocity, ω. The intrinsic binding constant is designated K_0 which is assumed to be equal for each ligand, if multiple ligand molecules are bound per mole of protein.

The average velocity of the protein, $\bar{v}_p$, in the equilibrium mixture of P and PL_i is given as a function of position, x, in the following equation:

$$\bar{v}_p(x) = v_p(x) + \bar{\nu}\omega \tag{10}$$

where $v_p(x)$ is the velocity of the protein at position x while $\bar{\nu}$ is the amount of ligand bound per mole of protein. The ligand binding isotherm is described by the equation

$$\bar{\nu} = \frac{nK_0C_L(x)}{1 + K_0C_L(x)} \tag{11}$$

where $C_L(x)$ is the equilibrium concentration of the free ligand at position x.

Since the pH gradients used in isoelectric focusing experiments are generally linear, and the electrophoretic mobility of the protein varies linearly in the region of the isoelectric point, $\bar{V}_p(x)$ is assumed to be a linear function of x; hence

$$\bar{V}_p(x) = a - bx \tag{12}$$

Substitution of Eq. (12) into Eq. (10) results in

$$\bar{V}_p(x) = (a + \bar{\nu}\omega) - bx \tag{13}$$

Once the ligand–protein complex has reached equilibrium and moved to the constituent isoelectric point, $V_p(x) = 0$ so $x_0 = a/b$. Substitution into Eq. (13) yields

$$\bar{x} = x_0 + \bar{\nu}(\omega/b) \tag{14}$$

Substituting Eq. (11) into Eq. (14) leads to

$$\bar{x} = \frac{x_0 + nK_0C_L(\bar{x})}{1 + K_0C_L(\bar{x})}(\omega/b) \tag{15}$$

When the concentration of the free ligand at $\bar{x}$ approaches infinity, the

observed isoelectric point approaches the position of the fully liganded protein, X_F. Equation (15) then reduces to

$$\omega/b = (X_F - X_0)/n \tag{16}$$

Substitution of Eq. (16) into Eq. (15) and rearrangement leads to the double reciprocal relationship

$$\frac{1}{\bar{X} - X_0} = \frac{1}{X_F - X_0} + \frac{1}{X_F - X_0}\frac{1}{K_0}\frac{1}{C_L(\bar{x})} \tag{17}$$

Plotting $1/(\bar{X} - X_0)$ versus $1/C_L(\bar{x})$ and extrapolation to infinite free ligand concentrations provides the value for $1/(X_F - X_0)$. Dividing the slope by this value results in the value for $1/K_0$ which is the reciprocal of the dissociation constant.

Systems may also be analyzed in which only one ligand is bound, and binding changes the frictional coefficient of the protein. In this case K_0 will be an apparent binding constant which depends upon the diffusion coefficients of the unliganded protein and the protein–ligand complex.

$$K_0' = K_0(D_{PL}/D_P) \tag{18}$$

K_0' is the apparent equilibrium constant, and D_{PL} and D_P are the diffusion coefficients of the complex and the unliganded protein, respectively. If the change in the diffusion coefficient is small, K_0' will be a good estimate of K_0.

Proteins which bind ligand cooperatively may be analyzed utilizing the isoelectric focusing technique, but in this case $C_L(x)$ must be replaced by $[C_L(x)]^h$ where h is the Hill coefficient. The validity of the analysis depends upon the assumption that the frictional coefficient of the protein does not change appreciably. The constant obtained for a cooperative interaction will require the acquisition of extensive binding and structural data to determine the significance of the experimentally derived equilibrium constant.

The stoichiometry cannot be obtained from the approach discussed thus far, but it may be estimated. If one can determine the amount of bound and free ligand present under saturating conditions for a known amount of protein, the molar ratio of ligand to protein will give an approximate value for the stoichiometry, as demonstrated by Park.[22]

Experimental Procedure. 1. The gels should be prepared as described by Wrigley[23] using 7.5% acrylamide and a pH range of Ampholines which

[22] C. M. Park, *Ann. N.Y. Acad. Sci.* **209**, 237 (1973).
[23] C. W. Wrigley, this series, Vol. 22, p. 559.

will encompass the pI of the protein as well as the pI of the protein–ligand complex.

2. The protein may either be included in the solution used to prepare the gels, or it may be loaded on top of the polyacrylamide gels after polymerization as discussed by Wrigley.[23]

3. The protein should then be focused in several different tubes. One of the tubes should be removed and the position of the protein, X_0, should be determined from a densitometric scan of the gel after staining.

4. Using the gels in which the protein has been prefocused, ligand should be added in the appropriate electrode compartment so that it will be driven through the region in which the protein focuses. The position of the protein after equilibration with the ligand, $\bar{x}$, should be determined from a densitometric scan of the stain gel. The value for $\bar{x}$ should be obtained using a range of ligand concentrations. A single set of gels may be used for the entire concentration range to be tested. The lowest concentration should be tested first, then one of the gels should be removed, additional ligand added to the electrode solution, and electrophoresis should be resumed. Several ligand concentrations may be tested in this manner, as long as the protein is stable under the conditions of the experiment for a sufficient length of time.

5. The gels may be stained as described by Wrigley[23] or Wilson[18] and then scanned. The values for X_0 and $\bar{X}$ can be ascertained by integration of the scans and determination of the position of the center of mass. The center of mass will be considered that point in the gel which has equal amounts of protein on either side of it. The position of the protein should be correlated with the pH gradient so that small changes in the length of the gel do not complicate the analysis.

6. The data should be plotted in a $1/(\bar{x} - x_0)$ versus $1/C_L(\bar{x})$ plot and the value for $1/(X_F - X_0)$ obtained by extrapolation to infinite $C_L(\bar{x})$. The value for the slope of the plot must then be divided by the value for $1/(X_F - X_0)$ to determine the dissociation constant.

7. The stoichiometry of the interaction may be determined utilizing radioactive ligand. The isoelectric focusing procedure should be performed using a concentration of ligand approaching saturation. After the protein and ligand have achieved equilibrium (the position of the protein is constant as a function of time), the gel should be removed from the apparatus and sliced. Each slice is then placed in a solubilizer and counted in a scintillation fluid. The counts should be corrected for quench, and the amount of ligand in each slice determined from the specific radioactivity of the ligand. The amount of ligand in the slices between the electrode compartment which originally contained the ligand, and x_0, should be constant and should represent the amount of free ligand associ-

ated with the protein. The difference between the total amount of ligand present in the slices containing protein and the amount of free ligand in these slices will be equal to the amount of bound ligand. Dividing the amount of ligand bound to the protein by the amount of protein in the slices will yield the stoichiometry.

8. The stability of the ligand in the electrode solution must be checked to assure that it is not degraded or modified.

9. The stability of the protein in the presence of Ampholines should also be checked for a period of time equal to or greater than the time required to perform the focusing experiments.

10. The temperature of the gel should be regulated over the course of the focusing experiment to avoid potential complications which might arise if the equilibrium binding constant is temperature dependent.

General Comments. The use of isoelectric focusing to determine the equilibrium binding constant for a protein–ligand interaction is based on several assumptions. (1) It must be assumed that the association and dissociation rates of complex formation are sufficiently fast to maintain equilibrium at every instant of electrofocusing. (2) The binding of the ligand must change the net charge of the protein, but must not significantly affect the frictional coefficient of the protein. (3) The binding of each ligand molecule is assumed to affect the electrophoretic velocity by the same increment.

A direct binding technique should be used to corroborate the results obtained with the isoelectric focusing technique. Several protein concentrations should be used in the determination of K_0 so that one can observe possible protein concentration effects on K_0.

This technique is limited to ligands which are ions and are capable of migrating through the pH gradient to a region beyond the region in which the protein is located. The ligand must not be bound by the Ampholines, since this will result in multiple equilibria which will be difficult to analyze. It is also essential that the Ampholines do not influence the affinity of the protein for the ligand.

This technique is potentially very powerful in studying ligand–protein interaction of a heterogeneous protein sample such as a solution of isozymes. The interaction between ligand and a specific isozyme can be studied quantitatively without having to purify the individual isozymes.

Acknowledgments

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