CHEMICAL ANALYSIS

A SERIES OF MONOGRAPHS ON
ANALYTICAL CHEMISTRY AND ITS APPLICATIONS

Editor
J. D. WINEFORDNER

VOLUME 146

A Wiley-Interscience Publication

JOHN WILEY & SONS, INC.

New York / Chichester / Weinheim / Brisbane / Singapore / Toronto

High-Performance Capillary Electrophoresis

Theory, Techniques, and Applications

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A Wiley-Interscience Publication

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CHAPTER

29

AFFINITY CAPILLARY ELECTROPHORESIS: USING CAPILLARY ELECTROPHORESIS TO STUDY THE INTERACTIONS OF PROTEINS WITH LIGANDS

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High Performance Capillary Electrophoresis, edited by Morteza G. Khaledi. Chemical Analysis Series, Vol. 146.
ISBN 0-471-14851-2 © 1998 John Wiley & Sons, Inc.

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29.1. BACKGROUND

Affinity capillary electrophoresis (ACE) is a procedure that uses shifts in electrophoretic mobilities of a receptor on association with a ligand to measure binding constants (1). ACE is especially useful in studying protein–ligand interactions because it requires only small quantities of proteins and provides thermodynamic and kinetic information about the complexes under physiological conditions (Table 29.1).

Affinity gel electrophoresis (AGE) (2–3) provides a historical and conceptual background for ACE. AGE uses gels that have ligands covalently attached; these ligands interact with proteins migrating in the gels. Because interaction of protein with immobilized ligand decreases the electrophoretic migration of the protein, analysis of the change in mobility of the protein as a function of the density of the immobilized ligand provides an estimate of the binding affinity. AGE can also be used to estimate the binding affinity of a soluble ligand by allowing it to compete for the protein with the immobilized ligand during electrophoresis. AGE uses small amounts of proteins, does not require radioactive or colorimetric ligands, and is applicable to a wide range of proteins. There are, however, several characteristics of the technique that have limited its use in studies of protein–ligand complexes: designing and preparing the affinity gels can be difficult; estimating the thermodynamic activity (the "effective concentration") of the ligands immobilized in the gels is not straightforward; understanding interactions between proteins and both gels and gel-immobilized ligands is complicated and difficult to relate to interactions between soluble ligands and proteins in solution; gel electrophoresis of proteins in nondenaturing conditions is also slow.

ACE (1) circumvents some of the limitations encountered in AGE (although ACE, of course, has its own limitations). ACE measures changes in the electrophoretic mobility of a soluble protein as a function of the concentration of a soluble, charged ligand in the buffer. Since ACE determines the affinity constants describing protein–ligand interactions in homogeneous solution, it is conceptually simpler than AGE and is compatible with a wider range of buffer conditions than is AGE. Further, the well-defined mechanism of separation on which CE is based makes it possible to design experiments rationally.

Table 29.1. Summary of Advantages and Disadvantages of CE and ACE in Analyzing Proteins and Protein–Ligand Interactions

Advantages	Disadvantages
• High resolution	• Narrow pathlength (~ 50 μm); requires (typically) > μM concentrations of analytes
• Easily automated	• Adsorption of proteins with high values of p*I* or high MW to the walls of the uncoated capillaries limits applicability
• *Uncoated* capillaries are inexpensive	• Identification of analytes can be difficult
• Use of internal standards to measure electroosmotic flow (EOF) allows very reproducible measurement of mobilities	• Analytical, not preparative scale
• Small quantities of proteins and ligands required	• Background UV adsorption of analytes may be problematic in ACE
• Mobilities can be measured with impure samples	
• A wide range of types of buffer can be used	
• Buffers can simulate physiologically relevant conditions	
• Applicable to screening libraries of ligands	
• Separation based on charge and mass; rational design of experiment is possible	
• Applicable to certain mixtures of isozymes	
• When used with charge ladders, provides detailed information about electrostatics of interactions	
• Can provide information about *rates* of association and dissociation	

29.2. PRINCIPLES OF AFFINITY CAPILLARY ELECTROPHORESIS

The velocity with which a protein migrates in an electric field at unit field strength is defined as its electrophoretic mobility (μ, in $cm^2\,kV^{-1}\,s^{-1}$). The value of μ for a protein is related linearly to the force on it in the electric field, and inversely to the hydrodynamic drag on it (4,5):

$$\mu \approx C_p \frac{Z}{M^{\alpha}} \tag{29.1}$$

The electrical force is proportional to the charge, Z, of the protein; this charge is the summation of charges carried by its electrostatic components, including the side chains of the protein, associated ligands, covalently attached saccarides, cofactors, and metal ions. The hydrodynamic drag is related to the mass (M) (or the molecular volume and shape) of a protein in solution (6). C_p is an electrophoresis constant and its value is dependent on protein shape and structure and conditions of the experiments; α is defined as the power constant of the molecular weight to which the electrophoretic mobility is inversely proportional. Recently, we have determined the values of C_P and α to be equal to $6.3\,cm^2\,min^{-1}\,kV^{-1}\,charge^{-1}\,kDa^{0.48}$ and 0.48, respectively, under the condition of 25 mM Tris/192 mM Gly (pH = 8.3; $T = 37\,°C$) (7).

The ACE experiment requires that the electrophoretic mobility of the protein–ligand complex differ from that of the protein (Figure 29.1). When rapid equilibration occurs between unbound and bound forms of the protein, the measured electrophoretic mobility is the *average* of the electrophoretic mobilities (appropriately weighted by the mole fractions) of the bound and unbound protein:

$$\mu = \theta\mu_{P,L} + (1 - \theta)\mu_P \tag{29.2}$$

Equation (29.2) describes this relationship for a monovalent binding system, where θ is the mole fraction of the protein bound with ligand, and $\mu_{P,L}$ and μ_P are the electrophoretic mobilities of the protein–ligand complex and the uncomplexed protein, respectively. Scatchard analysis of the change in electrophoretic mobility ($\Delta\mu_{P,L} = \mu - \mu_P$) as a function of the concentration of the ligand ([L]) in solution yields the binding constant (K_b) [Eq. (29.3)]:

$$\Delta\mu_{P,L}/[L] = K_b\,\Delta\mu_{P,L}^{max} - K_b\,\Delta\mu_{P,L} \tag{29.3}$$

The analysis based on Eq. (29.3) makes several assumptions: equilibrium is established between bound and unbound species; the rate constant for dissociation of the protein ligand complex is fast relative to the time for the experiment; the concentration of the ligand in the CE buffer is sufficiently high relative to protein in the sample that the total concentration changes negligibly upon binding to protein; the interaction of the ligand and the receptor with the wall of the capillary does not significantly alter the binding of the ligand to the receptor; and the electric field does not affect the binding. In experiments where changes in the electroosmotic flow with higher values of [L] are much smaller than the changes in $\mu_{P,L}$, Eq. (29.4) can be used to determine the binding affinity of the protein–ligand pair.

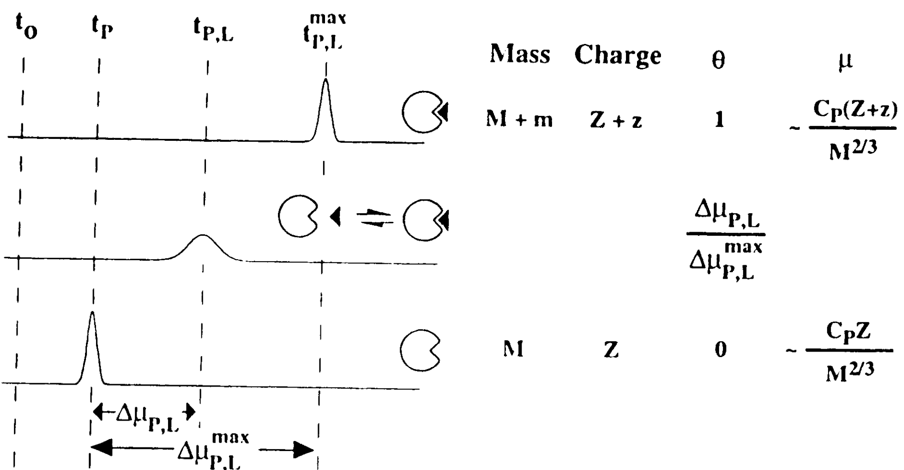


Figure 29.1. The electrophoretic mobility of a protein P (mass M and charge Z) varies with the occupancy of the binding site by the ligand L (▶, mass m and charge z). The molar fraction of the bound form of enzyme (θ) is calculated from the ratio of $\Delta\mu_{P,L}$ to $\Delta\mu_{P,L}^{max}$. Peaks broadening is observed when the value of the half-life of the protein–ligand complex is close to the migration time. The expressions for mobility assume that $(M + m)^{2/3} \approx M^{2/3}$ (that is, $M \gg m$) and that the values of C_P and α are the same for the protein and the protein–ligand complex.

$$\Delta t_{P,L}/[L] = K_b\,\Delta t_{P,L}^{max} - K_b\,\Delta t_{P,L} \tag{29.4}$$

Association of a ligand can change the electrophoretic mobility of a protein in three ways: (i) by changing the hydrodynamic drag, while leaving the charge unchanged (binding of large, neutral ligands); (ii) by changing the charge, while leaving the hydrodynamic drag unchanged (binding of small, charged ligands); and (iii) by changing both the charge and hydrodynamic drag (binding of large, charged ligands, i.e., protein–protein interactions). Any of these three strategies can be used in rationally designing ACE experiments; we and others have used the last two strategies.

29.3. TECHNICAL ISSUES

29.3.1. Correction for Changes in Electroosmotic Flow

In the absence of changes in electroosmotic flow in the ACE experiment, it has been possible to use migration times directly to determine association constants [Eq. (29.4)]. ACE experiments, however, are often complicated by changes in the electroosmotic flow (indicated by changes in the migration time of a neutral marker) with increasing concentrations of the ligand in the buffer. This behaviour may be due to adsorption of the ligand on the wall of the capillary, changes in the dielectric constant of the buffer, or changes in the viscosity of the buffer. This effect can be corrected by adding to the sample a species whose electrophoretic mobility does not change with increasing concentrations of ligands—this species can be either neutral molecule or a protein—and calculating electrophoretic mobilities from the times of migration of the noninteracting marker (t_{marker}) and the receptor (t_{receptor}) using the following equation:

$$\mu = \frac{L_t L_d}{V}\left(\frac{1}{t_{\text{marker}}} - \frac{1}{t_{\text{receptor}}}\right) \tag{29.5}$$

where L_t is the total length of the capillary; L_d is the length of the capillary from the injection end to the detector; and V is the voltage that is applied across the capillary. We have used this strategy employing mesityl oxide as neutral noninteracting marker to measure association constants for the binding of CA to benzenesulfonamide ligands (8), SH_3 to peptides (9), and IgG_{2b} to DNP (10).

29.3.2. Choice of Buffers

Proteins with high values of pI(p$I > 8$) or with high molecular weight (> 50 kDa) tend to adsorb to the walls of the uncoated fused-silica capillaries when the pH of the buffer is below ~ 7. Several strategies help to overcome this problem. The simplest method is to increase the pH of the electrophoresis buffer (while remaining in the range in which the protein is still in its native conformation). For values of pH higher than the pI of the protein, the protein will carry a net negative charge and will have a smaller tendency to adsorb electrostatically to the negatively charged wall. Buffer additives, such as the organic zwitterions in combination with inorganic salts developed by Bushey and Jorgenson (11), decrease the adsorption of proteins. Some of these zwitterions (e.g., betaine) are commercially available and inexpensive. The requirement that these zwitterions be used at high concentrations (> 0.5 M) makes it necessary to examine the possibility that they might influence ligand receptor interactions. We note, however, that a high concentration of zwitterionic materials and organic materials may be more representative of intracellular conditions than are the simple buffers often used in binding assays (10). For proteins with high values of pI, noncovalent adsorption of polycations on the wall of the capillary can reduce adsorption (12). Although covalently modified capillaries can markedly reduce adsorption in some instances, they are expensive and are often not stable over the course of many experiments.

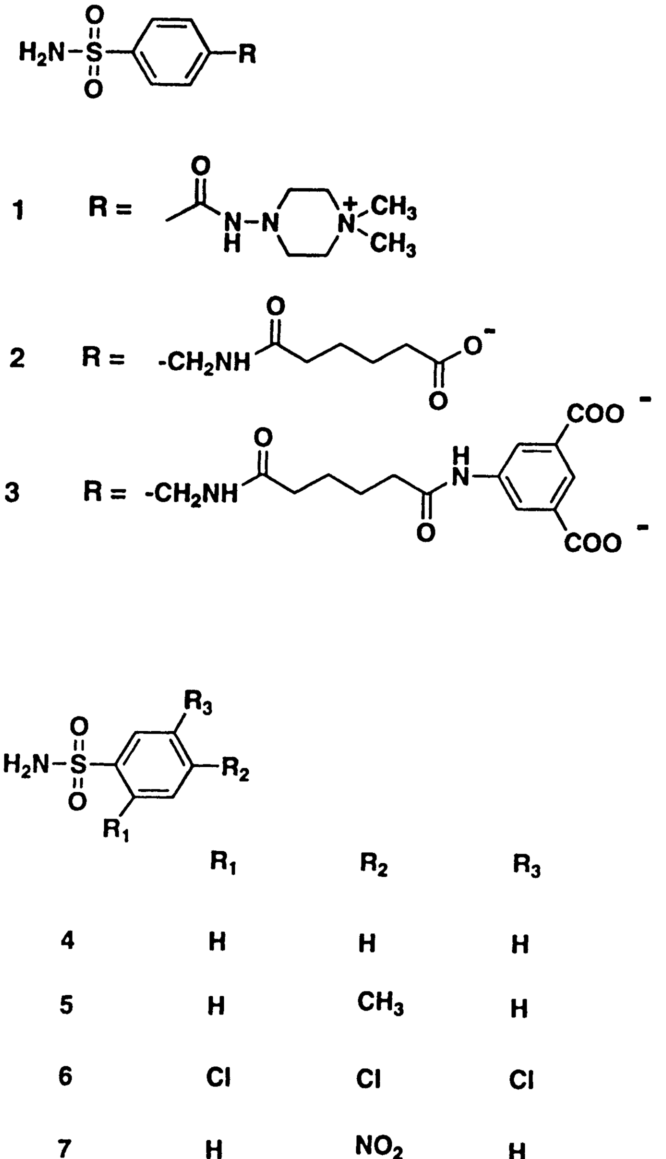
29.4. CARBONIC ANHYDRASE AS A MODEL PROTEIN

We have used carbonic anhydrase (CA;EC 4.2.1.1, from human and bovine erthrocytes) (13) as a model protein for developing and testing new techniques in ACE. Several crystal structures of CA and CA ligand complexes are known (14). CA has a molecular weight of 30 kDa, and a number of isozymes that differ in pI are commercially available. CA is inhibited by para-substituted arylsulfonamides with values of K_b ranging between 10^5 and $10^9\,M^{-1}$; charged derivatives of these ligands are easily synthesized (Scheme I). Arylsulfonamide ligands bind CA in a 15 Å deep, conically shaped cleft; the protein does not undergo significant conformational changes upon binding the ligands (14).

29.5. DETERMINATION OF BINDING AFFINITY

29.5.1. Using Affinity Capillary Elecrtrophoresis to Determine the Binding Affinity of Benzenesulfonamide for Carobonic Anhydrase

We used ACE to determine the association constants of several benzenesulfonamide ligands [1 7 (see Scheme I)] to CA. Binding of the charged ligands **1** **3** to CA results in changes in electrophoretic mobility that correlate with the charge of the ligand. Ligands **1** and **2** carry opposite charges; upon binding of these ligands to CA, the electrophoretic mobility shifts by equal amounts but in opposite directions. Ligand **3** has a charge of -2 and results in a shift in the electrophoretic mobility of CA that is twice the magnitude of that observed upon binding of ligand **2**. Scatchard analysis of these changes in electrophoretic mobility as a function of the concentration of ligand added to the buffer yields association constants for the complexes [Eq. (29.3)].



Scheme I. Structures of arylsulfonamide ligands having different net charges that bind to CAII.

29.5.2. Binding of Families of Derivatives of Carbonic Anhydrase to a Positively Charged Ligand

Random acetylation of the ε-amino groups of lysine side chains of CA generates a mixture of derivatives of the protein. Conversion of each charged ε-ammonium group ($-CH_2NH_3^+$, pK_a ~ 11; a total of 18) to a neutral *N*-acetyl group ($-CH_2NHCOCH_3$) changes the charge of CA by approximately 1 unit (at pH 8.3). Making the assumption (well supported by experiment) that the hydrodynamic drag on the protein changes relatively little upon acetylation, we find that each peak in the electropherogram corresponds to a heterogeneous family of derivatives having the same *number* of *N*-acetylated lysine groups. The set of acylated proteins appear in the electropherogram as a series of peaks differing in mobility (and charge) by regular intervals; we call the electropherogram of this set of proteins a *charge ladder* (Figure 29.2). Upon addition of a sulfonamide ligand to the electrophoresis buffer, the electrophoretic mobilities of all the derivatives of CA change. Scatchard analysis of the changes in electrophoretic mobility as a function of the concentration of the sulfonamide ligand gives the binding affinity of the ligand to each family of derivatives simultaneously. This example demonstrates two strengths of CE: derivatives of proteins that differ only by 1 unit of charge can be prepared and resolved; and binding affinites of a single ligand to multiple proteins can be measured simultaneously. The combination of protein charge ladders and ACE is also usefull in quantitating the influence of charge on protein ligand interactions (15).

29.5.3. Using Competitive Affinity Capillary Electrophoresis to Study Binding of Small Neutral Ligands to a Receptor

Since a small, electrically neutral ligand will not change the electrophoretic mobility of a protein upon binding [Eq. (29.1)], CE cannot differentiate between bound and unbound forms of the protein and therefore cannot measure directly the binding constants of these type of ligands. This limitation can, however, be overcome by using a charged ligand with a known binding affinity to compete with the neutral ligand for the protein. Typically, a sufficient concentration of the charged ligand is added to the sample and electrophoresis buffer to saturate the binding site of the protein; electropherograms are obtained with increasing concentrations of the neutral ligand in the electrophoresis buffer to compete with the charged ligand. Analysis of the average mobility of the protein as a function of the concentration of the neutral ligand yields its binding affinity relative to the charged ligand (16). Table 29.2

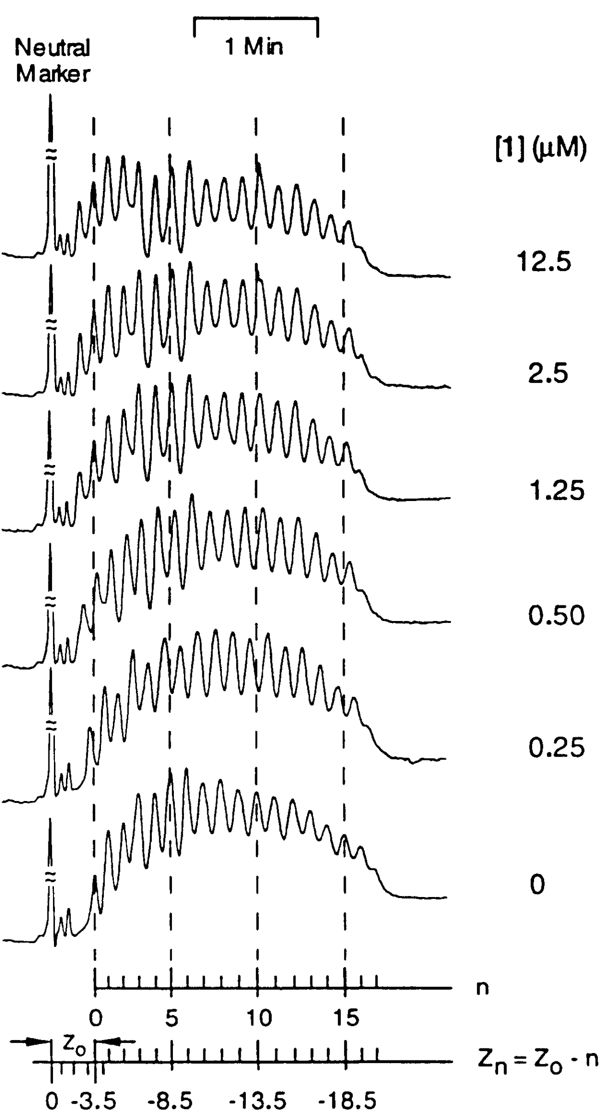


Figure 29.2. Electropherograms of binding a positively charged ligand (**1**) to the families of derivatives of CAII. The derivatives were obtained by nonspecific modification of ε-amino groups of Lys on CAII using acetic anhydride. Increasing concentrations of **1** (with charge + 1) was added to the electrophoresis buffer (25 mM tris/192 mM Gly, pH = 8.3). The neutral marker was *p*-methoxybenzyl alcohol. The number of modified ε-amino groups (n) and net charge of the modified proteins (Z_n) are indicated below the electropherograms. The timescale shown on top of the figure applies to all electropherograms.

Table 29.2. Comparison of Binding Constants of Ligands 4–7 to CA Obtained by ACE and Other Methods

Ligand	ACE[a] (10^6 M^{-1})	Values from the Literature[b] (10^6 M^{-1})	Values form the Literature[c] (10^6 M^{-1})
4	1.1	0.3	0.7
5	1.9	0.7	2.0
6	4.5		6.8
7	7.0	2.6	16.0

[a] The conditions for ACE were 192 mM Gly and 25 mM Tris at 25 °C (pH 8.3).
[b] Literature values are for bovine CA II (BCA II) measured at 25 °C in 100 mM Tris–HCl Buffer, pH 7.2 (17).
[c] Literature values are for human CA II (HCA II) measured by fluorescence at 25 °C in 20 mM phosphate buffer, pH 7.5 (18).

compares values of K_b of arylsulfonamides to CA measured by ACE and other techniques (16). The values obtained from ACE agree well with those obtained from other measurements.

29.5.4. Binding of a Protein to Two Ligands: Immunoglobulin G_{2b}–*N*-Dinitrophenol Complexes

We used ACE to quantify the interaction between a bivalent anti-DNP rat monoclonal IgG_{2b} antibody and charged, monovalent ligands containing an *N*-dinitrophenyl (DNP) group (Figure 29.3.) (10). Since the antibody has two binding sites for the DNP ligands, three forms of protein are possible: IgG not bound to ligand, IgG bound to one ligand, and IgG bound to two ligands. Analysis of these equilibria using ACE presented three challenges: (i) the change in mobility of the IgG on complexing a ligand with a single charge was small because of the high molecular weight of the IgG [Eq. (29.1)]; (ii) the IgG tended to adsorb slowly to the walls of the uncoated quartz capillary during the course of the experiment; and (iii) the independence of the two sites on IgG could not be assumed a priori. We overcame these problems using three strategies: (i) we synthesized ligands that were multiply charged (up to −9) at pH 8.3 (the pH of the experiment); (ii) we used buffers containing zwitterionic additives in combination with inorganic salts to reduce adsorption of proteins [a strategy first used by Bushey and Jorgenson (11)]; and (iii) we devised an analysis that could yield both association constants in the bivalent system simultaneously (without assuming any cooperativity between the two sites).

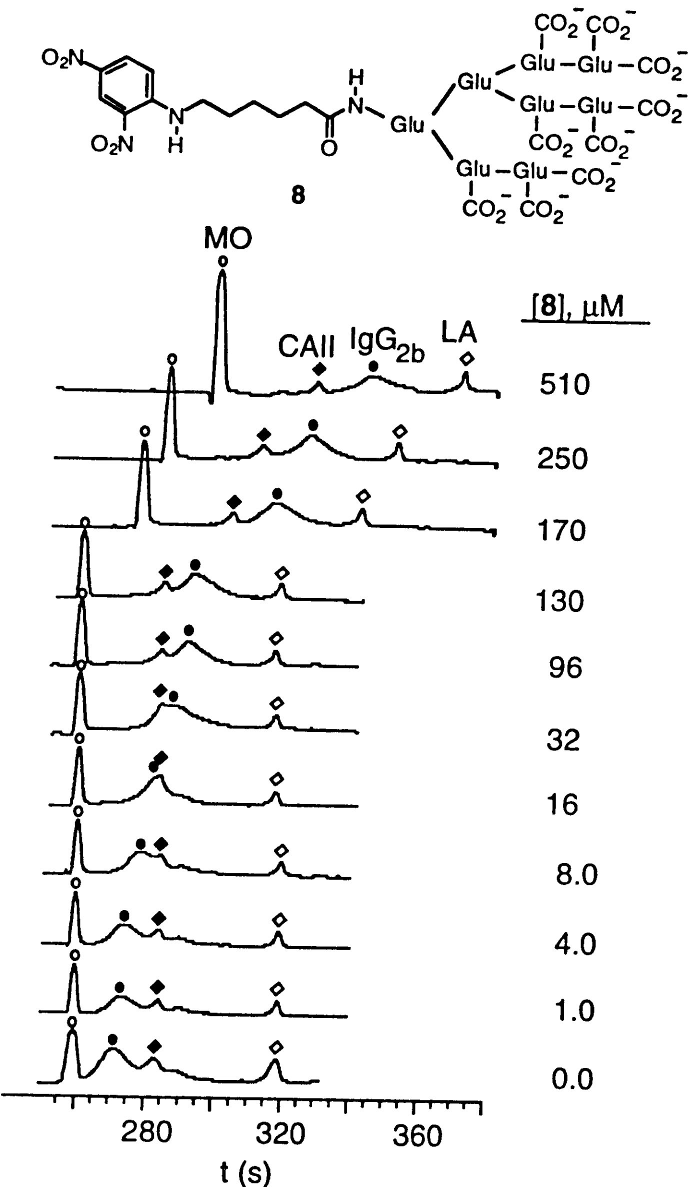


Figure 29.3. Electropherograms of IgG_{2b} as a function of the concentration of ligand **8** in 25 mM Tris/192 mM Gly/10 mM K_2SO_4/0.5 M 3-quinuclidinopropanesulfonate, pH = 8.3. Two charged ligands containing DNP groups (**8**) bind to IgG_{2b}, thus changing its electrophoretic mobility. The peak of IgG_{2b} is broad, probably due to different patterns of glycosylation (microheterogeneity). Ligand **8** has a total charge of −9 at pH 8.3. Mesityl oxide (MO), CAII, and α-lactalbumin (LA) were used as internal standards.

With increasing concentration of ligand in the buffer, the average electrophoretic mobility of the IgG changed sigmoidally. Because only a single peak was observed for all concentrations of ligand, equilibration was fast on the timescale of the experiment (10). We were able to determine association constants for a DNP ligand binding to each site of the IgG and showed that these binding events were noncooperative.

We believe that ACE can be used to quantitate many antibody ligand interactions rapidly and conveniently. The use of ligands having high net charges will be useful in analyzing shifts in electrophoretic mobility for large proteins (> 100 kDa).

29.5.5. Simultaneous Determination of Relative Binding Affinities of Several Isozymes of Carbonic Anhydrase to a Charged Ligand

Figure 29.4 shows the electropherograms of a mixture of four isozymes of CA (from human and bovine erythrocytes) obtained using an electrophoresis buffer containing a sulfonamide inhibitor having a charge of −1. The electrophoretic mobilities of the isozymes of CA increased with increasing concentrations of the charged ligand in the electrophoresis buffer, while the migration times of MO, as well as horse heart myoglobin and soybean trypsin inhibitor (used as protein markers), remained constant (up to 140 μM). Scatchard analysis of the changes in electrophoretic mobilities of the isozymes gave binding affinities of the ligand for each isozyme. This procedure may be useful to rapidly identify ligands that bind a single isozyme from a pool of several.

29.5.6. Simultaneous Determination of Relative Binding Affinities of Several Ligands to One Receptor

By analyzing the electrophoretic mobilities of several ligands in a sample using an electrophoresis buffer containing a single protein, ACE can provide the binding affinities of the receptor for each ligand in a single experiment. We used vancomycin and four peptidyl ligands as a model system (19). We measured changes in the electrophoretic mobility of the four ligands with increasing concentrations of vancomycin in the electrophoresis buffer to obtain four independent binding constants (Figure 29.5). Karger's group applied this approach to the screening of tight-binding ligands for vancomycin from a library comprising 100 peptides [see Chu et al. (20)]. The electrophoretic mobilities of the tighter-binding peptidyl ligands were retarded in the capillary, and their structures were identified by on-line mass spectrometry (MS) (21,22). This work demonstrates the usefulness of ACE–MS in screening and identifying tight-binding ligands (leads) from a library of compounds, although MS is still not a routine method of detection in CE.

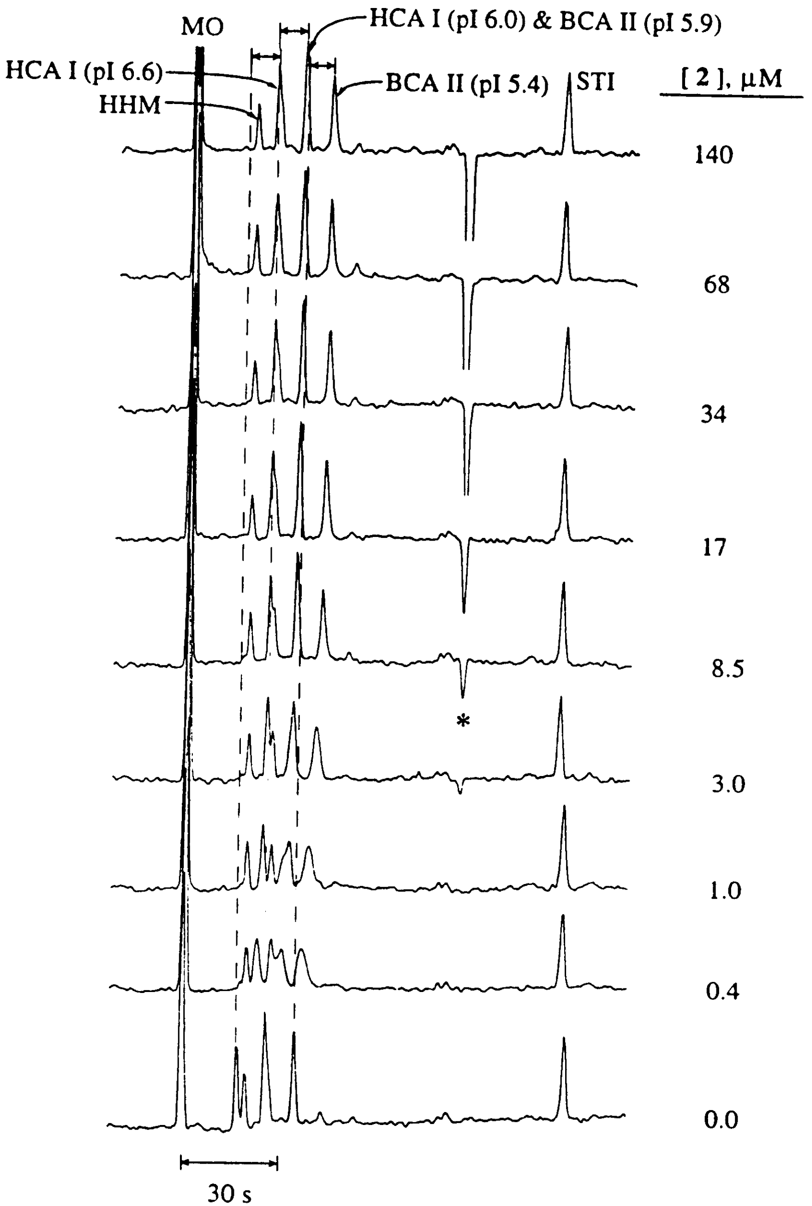


Figure 29.4. ACE of a mixture of isozymes of carbonic anhydrase: HCA I (human carbonic anhydrase, p*I* 6.6), HCA I (p*I* 6.0), BCA II (bovine carbonic anhydrase, p*I* 5.9), and BCA II (p*I* 5.4). The electrophoresis buffer used was Tris (25 mM)/Gly (192 mM) at pH 8.3. MO was added to measure EOF; HHM (horse heat myoglobin) and STI (soybean trypsin inhibitor) are protein markers that do not interact with affinity ligand **2**. The inverted peak migrates at the migration time of free **2** and is due to a lower free concentration of **2** in the migrating plug relative to the electrophoresis buffer because of binding of the ligand to CA.

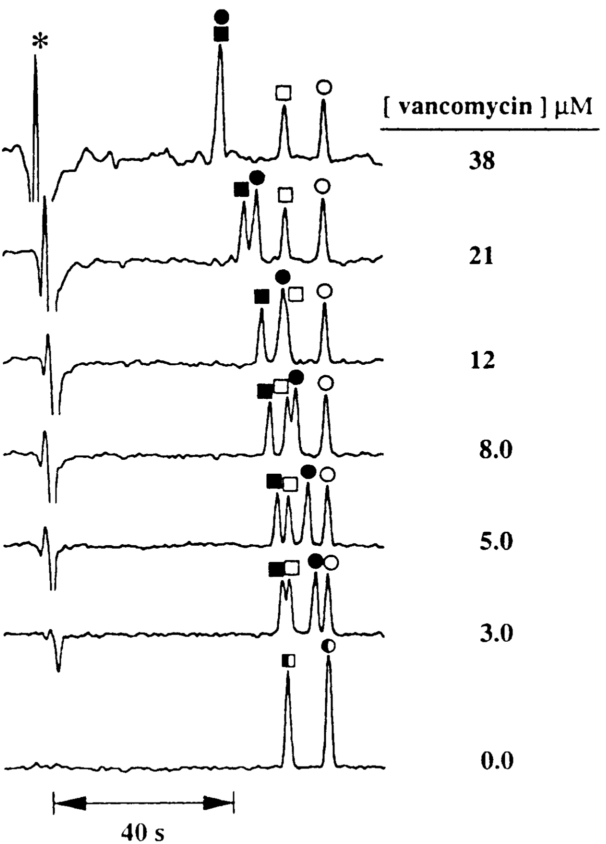


Figure 29.5. ACE of *N*-Fmoc-Gly-D-Ala-D-Ala (●), *N*-Fmoc-Gly-D-Ala-D-Ala (■), *N*-Fmoc-Gly-L-Ala-L-Ala (○), *N*-Fmoc-Gly-L-Ala-L-Ala (□) in 20 mM sodium phosphate buffer, pH 7.5, containing various concentrations of vancomycin. The asterisk (*) indicates the position of the peak for unidentified neutral species carried through the capillary by EOF.

29.5.7. Protein Protein Interactions: Dimerization of Insulin

Insulin is a peptide of MW = 5.7 kDa that dimerizes in aqueous solution (23). Based on Eq. (29.1) and a value of $\alpha = 2/3$, the electrophoretic mobility of monomeric insulin is a factor of 1.25 smaller than that of the dimer (we assume negligible changes in the values of pK_a of the charged side chains upon dimerization). We measured the electrophoretic mobility of insulin in Tris/Gly buffer at pH 8.3 as a function of its concentration over the range from 2 to 400 μM. The electrophoretic mobility of insulin increased with increasing

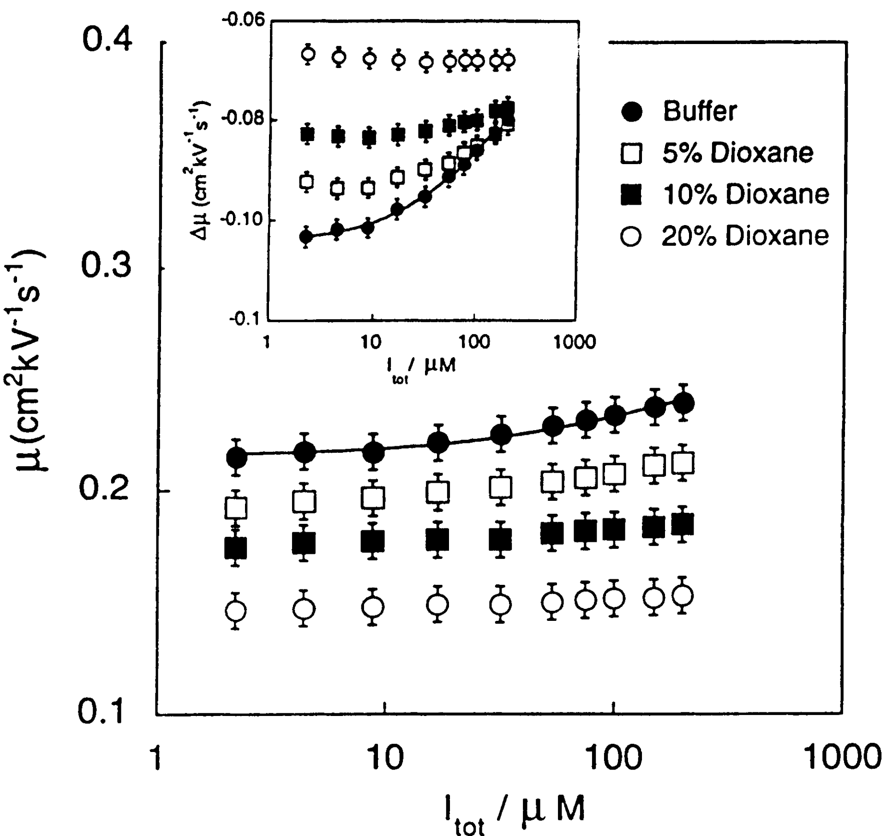


Figure 29.6. Measurement of the dimerization constants of bovine insulin. Plots of the electrophoretic mobility of insulin as a function of the concentration of insulin in several different buffers: (●) 25 mM Tris/192 mM Gly, pH = 8.4 (buffer A); (□) 5% dioxane in buffer A; (■) 10% dioxane in buffer A; (○) 20% dioxane in buffer A. Soybean trypsin inhibitor (STI) was used as protein marker to correct for changes in EOF; the inset shows plots of corrected mobilities after subtracting the mobility of STI.

concentration of insulin in the sample (Figure 29.6), while the mobility of the protein marker, soybean trypsin inhibitor, remained constant.

The determination of a dimerization constant is based on the assumption that the measured electrophoretic mobility of insulin is a weighted average of the electrophoretic mobilities of the monomer (μ_M) and dimer (μ_D) [$\mu = \mu_M(1 - \theta_D) + \mu_D\theta_D$], where θ_D is the mole fraction of dimer. Analysis of the electrophoretic mobility of insulin as a function of its concentration yields a dimerization constant of 6000 M^{-1} at pH 8.3, in good agreement with

previously reported values (23). In agreement with work by Frederiq, the dimerization of insulin (as inferred by changes in μ) was completely inhibited in an electrophoresis buffer containing 20% dioxame (23).

29.6. DETERMINATION OF KINETIC PARAMETERS FOR BINDING

The shapes of peaks in the electropherogram depend, in part, on the kinetic parameters for complexation (k_{on} and k_{off}) and the migration time of the protein ligand complex. We used CA and a charged sulfonamide ligand as a model system to illustrate how ACE can be used to obtain kinetic constants for association of ligands with proteins (16). We simulated the shapes of peaks using k_{on} and k_{off}, the concentration of the ligand in the buffer, and the relative mobilites of the protein and its complex as variables. By comparing qualitatively the shapes of the experimental peaks to the shapes of simulated peaks, we estimated the values of k_{on} and k_{off} for the binding. These estimated values were consistent with those obtained from studies using fluorescence spectroscopy (16). This method is useful only for systems where the rate constant for dissociation of the complex is comparable to the time of the electrophoresis experiment. The method will also be useful for analyzing the rate constants for association in systems where binding is slow but effectively irreversible during the course of the experiment, that is, when peaks for both the protein and protein–ligand complexes are resolved and sharp.

29.7. USING AFFINITY CAPILLARY ELECTROPHORESIS TO MEASURE THE EFFECTIVE CHARGE OF A PROTEIN

Protein charge ladders can be used to estimate the charge on a protein. In Section 29.5.2, we described acetylation as one method of generating charge ladders; another method relies on association of several small, differently charged ligands to a protein. In this latter method, a sufficient concentration of ligand (~ 100 times the K_d) is added to the buffer to saturate the binding site of the protein. Multiple experiments using differently charged ligands generate each peak of the charge ladder independently.

In both methods, the analysis assumes that the values of C_p, α, and M are constants that do not change upon modification of the protein. This assumption is reasonable for acylating agents or charged ligands that are small relative to the protein. The charge of a protein changes from a value of Z_0 to Z_n on modification or association with ligands. If the change in hydrodynamic drag on modification is small compared to the changes in mobility due to charge, the ratio of the mobilities of the modified and unmodified proteins

eliminates the variables C_p, α, and M [Eq. (29.6)]:

$$\frac{\mu_0}{\mu_n} = \frac{Z_0}{Z_n} \tag{29.6}$$

$$\Delta Z = Z_n - Z_0 = Z_0 \frac{\mu_n - \mu_0}{\mu_0} = Z_0 \left[\frac{(\Delta t/t)_n}{(\Delta t/t)_0} - 1 \right] \tag{29.7}$$

Equation (29.7) relates the increments of charge to the electrophoretic mobilities (and hence migration times) of the derivatives of the protein. Analysis using Eq. (29.7) yields the total charge of the protein in the electrophoresis buffer (Figure 29.7) (24). This type of analysis on a charge ladder of CA indicates the total charge of CA to be −3.5 at pH 8.3 (24). Similar experiments found the charge of IgG_{2b} to be −8.0 at pH 8.3 (10) and of insulin to be −4.2 at pH 8.3 (23).

The alternate method of generating charge ladders using noncovalent modification has the disadvantages that it requires knowledge of the binding stoichiometry of the ligands and protein, and the syntheses of ligands having different charges. This method is useful, however, when covalent modification of the protein caused changes in structure or binding properties or the derivatized proteins cannot be resolved by CE (e.g., for proteins having high molecular weights).

29.8. DETERMINATION OF STOICHIOMETRY OF BINDING

ACE can be used to determine the stoichiometry in receptor–ligand complexes. One method analyzes the changes in concentration of free ligand in the sample containing protein caused by association of the ligand with protein. For example, when the same *total* concentration of ligand **2** was maintained in both the electrophoresis buffer and sample containing CA (4.2 μM), a negative peak appeared at the migration time of the free ligand. This negative peak was due to a lower *free* concentration of **2** in the migrating plug relative to the electrophoresis buffer due to binding of the ligand to CA. As the concentration of **2** was increased in the sample, the amplitude of the negative peak decreased until a positive peak (due to excess ligand) appeared. The increase in concentration of ligand **2** in the sample required to compensate the negative peak is equal to the amount of **2** that bound CA. The ratio between the increase in concentration of **2** in the sample and the concentration of CA gave a binding stoichiometry of 1 : 1 for CA binding **2** (25). The binding stoichiometries of several protein–ligand systems have been detemined by this method, including mouse monoclonal IgG–human serum albumin and streptavidin–biotin (25).

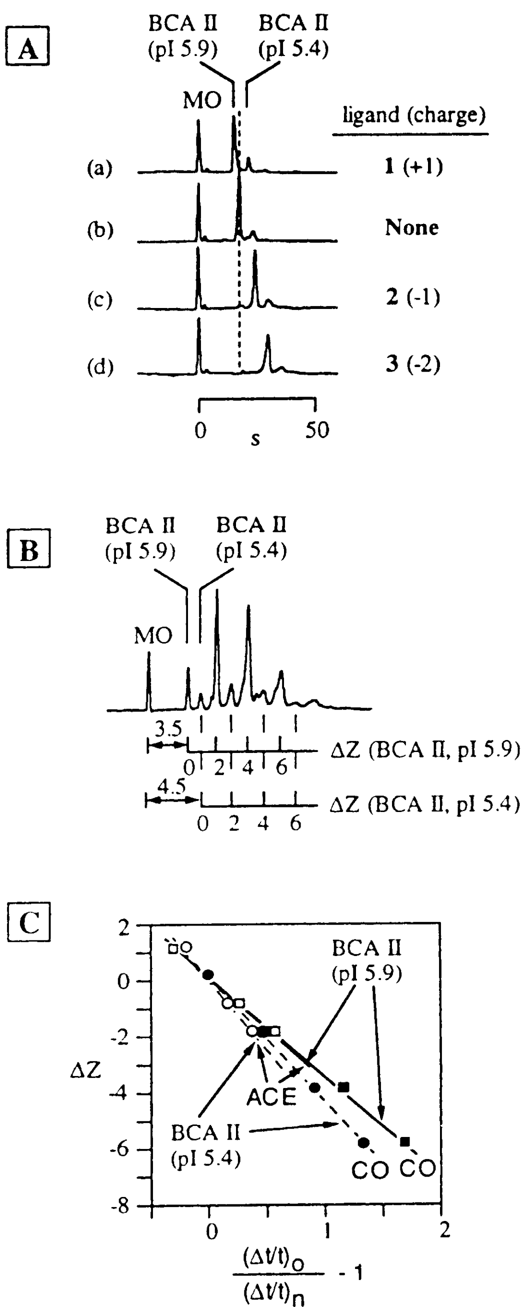


Figure 29.7. Measurement of the effective charge of bovine carbonic anhydrases (BCA II) by measuring mobilities of aggregates with ligands having different values of charge and using a charge ladder. The BCA II sample (from Sigma) contains two isozymes: BCA II (p*I* 5.9) and BCA II (p*I* 5.4) in about a 10:1 ratio; these isozymes differ by 1 unit of charge. (A) The charge ladder was generated by complexation of BCA II with charged ligands: (a) [**1**] = 1.0 mM ($Z = +1$); (b) buffer; (c) [**2**] = 1.0 mM ($Z = -1$); and (d) [**3**] = 0.5 mM ($Z = -2$). (B) Covalent modification of lysine ε-amino groups on BCA II by 4-sulfophenyl isothiocyanate generated two independent charge ladders of BCA II (p*I* 5.9) and BCA II (p*I* 5.4) [buffer = Tris (25 mM)/Gly (192 mM), pH = 8.3, $t_{t,0} \approx 170$ s]. The charge increment for covalent modifications is −2 for this acylating agent. The migration times of the unmodified proteins are indicated and provide a scale in ΔZ. The timescale applies to electropherograms in both parts A and B. (C) Determination of effective charge from the analysis of charge ladders. The data shown for BCA II (p*I* 5.9) and BCA II (p*I* 5.4) were estimated using covalent and noncovalent charge ladders generated by ACE and covalent (CO) methods, respectively.

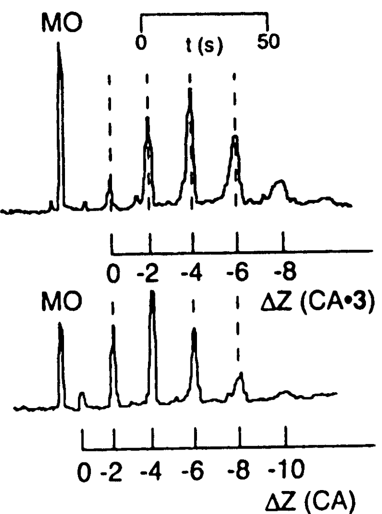


Figure 29.8. Determination of binding stoichiometry using charge ladders. The charge ladder of CA II (from human erythrocyte) was formed by acylation of its ε-amino groups of Lys using succinic anhydride ($\Delta Z = -2$). Treatment of this charge ladder with ligand **3** (charge of -2) at a concentration that saturates the active site of CA II shifted the mobility of each protein by an amount corresponding to a change in charge of -2. Comparison of the charge increments obtained by covalent modification and upon binding of ligand **3** indicated a 1 : 1 stoichiometry of binding of **3** to CA II.

More recently, we have used charge ladders of proteins to determine stoichiometries of binding (26). The charge ladder provides an internal scale in integral units of charge. Saturation of the derivatized proteins with a charged ligand results in a shift of the peaks in the ladder by an amount that is related to the net change in effective charge upon binding. The ratio between the total change in charge of members of the ladder and the charge of a single ligand provides the number of ligands that bind each protein (Figure 29.8). This method has the advantage that it does not require knowledge of the molecular weight of the protein; hence, proteins from impure samples can be analyzed without purification, provided that the peaks are resolved in the electropherogram.

29.9. PROSPECTS AND LIMITATIONS OF AFFINITY CAPILLARY ELECTROPHORESIS

ACE is a useful analytical method in biochemistry (Table 29.3) (23,25,27–56). It is a simple technique experimentally: it does not involve radioactive

Table 29.3. Studies on Binding of Ligands to Receptors Using ACE

Example	Reference
A. Protein–protein interactions	
• Human serum albumin (HSA) and anti-HSA	25
• Human growth hormone (hGH) and anti-hGH (or its fragment)	27
• IgG with protien A	28
• Insulin dimerization	23
B. Protein–DNA interactions	
• EcoR1 and oligonucleotide; peptide and oligonucleotide	29
• Transcription factor–oligonucleotide	30
C. Protien–peptide interactions	
• SH_3 domain and proline-rich peptides	31
• Antibody–antigen interaction	32,33
D. Protein–drug interactions	
• Bovine carbonic anhydrase (CA) and arylsulfonamides; glucose-6-phosphate dehydrogenase (G6PDH) and $NADP^+$; G6PDH and NADPH; IgG_{2b} and 2,4-DNP	8,10,16,34
• Bovine serum albumin (BSA); bacteiral cellulase and tryptophan benzoin; pindolol; promethazine; warfarin	35
• HSA and kynurenine; tryptophan; 3-indolelactic acid; 2,3-dibenzoyl tartaric acid; 2,4-dinitrophenyl glutamate	36
• Cellulase and β-blockers	37
• BSA and warfarin; leucovorin	38,39
• Albumin–ofloxacin	40
E. Protein–metal ion interactions	
• Calmodulin, parvalbumin, thermolysin, and Ca (II); carbonic anhydrase, thermolysin, and Zn(II)	41,42
• C-reactive protein and Ca(II)	43
F. Protein–carbohydrate interactions	
• Concanavalin A–monosaccharides	44
• Lectin–carbohydrate	45–47
G. Peptide–peptide interactions	
• Vancomycin and peptides	19,20,48,49
H. Peptide–carbohydrate interactions	
• Synthetic peptide and anionic carbohydrates	50
I. Peptide–dye interactions	
• Synthetic peptide and Congo Red	51
J. Carbohydrate–drug interactions	
• Methyl-β-cyclodextrin and propranolol	52
• β-Cyclodextrin and salbutanol	53
K. Oligonucleotide–oligonucleotide interactions	
• $d(A)_n$–$d(T)_n$	54–56

materials or chemically immobilized ligands, and the analyses are fast, reproducible, and capable of high resolution. Because ACE permits a wide range of buffer conditions, proteins can be studied in their native conformations under physiological conditions. Even complex and impure samples can be analyzed if the analyte of interest is well resolved in the electropherogram. The quantitative relationships between the electrophoretic mobility of a protein and the charge and mass of the protein make this technique useful for both fundamental studies of biomolecular recognition and applications in bioanalytical chemistry.

ACE currently has certain limitations (Table 29.1); the primary limitation is that many proteins do not elute through the capillary to give sharp peaks but interact strongly with the walls of the capillary to give broadened peaks. Uncoated capillaries (with or without zwitterionic and inorganic additives) and coated capillaries (with either covalent or noncovalent coatings) handle only a limited subset of proteins and have their own set of drawbacks. The mechanisms of adsorption of protein to the walls of the capillary are not yet well established but are under active investigation. Other technical problems with ACE are the high-background UV absorbance present when protein–protein or protein–DNA complexes are being analyzed and difficulties in identifying the analytes that emerge from the capillary when complex samples are used.

Many of the applications of ACE described in this chapter can be applied to problems in bioanalytical and medicinal chemistry. Although the theoretical basis for ACE is general and applicable to most receptor–ligand pairs, the aforementioned limitations still prevent wider application of the technique. Despite these limitations, CE is well suited for fundamental studies of the roles of electrostatic interactions in protein–ligand complexes. We believe this technique will permit detailed studies of electrostatic influences in biomolecular recognition using the CA–sulfonamide and other model systems.

ADDENDUM

After submission of this chapter, several advances have been reported in ACE that have broadened its application in studying the influence of electrostatics on molecular recognition. Mammen et al. have applied the principles of ACE to examine shielding of the charged capillary surface by small ions in solution (57). CE was used to measure the rate of EOF as a function of the concentration of various monovalent and divalent cations in aqueous solution. A model was described that treats the interactions between cations in solution and negatively charged siloxide groups on a surface in terms of discrete dissociation equilibria with characteristic dissociation constants, then interprets these

dissociation constants in terms of well-recognized physical characteristics of the ions. In another example, Rao and coworkers used vancomycin and D-Ala-D-Ala (DADA)-containing ligands as a model system to examine the influence of electrostatics on the net charge of vancomycin and on the pK_a values of ionizable residues on vancomycin (58). They confirmed that the electrostatic interaction between the $-NH_2^+CH_3$ group of vancomycin and the $-CO_2^-$ group of DADA contributes ~1.4 kcal/mol to the free energy of binding. In addition, the interaction increases the value of pK_a of the N-terminal ammonium group from 7.1 to 8.8 and thus influences the net charge on vancomycin. These examples demonstrate that ACE/CE provides a useful tool with which to investigate electrostatic effects in physiologically relevent media.

In another application, ACE has been used to separate racemic mixtures of drugs via interactions with a chiral selector. For example, Liu and coworkers have used β-cyclodextrin and its derivatives as chiral selectors to distinguish water-soluble drug enantiomers (59). The separation was based on different affinities of the drug enantiomers for the cavity of the β-cyclodextrin. Tanaka et al. have used α_1-acid glycoprotein to separate a variety of racemic basic drugs (60). These applications demonstrate the power of ACE to analyze racemic drugs and may also be useful in examining the purity of drugs.

LIST OF ACRONYMS OR ABBREVIATIONS

Acronym or Abbreviation	Definition
ACE	affinity capillary electrophoresis
AGE	affinity gel electrophoresis
BCA	bovine carbonic anhydrase
CA	carbonic anhydrase
CE	capillary electrophoresis
DADA	D-Ala-D-Ala
DNP	*N*-dinitrophenol
EOF	electroosmotic flow
HCA	human carbonic anhydrase
HHM	horse heart myoglobin
IgG_{2b}	immunoglobulin G_{2b}
MO	mesityl oxide
MS	mass spectrometry
STI	soybean trypsin inhibitor
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (GM 51559). Milan Mrksich was supported by an American Cancer Society postdoctoral fellowship. Mathai Mammen was supported by an Eli Lilly predoctoral fellowship.

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CHAPTER

30

DETERMINATION OF PHYSICOCHEMICAL PARAMETERS BY CAPILLARY ELECTROPHORESIS

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30.1. INTRODUCTION

The extraordinary usefulness of electrophoretic techniques for the analysis of macromolecules was first demonstrated by Tiselius (1) with his elegant moving-boundary apparatus, which resolved human serum proteins into albumin and the four globulin fractions, α_1, α_2, β, and γ. This original discovery gave rise to a steady development of new instruments and techniques with ever-increasing resolution. Currently, two-dimensional electrophoresis, combined with sophisticated computer-image analysis, can

High Performance Capillary Electrophoresis, edited by Morteza G. Khaledi. Chemical Analysis Series, Vol. 146.
ISBN 0-471-14851-2

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