Using Affinity Capillary Electrophoresis To Determine Binding Stoichiometries of Protein–Ligand Interactions†

Yen-Ho Chu, Watson J. Lees, Adonis Stassinopoulos, and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115

Received February 17, 1994; Revised Manuscript Received May 25, 1994®

ABSTRACT: We have developed a new method utilizing affinity capillary electrophoresis (ACE) for the determination of binding stoichiometries in biochemical systems. Using the same concentration of a ligand in the sample and the electrophoresis buffer, the appearance of an inverted peak corresponding to the free ligand in the resulting electropherogram provides a criterion of binding of a ligand to its receptor protein. For both low (fast off rates) and high (slow off rates) affinity systems, analysis of the integration of free ligand peak in electropherograms as a function of the total concentration of a ligand in samples at constant concentration of receptor protein yields the binding stoichiometry of the ligand to the protein. Applications of this technique to studies of (i) the inhibition of carbonic anhydrases (CA, EC 4.2.1.1, from human and bovine erythrocytes) by 4-alkylbenzenesulfonamide **1**, (ii) the interaction of a monoclonal antibody to human serum albumin (anti-HSA) with its antigen HSA, and (iii) the binding of streptavidin (from *Streptomyces avidinii*) to biotin derivatives (monobiotinylated oligodeoxyribonucleotide **2**, fluorescein biotin, or Lucifer Yellow biotin) yield stoichiometries of 1:1, 1:2, and 1:4, respectively. For multivalent, tight-binding systems, this ACE method can readily separate stable intermediate species. This method is generally applicable to both tight- and weak-binding systems, requires only nanograms of proteins and ligands, involves no radioactive materials, and does not require changes in electrophoretic mobilities of receptor proteins upon binding with ligands. It thereby provides a rapid, sensitive, and convenient method for measuring binding stoichiometries of ligands to proteins.

This paper reports the use of affinity capillary electrophoresis (ACE) as a technique to determine stoichiometries of ligand binding to proteins. Binding interactions of ligands to receptor proteins are of fundamental importance in chemistry, biochemistry, and molecular medicine (Connors, 1987; Hulme, 1992). To understand the mechanism and specificity of these interactions, measurements of both binding stoichiometries and equilibrium constants are important. For example, the determination of stoichiometry of interrelationship between Bcl-2 and Bax proteins has begun to reveal the regulation of programmed cell death (Oltvai et al., 1993). Studies of stoichiometries on a signal transduction pathway involving a sensory protein Van S, a regulatory protein Van R, and structural genes will gain insights on molecular mechanisms of vancomycin resistance in bacteria (Walsh, 1993). For a new biochemical system, the stoichiometries of receptor–ligand interaction are valuable to measure concentrations of active receptors and to probe specificity (Biemann & Koshland, 1994; Fountoulakis et al., 1992; Boudier & Bieth, 1992).

Common methods in current use for determining stoichiometries of protein–ligand interactions are based on (i) separation of the receptor protein and its ligand by gel filtration (Hummel & Dreyer, 1962), (ii) changes in absorption or emission spectrum (Chriswell & Schilt, 1975; Meyer & Ayres, 1957) of the receptor protein upon binding (the mole ratio method), (iii) determination of the diffusion coefficient or the sedimentation coefficient of the titrated complex by analytical ultracentrifugation (Boudier & Bieth, 1992), or (iv) active site titration of enzyme by its inhibitor in the presence of substrate (Ehlers & Riordan, 1991). The Hummel–Dreyer method is only suited for binding systems in which the two components are of very different sizes. The mole ratio method is carried out in solutions but requires change in the spectrum of the receptor protein or the ligand upon binding. The method of analytical ultracentrifugation is useful for tight-binding macromolecule–macromolecule interactions, requires relatively large sample volumes and long analysis time, and involves a sophisticated instrument. The method of active site titration is not suited for proteins having no enzymatic activities.

We (Avila et al., 1993; Chu et al., 1992; Gomez et al., 1994) and others (Kraak et al., 1992) have recently demonstrated that affinity capillary electrophoresis (ACE) is a useful and sensitive method for measuring binding constants of ligands to proteins. We demonstrate that ACE is also applicable to the determination of binding stoichiometry. Capillary electrophoresis (CE) is a relatively new analytical technique that allows rapid (typically minutes), efficient ($\sim 10^6$ theoretical plates) separation of minute quantities (nanoliter injection volumes, picograms of proteins) of analytes such as nucleic acids, proteins, and polysaccharides (Engelhardt et al., 1993; Grossman & Colburn, 1992). CE measures the mobility of charged species under the influence of an electric field gradient. The mechanism of separation by this technique is well-established; the observed migration time of a species is determined by a combination of its electrophoretic mobility and the velocity of electroosmotic flow (EOF) (Grossman & Colburn, 1992). EOF is due to the low pK_a of the silanol groups on the wall of the fused silica capillary (Grossman & Colburn, 1992). The negatively charged walls attract positively charged ions that drag the electrophoresis buffer along. At pH values greater than 6, the EOF in capillaries of uncoated fused silica is sufficiently high to ensure a net migration of the electrophoresis buffer as a plug flow toward the cathode for most analytes, regardless of their charges (Grossman &

† This work was supported in part by NIH Grant GM 20011 to C.T.W. W.J.L. was a recipient of a fellowship from the Natural Sciences and Engineering Research Council of Canada.

* Corresponding author. Telephone: (617) 432-1715; Fax: (617) 432-2452.

® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

0006-2960/94/0433-10616$04.50/0

Colburn, 1992). The principle underlying ACE, a specific application of CE, is straightforward: the electrophoretic mobility of a receptor protein changes on binding to the charged ligand present in the electrophoresis buffer due to the change in its charge-to-mass ratio. Scatchard analysis of the change of the electrophoretic mobility of the receptor protein as a function of the concentration of the ligand in the electrophoresis buffer allows equilibrium constants to be determined (Chu et al., 1992).

We describe here an application of ACE in stoichiometry measurement of protein–ligand binding that is suited for studying a wide range of binding interactions, including those that are difficult to study using other methods.

MATERIALS AND METHODS

Materials. All chemicals used as internal standards for CE experiments in this study were of analytical grade and purchased from Sigma: dinitrophenylglycine (DNP-G), dinitrophenylaspartic acid (DNP-D), 2-iodobenzoic acid (S), and dansylglutamic acid (DNS-E). Fluorescein biotin and Lucifer Yellow biotin were obtained from Molecular Probes. Carbonic anhydrases II (EC 4.2.1.1) from bovine erythrocytes (BCA, p*I* 5.9; BCA, p*I* 5.4), human serum albumin (HSA), and streptavidin from *Streptomyces avidinii* were purchased from Sigma. A mouse monoclonal IgG antibody to HSA (anti-HSA) was purchased from Pierce. Concentrations of proteins and ligands used were determined gravitometrically, except that of anti-HSA and **2** were measured spectrometrically. Molecular masses of proteins used were as follows: BCA, 30 kDa (Botre et al., 1991; Chu et al., 1992); HSA, 67 kDa (Peters, 1985); streptavidin, 60 kDa (Green, 1990, 1970); anti-HSA, 150 kDa. The affinity ligand **1** was synthesized and purified following the protocol of Chu et al. (1992).

Synthesis of 5′-O-[[1-Hydroxy-3-[10-(N-biotinyl-3-aminopropyl)-1,4,7,10-tetraoxadecyl]glycer-2-yl]-phosphonyl]-d[CAGCTGGCACTACAG] (**2**). The first 15 deoxyribonucleotide residues of **2** (d[CAGCTGGCACTACAG]) were synthesized on an automated expedite nucleic acid synthesizer (Millipore) using the standard phosphoramidite chemistry on a 1-μmol scale. The biotin group was introduced at the 5′ end of this 15 mer through the use of a BioTEG phosphoramidite [2-cyanoethyl[(1-[(dimethoxytrityl)oxy]-[10-(*N*-biotinyl-3-aminopropyl)-1,4,7,10-tetraoxadecyl]glycer-2-yl]-*N*,*N*-diisopropylphosphoramidite] (Glen Research), containing a 15 atom spacer based on triethylene glycol. The coupling of the BioTEG phosphoramidite was carried out manually, using the double syringe method.

The 3′CPG (controlled pore glass)-15 mer was allowed to react with a mixture of tetrazole activator solution (Glen Research) (100 μL) and BioTEG phosphoramidite (60 mM, 100 μL), both in acetonitrile, for 1 h at ambient temperature. The resulting phosphite ester was treated with iodine/THF/H_2O/pyridine solution mixture (Glen Research) to yield the 3′-*O*-CPG-**2**-1-*O*-DMT (dimethoxytrityl) ester. This product was treated with concentrated NH_4OH (1 mL) for 16 h at 55 °C to cleave the oligonucleotide from the CPG support and deprotect the bases. The solution was filtered, and the resulting 3′-HO-**2**-1-*O*-DMT ester was precipitated by the addition of 1-butanol (1:9, v/v). The crude product was isolated by centrifugation (14000*g*, ambient temperature) and subsequently dried in a speed evacuator. The white powder was dissolved in triethylammonium acetate (TEAA) solution (100 mM, pH 7) and subjected to purification by HPLC on an analytical reverse-phase C18 column (Dynamax 300A, Rainin Instruments): a linear gradient from 10% to 40% acetonitrile in 0.1 M TEAA, pH 7.0, was used with a gradient time of 30 min. The desired product eluted at 24 min (70% of total area of peaks). The proper fractions were collected, combined, and dried in vacuo. The resulting residue was dissolved in 1% aqueous trichloroacetic acid for 3 min (to remove the DMT protecting group) and neutralized with NH_4-OH. HPLC analysis of the resulting solution showed complete conversion of the protected product to the final form **2**, which eluted at 14 min as a single peak. Gel electrophoresis of **2** on a 20% polyacrylamide gel, in the presence of 7 M urea, also showed one band. As expected, **2** has a longer retention time than the 15 mer lacking the biotin-spacer moiety, which elutes after 8 min. The total isolated yield of **2** was 40%.

CE Instrumentation. The capillary electrophoresis system used in this study was an automated Beckman Model 2210. The capillary tubing (Polymicro Technologies) was of uncoated fused silica with an internal diameter of 50 μm, a total length of 27 cm, and a length from inlet to detector of 20 cm. The conditions used for the measurement of binding stoichiometry are given in detail for each figure. The temperature for the CE experiments was kept at 20.0 ± 0.2 °C. The duration of pressure injection of samples was 3 s.

General ACE Procedures Used in Measurement of Binding Stoichiometry of Slow Off Rate Systems. Using constant concentrations of the receptor protein and an internal standard, samples of various concentrations of the ligand in the buffer were prepared and injected into a capillary containing the electrophoresis buffer with no ligand present. Determination of the integration of normalized free ligand was straightforward: peaks of free ligand and of the internal standard in electropherograms were cut and weighed. A sharp change in the slope of the plot of the integration of the normalized free ligand peak vs the total ligand concentration for each sample allowed determination of the binding stoichiometry.

General ACE Procedure Used in Measurement of Binding Stoichiometry of Fast Off Rate Systems. Procedures similar to those in slow off rate systems were followed, except that the ligand was included in the running buffer, and the concentrations of the ligand in both samples and running buffer were high enough to fully saturate the receptor protein in samples (before injection) as well as in the capillary (after injection) during the experiments. The integration of the peak at the electrophoretic mobility of the ligand in electropherograms thereby represented the concentration of the free ligand present in samples. The zero value for integration of the peak area of normalized free ligand vs total ligand concentration determined the total ligand concentration of a sample where free ligand concentration was identical to the ligand concentration present in the running buffer. The bound ligand concentration was thus obtained, and the binding stoichiometry of the system was determined.

RESULTS AND DISCUSSION

Weak-Binding Systems (Fast Off Rates): Principle. Using the ACE method previously developed for measurements of equilibrium constants (Chu et al., 1992), we studied protein–ligand interactions of low affinities (fast on–off regimes) and demonstrated that this ACE assay of binding stoichiometry is suited for weak-binding systems. The method is based on the simple interaction of eq 1. In order to simplify the

$$R{\cdot}L_n \overset{K_d}{\rightleftarrows} R + nL \qquad (1)$$

determination of binding stoichiometry (n), the receptor protein (R) is kept in its fully bound form by using concentrations of the ligand (L) much greater than the dissociation constant (K_d), both in samples and in the

electrophoresis buffer (i.e., $[L]_{sample}$ and $[L]_{buffer} \gg K_d$). In electropherograms, the integration of the peak at the migration time of the ligand L represents the concentration of the free ligand ($[L]_{free}$). Zero integration in the plot of the area of the free ligand peak vs the total ligand concentration present gives a value of ligand ($[L]_{sample}$) in a sample where the free ligand concentration equals the concentration of the ligand in the electrophoresis buffer (i.e., $[L]_{free} = [L]_{buffer}$). The difference between the total and free ligand concentrations is the amount of the ligand associated with the receptor ($[L]_{bound} = [L]_{sample} - [L]_{buffer}$). The concentration ratio of the bound ligand to the receptor yields the binding stoichiometry ($n = [L]_{bound}/[R]$).

Weak-Binding Systems (Fast Off Rates): Examples. From Figure 1, we determine that bovine carbonic anhydrase B (CAB) binds the arylsulfonamide affinity ligand **1** (see Chart 1) with a stoichiometry of 1:1. In Figure 1A, the inflection point from the negative area of free **1** to the positive area is between 69 and 73 μM. A plot of the normalized area of free **1** vs its total concentration is shown in Figure 1B. The zero area of free **1** (i.e., zero absorbance in the y-axis) is the stoichiometric titration point and can be read on the horizontal axis as 71 μM. To obtain the stoichiometry n involves subtracting 67 μM (concentration of **1** in buffer) from 71 μM and dividing the resulting 4 μM by the concentration of CAB (4.2 μM in the experiment) to yield a stoichiometry of 0.9. Under the experimental conditions, the protein CAB was fully saturated (K_d = 2 μM) (Chu et al., 1992) and in rapid equilibrium with ligand **1** (Maren, 1992). In Figure 1, the determination of stoichiometry involves the subtraction of two concentrations of the ligand ($[L]_{sample} - [L]_{buffer}$). To access the experimental error from this subtraction, we measured the binding stoichiometry of the protein CAB to a control ligand, dinitrophenylglycine, which does not interact with the protein, and determined the stoichiometry to be zero. We initially anticipated instrumental error in injection volumes and used an internal standard to compensate for it. Experimentally, we found no significant difference both in the presence and absence of an internal standard in integrating the peak area of free **1** and in measuring binding stoichiometry (within 10%).

Tight-Binding Systems (Slow Off Rates): Principle. In cases of high-affinity systems such as antibody–antigen and streptavidin–biotin interactions (Figures 2A, 3A, and 4A) where off rates from $R{\cdot}L_n$ complexes are slow, for each mole of the receptor protein in the sample solution, the addition of n moles of a ligand results in the formation of exactly 1 mol of the complex (eq 1). Using CE, samples having a fixed concentration of a receptor protein and various concentrations of the ligand are prepared and injected into a capillary containing the electrophoresis buffer alone. In electropherograms as the concentration ratio $[L]_{total}/[R]$ increases, the concentration of the complex peak increases until $[L]_{total}/[R] = n$; beyond this value no more complex can form because all the receptor protein has been fully titrated and associated with the ligand. Excess ligand will migrate at the free ligand position. An abrupt change in the slope, in a plot of the integration of free ligand vs the ratio of $[L]_{total}/[R]$ in samples, corresponds to the binding stoichiometry n of the system studied (Figures 2A, 3A, and 4A) (Rose, 1993).

Tight-Binding Systems (Slow Off Rates): Examples. It is well-established that monoclonal antibodies possess two antigen-binding sites. The results (Figure 2) on binding interactions of a monoclonal anti-human serum albumin (anti-HSA) to its antigen HSA showed that the antibody elutes readily from the untreated fused silica capillary under the

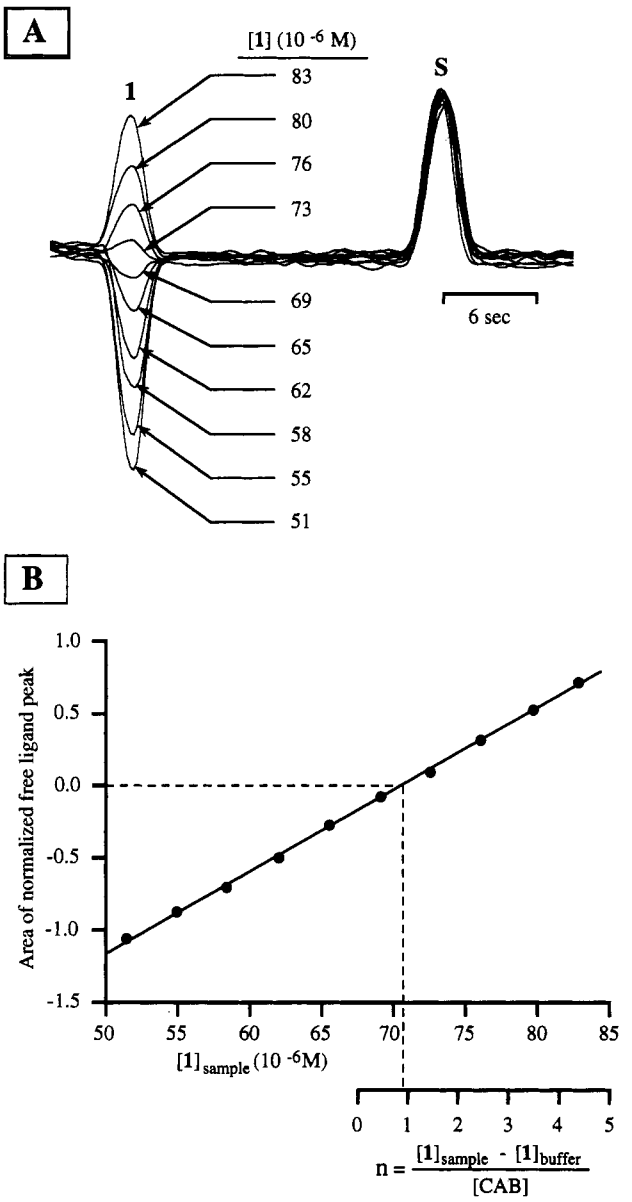
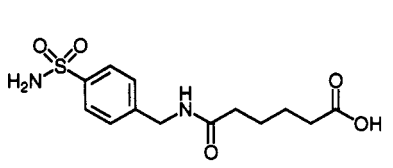


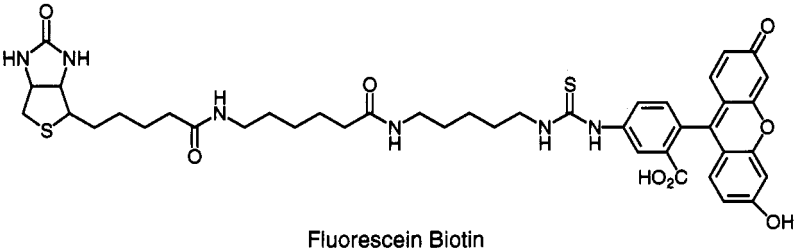
FIGURE 1: Determination of binding stoichiometry of a low affinity system: the carbonic anhydrase–arylsulfonamide **1** interaction. (A) Affinity capillary electrophoresis of samples having a constant concentration of bovine carbonic anhydrase B (CAB, 4.2 μM) and various concentrations of affinity ligand **1** ($[\mathbf{1}]_{sample}$ = 51, 55, 58, 62, 65, 69, 73, 76, 80, and 83 μM) in 192 mM glycine–25 mM Tris buffer (pH 8.3) containing $[\mathbf{1}]_{buffer}$ = 67 μM. The total electrophoresis time in each experiment was 2.5 min at 12 kV using 200 nm as the detection wavelength. 2-Iodobenzoic acid (S) was used as the internal standard for integration calibration. (B) The graph is the experimental data according to panel A. Zero peak area of **1** in the plot determined the total ligand concentration ($[\mathbf{1}]_{sample}$ = 71 μM) of a sample having free ligand concentration identical to the ligand concentration present in the running buffer ($[\mathbf{1}]_{free} = [\mathbf{1}]_{buffer}$ = 67 μM). The ratio of ($[\mathbf{1}]_{sample} - [\mathbf{1}]_{buffer}$)/[CAB] gave the binding stoichiometry n: (71–67)/4.2 = 0.9.

experimental conditions and associates tightly with its antigen to form complexes having different electrophoretic mobilities, with a final stoichiometry ratio of 1:2. In Figure 2A, the concentration of free anti-HSA decreases accompanied by the increases of new peaks representing complexes (1:1 and 1:2 stoichiometries) when the ratio of [HSA]/[anti-HSA] increases. When the appearance of the free HSA peak is detected (upper five traces of Figure 2A), it indicates that the anti-HSA (0.33 μM) has been fully titrated by its antigen (electropherograms of [HSA] > 0.76 μM in Figure 2A). Since the complexes do not dissociate significantly under these

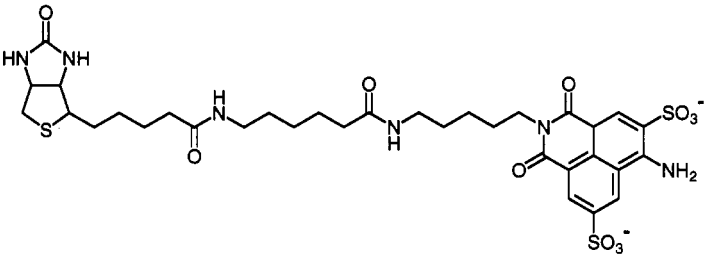
Chart 1



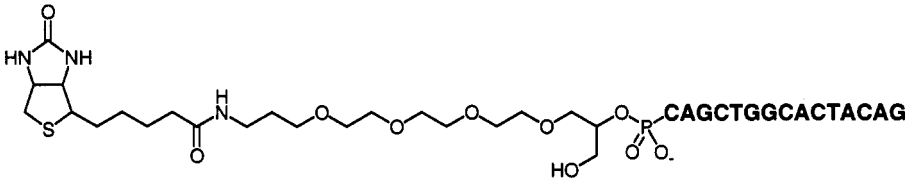
1



Fluorescein Biotin



Lucifer Yellow Biotin



2

conditions, a plot of the integration of the free HSA peak vs the ratio of [HSA]/[anti-HSA], with the concentration of anti-HSA held constant ([anti-HSA] = 0.33 μM), gives a horizontal line of zero concentration of free HSA ([HSA] < 0.61 μM), breaks sharply at a point (0.65 μM) corresponding to the binding stoichiometry n, and rises steeply as a straight line that follows the Beer–Lamberts law ([HSA] > 0.76 μM). Here n clearly equals 2 (0.65/0.33 = 1.9).

We also examined streptavidin–biotin interactions and obtained the expected binding stoichiometry of 1:4 by CE. Streptavidin from *S. avidinii* is an acidic (p*I* 5.0), tetrameric protein that binds four biotin molecules with exceptionally high affinity ($K_d = 4 \times 10^{-14}$ M and $t_{1/2}$ = 2.9 days at pH 7) (Green, 1990, 1970). Fluorescein biotin and Lucifer Yellow biotin were used initially as ligands for streptavidin because of their high molar extinction coefficients and commercial availability. Although correct 1:4 stoichiometries were determined upon using these chromophoric biotins, peak shapes of partially saturated stretptavidin species were somewhat broad and unresolved. Detectable, but small, changes in electrophoretic mobility of streptavidin were observed upon binding with its ligands (Figure 3). We rationalized this minimal mobility shift as a result of insufficient contribution of charges from biotins to the resulting streptavidin–biotin complex (i.e., if the protein binds a charged ligand of relatively small mass, the change in electrophoretic mobility of the protein due to the change in mass is small relative to the change in electrophoretic mobility due to the change in charge) (Chu et al., 1992; Chu & Whitesides, 1992) and decided to prepare a highly charged derivative of biotin, the monobiotinated oligonucleotide (**2**). As shown in Figure 4A, excellent peak shapes of streptavidin species that associated with **2** were obtained, and a complete transition of the protein states from its free form through intermediate loading states to its fully saturated species was observed. Using dinitrophenylaspartic acid (DNP-D) as the internal standard for integration calibration, the binding stoichiometry n = 4 was determined from a sharp change of slope in a plot of the concentration of free ligand **2** vs the ratio of [**2**]/[streptavidin] (Figure 4B).

Tight-Binding Systems (Slow Off Rates): Detection of Stable Intermediates. One qualitative observation is important to understanding the interactions of ligands with high affinities to their receptor proteins. Using CE, stable intermediate species are separated and obtained upon titrating the receptor protein with its ligand for binding systems of n > 1 (Figures 2 and 4). We propose, without definitive evidence, that the intermediate in Figure 2A is the 1:1 complex of anti-HSA and HSA, and intermediates 1–3 in the streptavidin-biotinated oligonucleotide **2** interactions are 1:1, 1:2, and 1:3 complexes, respectively (Figure 4A). Because stable intermediates are always observed and separated between peaks of free receptor protein and its free ligand in electropherograms (i.e., electrophroetic mobility:free ligand > intermediates, the final $R{\cdot}L_n$ complex > free protein), these species should not interfere with the determination of binding stoichiometry n. The number of stable intermediate species observed in capillary electropherograms on protein–ligand interactions further supports the correct measurement of their binding stoichiometries.

Conclusion. In summary, we have developed methods using ACE for the determination of binding stoichiometries of proteins to ligands. Although adsorption on the wall of the uncoated capillary is a potential problem for proteins (Engelhardt et al., 1993; Grossman & Colburn, 1992), any system comprised of different electrophoretic mobilities for the receptor protein and the ligand can, in principle, be examined by this method. We have demonstrated that these ACE protocols are applicable to binding systems of both low and high affinities (Figures 1–4). For systems of intermediate affinities, when half-lifes of protein–ligand complexes are compatible with electrophoresis times, the conditions under which electrophoresis experiments are performed can be

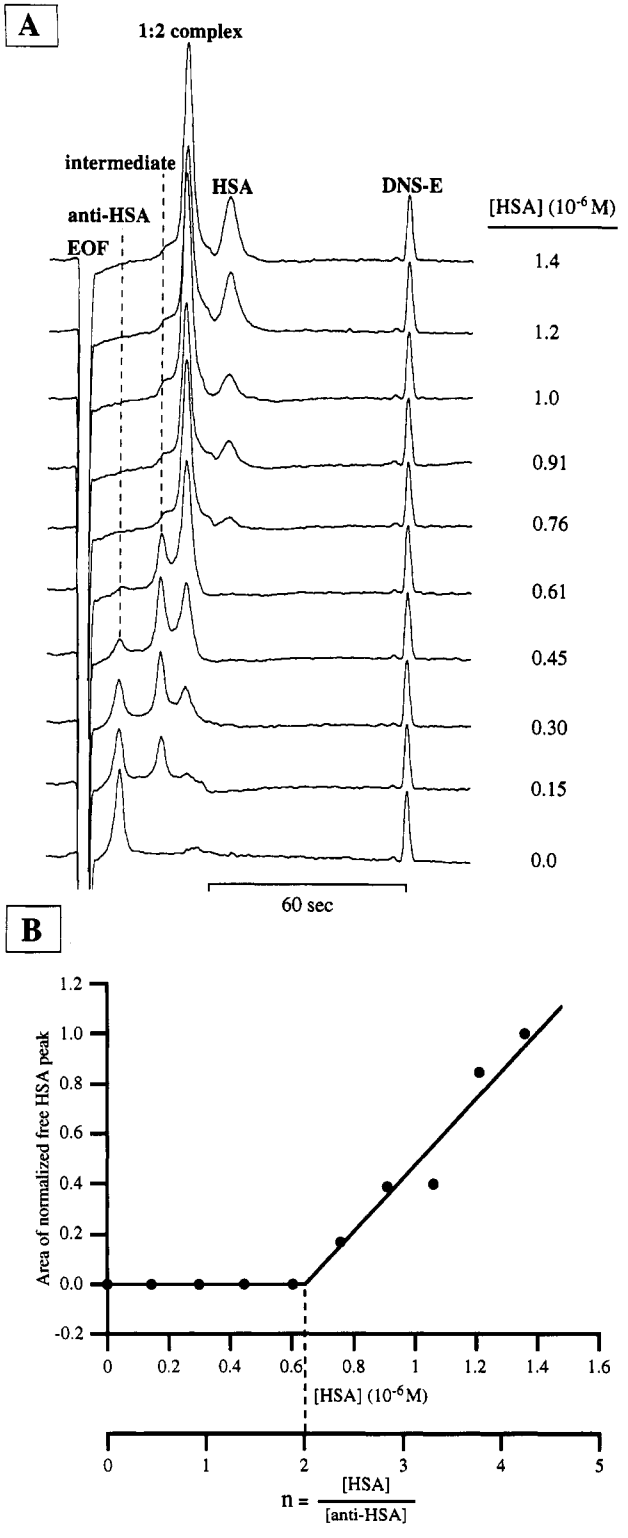


FIGURE 2: Determination of the binding stoichiometry of human serum albumin (HSA) to its mouse monoclonal IgG antibody (anti-HSA) using affinity capillary electrophoresis in 192 mM glycine–74 mM Tris buffer (pH 8.7). (A) The total electrophoresis time in each experiment was 4.0 min at 10 kV using 200 nm as the detection wavelength. The concentration of anti-HSA used in these experiments was 0.33 μM, and the HSA concentrations studied were 0.0, 0.15, 0.30, 0.45, 0.61, 0.76, 0.91, 1.0, 1.2, and 1.4 μM. DNS-E was dansylglutamic acid used as an internal standard. We tentatively assign the intermediate species to be the 1:1 complex. (B) A plot of the concentration of free ligand vs the ratio of [HSA]/[anti-HSA] gives a sharp break at the stoichiometric point as described in the text.

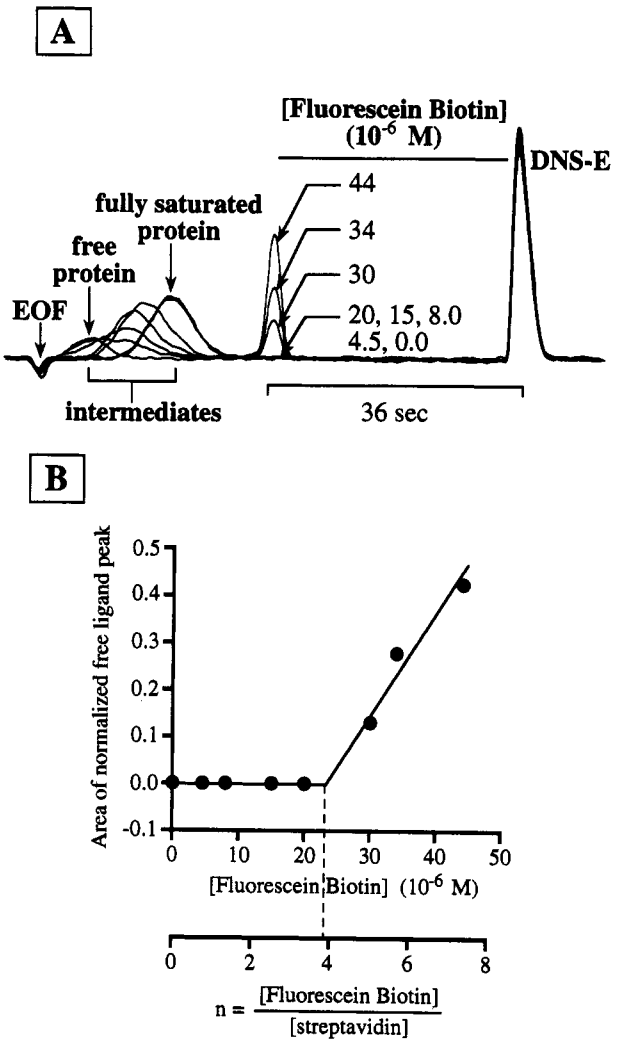


FIGURE 3: Determination of the binding stoichiometry of streptavidin–fluorescein biotin interaction. (A) Affinity capillary electrophoresis of samples having a constant concentration of streptavidin (5.8 μM) from *S. avidinii* and various concentrations of fluorescein biotin (0.0, 4.5, 8.0, 15, 20, 30, 34, and 44 μM) in 192 mM glycine–25 mM Tris buffer (pH 8.3). The total electrophoresis time in each experiment was 5 min at 10 kV using 280 nm as the detection wavelength. Dansylglutamate (DNS-E) was used as the internal standard for integration calibration. (B) The graph is the experimental data according to panel A.

adjusted (voltage, length of capillary, pH, or concentration of electrophoresis buffer) so that these systems can be analyzed using the protocol for either weak-binding or tight-binding systems. This method requires no change in absorption or emission spectra upon binding, which clearly offers distinct advantages over both the mole ratio method and the method of continuous variations as widely used techniques for identifying complexes in solutions (Huang, 1982; Chriswell & Schilt, 1975; Meyer & Ayres, 1957). This ACE stoichiometry assay is experimentally straightforward and versatile and has several advantages over the conventional Hummel–Dreyer assay (Hummel & Dreyer, 1962), which is limited to macromolecule–low molecular weight ligand interactions with poor resolution and requires large amounts of materials and long separation times. ACE requires only small quantities of proteins and ligands:[1] the complete series of experiments in Figure 1, 2, and 4 consumed 11.1 ng of carbonic anhydrase

[1] Stoichiometry of protein binding with an electrically neutral ligand can, in principle, be determined by measuring the free protein peak (proteins are charged in most cases). In systems of slow off rate (low K_d), this method consumes very small amounts ($\sim$10 ng) of proteins. For systems of fast off rates (high K_d), this method will likely consume larger but still acceptable amounts ($\sim$100 μg) of proteins, since it requires adding the protein into the electrophoresis buffer.

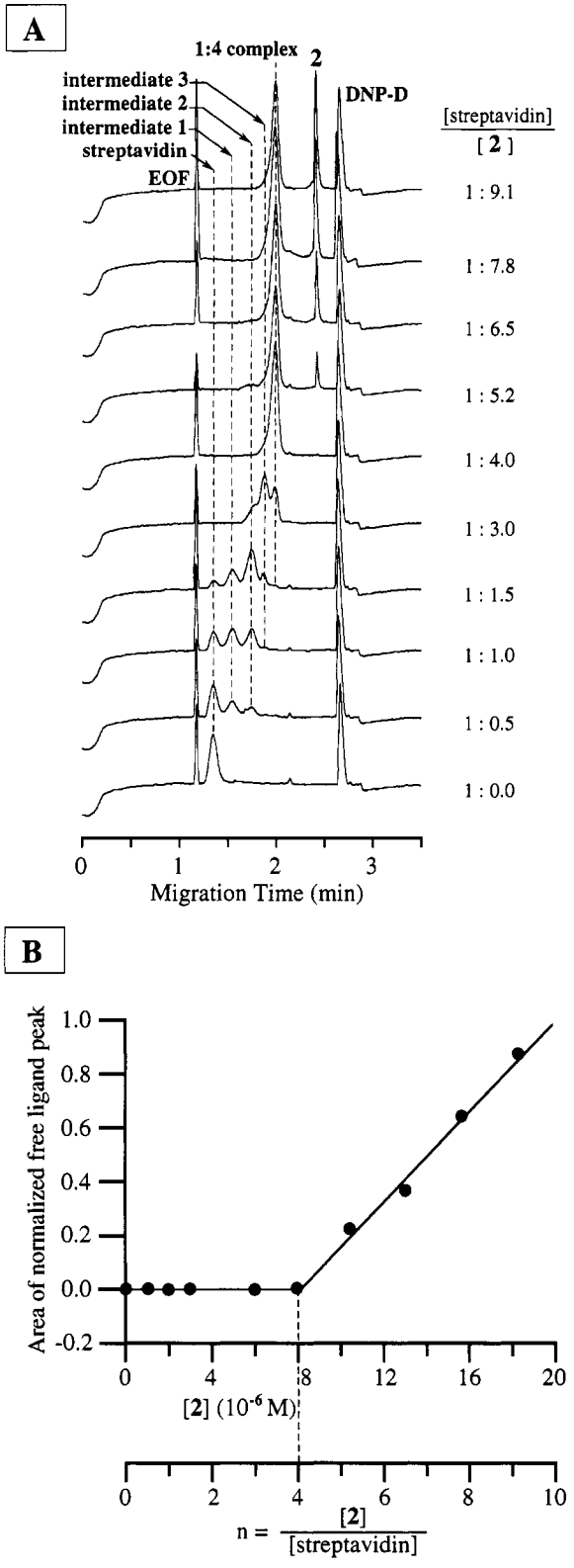


FIGURE 4: Measurement of the binding stoichiometry (n) of streptavidin–biotin **2** interactions in 192 mM glycine–96 mM Tris buffer (pH 8.9) by affinity capillary electrophoresis. (A) The total electrophoresis time in each experiment was 3.5 min at 15 kV using 214 nm as the detection wavelength. In sample preparations, the concentration of streptavidin used was 1.7 μM, and the concentrations of **2** studied were 0.0, 0.89, 1.7, 2.5, 5.1, 6.8, 8.9, 11, 13, and 16 μM. DNP-D was dinitrophenylaspartic acid used as the internal standard. We propose that intermediates 1–3 were the streptavidin–**2** complexes of 1:1, 1:2, and 1:3 stoichiometric ratios, based on their relative electrophoretic mobilities. (B) The graph is a plot of the experimental data in panel A.

and 2.1 ng of **1**, 5.0 ng of anti-HSA and 4.5 ng of HSA, and 10.2 ng of streptavidin and 3.4 ng of **2**, respectively. The assay is rapid: the individual run time for each experiment shown in Figure 1 was 2.5 min; the total analysis time of 10 data points used in Figure 1 was finished in 30 min. Because capillary electrophoresis is a high-resolution technique, this affinity method allows detection and, in principle, characterization of stable intermediates in the binding process. This ACE assay have obvious applications in stoichiometry measurement where the receptor molecule coelutes with the ligand (protein–protein interaction, for example) from a gel filtration column that interferes with measurements of the ligand.

ACKNOWLEDGMENT

We thank Dr. Bernard Cuenoud for his assistance and advice regarding the preparation of **2** and Dr. Ahamindra Jain for his helpful discussion of the manuscript.

REFERENCES

Avila, L. Z., Chu, Y.-H., Blossey, E. C., & Whitesides, G. M. (1993) *J. Med. Chem. 36*, 126–133.

Biemann, H.-P., & Koshland, D. E., Jr. (1994) *Biochemistry 33*, 629–634.

Botre, F., Gros, G., & Storey, B. T. (1991) *Carbonic Anhydrase: From Biochemistry and Genetics to Physiology and Clinical Medicine*, VCH Publishers, New York.

Boudier, C., & Bieth, J. G. (1992) *J. Biol. Chem. 267*, 4370–4375.

Chriswell, C. D., & Schilt, A. A. (1975) *Anal. Chem. 47*, 1623–1629.

Chu, Y.-H., & Whitesides, G. M. (1992) *J. Org. Chem. 57*, 3524–3525.

Chu, Y.-H., Avila, L. Z., Biebuyck, H. A., & Whitesides, G. M. (1992) *J. Med. Chem. 35*, 2915–2917.

Connors, K. A. (1987) *Binding Constants: The Measurement of Molecular Complex Stability*, John Wiley & Sons, Inc., New York.

Ehlers, M. R., & Riordan, J. F. (1991) *Biochemistry 30*, 7118–7126.

Engelhardt, H., Beck, W., Kohr, J., & Schmitt, T. (1993) *Angew. Chem. Int. Ed. Engl. 32*, 629–649

Fountoulakis, M., Zulauf, M., Lustig, A., & Garotta, G. (1992) *Eur. J. Biochem. 208*, 781–787.

Gomez, F. A., Avila, L. Z., Chu, Y.-H., & Whitesides, G. M. (1994) *Anal. Chem. 66*, 1785–1791.

Green, N. M. (1970) *Methods Enzymol. 18A*, 418–424.

Green, N. M. (1990) *Methods Enzymol. 184*, 51–67.

Grossman, P. D., & Colburn, J. C. (1992) *Capillary Electrophoresis: Theory and Practice*, Academic Press, San Diego.

Huang, C. Y. (1982) *Methods Enzymol. 87*, 509–525.

Hulme, E. C. (1992) *Receptor-Ligand Interactions, A Practical Approach*, Oxford University Press, New York.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Kraak, J. C., Busch, S., & Poppe, H. (1992) *J. Chromatogr. 608*, 257–264.

Maren, T. H. (1992) *Mol. Pharmacol. 41*, 419–426.

Meyer, A. S., & Ayres, G. H. (1957) *J. Am. Chem. Soc. 79*, 49–53.

Oltvai, Z. N., Milliman, C. L., & Korsmeyer, S. J. (1993) *Cell 74*, 609–619.

Peters, T., Jr. (1985) *Adv. Protein Chem. 37*, 161–245.

Rose, D. J. (1993) *Anal. Chem. 65*, 3545–3549.

Walsh, C. T. (1993) *Science 261*, 308–309.