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Journal of Chromatography A, 727 (1996) 291–299

JOURNAL OF CHROMATOGRAPHY A

Multiple-plug binding assays using affinity capillary electrophoresis

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Received 18 July 1995; revised 2 October 1995; accepted 9 October 1995

Abstract

This work evaluates the concept of a multiple-plug binding assay to estimate binding constants of proteins to ligands using affinity capillary electrophoresis (ACE). This concept is demonstrated using two model systems: carbonic anhydrase B (CAB, EC 4.2.1.1) and vancomycin from *Streptomyces orientalis*. Multiple plugs of protein, and non-interacting neutral and protein standards, are injected and analysis of the electrophoretic mobilities of the individual protein plugs, relative to the non-interacting neutral standard, as a function of the concentration of ligand yields values for their binding constants to the protein. These values agree well with those estimated using other assay and ACE techniques. This technique offers a new and expeditious approach to estimating binding constants of ligands to proteins.

Keywords: Affinity electrophoresis; Binding constants; Protein–ligand interactions; Multiple-plug binding assays; Proteins; Carbonic anhydrase B; Vancomycin; Mesityl oxide; Myoglobin

1. Introduction

This paper describes a multiple-plug binding assay for obtaining binding constants (K_b) of proteins for ligands using affinity capillary electrophoresis (ACE). We illustrate this method by analysis of the binding of carbonic anhydrase B (CAB, EC 4.2.1.1) to charged arylsulfonamides, and vancomycin to the dipeptide N-acetyl-D-Ala-D-Ala.

The characterization of specific interactions is the focus of much biochemical research [1]. There are a number of methods to measure binding parameters for non-covalent interactions; however, they are frequently based on the separation and quantitation of free or complexed molecules in an equilibrium mixture [2]. Assuming that there are methods for quantitating the amount of bound and free ligand in solution, these techniques all give unequivocal estimates of K_b. Capillary electrophoresis (CE) is a new technique that may often be used advantageously for studying protein–ligand interactions [3–6]. Recently ACE has been demonstrated to be a useful approach for the study of biomolecular non-covalent interactions and determination of the binding and dissociation constants of the complexes formed [4–29]. For example, binding of the SH3 domain of a tyrosine kinase to proline-rich peptides resulted in a shift in mobility of the SH3 domain upon increasing concentration of the peptide in the running buffer [6]. Affinity interaction between protein–protein [7–9], protein–DNA [1,10], protein–drug [4,11–17], protein–carbohydrate [18,19], peptide–peptide [20–25],

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SSDI 0021-9673(95)01155-2

peptide–carbohydrate [26], peptide–dye [27], carbohydrate–drug [28], and antibody–antigen [29] have also been demonstrated.

In ACE, changes in the electrophoretic mobility μ_P of a protein (P) on complexation with a ligand (L) present in the buffer can be correlated to the binding constant K_b [16,30–32]. Analysis of the magnitude of the change in mobility $\Delta\mu_{P,L}$ as a function of the concentration [L] of ligand yields K_b (Eq. 1 is used for Scatchard analysis).

$$\Delta\mu_{P,L}/[L] = K_b\Delta\mu_{P,L}^{max} - K_b\Delta\mu_{P,L} \quad (1)$$

ACE has several advantages as a method for measuring biomolecular non-covalent interactions. First, it requires only small quantities of both protein and ligand. Second, purification of the sample prior to injection is not necessary as long as the component to be analyzed can be separated from other species. Third, it does not require radiolabelled or chromophoric ligands. Fourth, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient.

In this paper, we test the hypothesis that binding constants between ligands and proteins may be obtained in a series of ACE experiments by injecting multiple plugs (Fig. 1) of sample containing both protein and non-interacting standards followed by analysis of each individual protein plug over a series of increasing ligand concentrations.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade. 4-Carboxybenzenesulfonamide (**2**), carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes), vancomycin from *Streptomyces orientalis*, N-acetyl-D-Ala-D-Ala (**4**), and horse heart myoglobin were purchased from Sigma (St. Louis, MO, USA) and used without

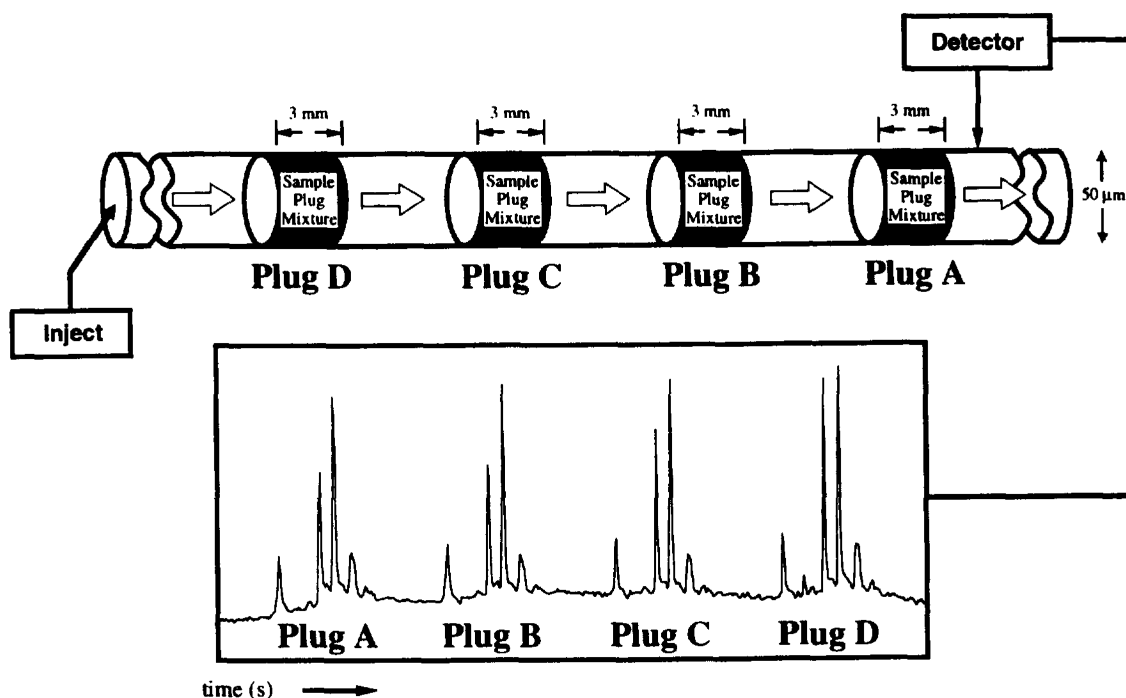

Fig. 1. Schematic of a multiple-plug binding assay.

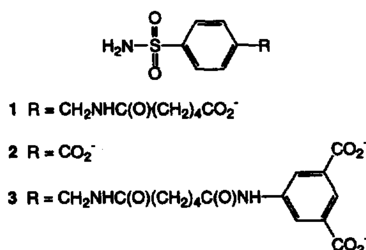

Fig. 2. Structures of compounds **1-3**

further purification. Mesityl oxide was purchased from Calbiochem (San Diego, CA, USA). Compounds **2** and **3** (see Fig. 2) were gifts of J. Gao. Stock solutions (1 mg/ml) of bovine carbonic anhydrase B, horse heart myoglobin (1 mg/ml), and vancomycin (4 mg/ml) were each prepared by dissolving the lyophilized protein in buffer (192 m*M* glycine–25 m*M* Tris; pH 8.3).

2.2. Apparatus

The capillary electrophoresis (CE) system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was of uncoated fused-silica with an internal diameter of 50 μm, length from inlet to detector of 60.5 cm, and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 30 kV; current, 5.2 μA; detection, 200 nm; temperature, 25±2°C.

2.3. Procedures

A sample (3.6 nl) of solution containing 0.2 mg/ml of carbonic anhydrase B, 0.1 mg/ml of horse heart myoglobin, and 0.08 mg/ml of mesityl oxide in buffer was introduced into the capillary by vacuum injection. The electrophoresis was carried out using a Tris–Gly buffer and appropriate concentrations of the arylsulfonamide ligand (0–120 μM). For vancomycin, a sample (3.6 nl) of solution containing 0.14 mg/ml of vancomycin, 0.2 mg/ml of carbonic anhydrase B, and 0.17 mg/ml of mesityl oxide in buffer was introduced into the capillary by vacuum injection. The electrophoresis was carried out using a sodium phosphate buffer and appropriate concentrations of the N-acetyl-D-Ala-D-Ala ligand (0–1150 μM).

3. Results and discussion

A typical form of ACE frequently requires multiple experimental runs at a series of increasing ligand concentrations prior to obtaining a binding constant. This allows for the establishment of constant electroosmotic flows and consistent peak migration times. This type of experiment can be time-consuming depending on the length of time required to obtain a stable electroosmotic flow and/or the length of each experimental run. Hence, the evaluation of binding constants of ligands to proteins is slowed. Our goal was to demonstrate the utility of a multiple-plug binding assay for evaluating binding constants of ligands to proteins, thereby circumventing the need for multiple runs at a given concentration of ligand in the buffer and yielding rapidly values of K_b.

We tested our multiple-plug binding assay using three types of experiments. In the first (E_1), four plugs of sample containing carbonic anhydrase B (CAB), horse heart myoglobin (HHM), and mesityl oxide (MO), were vacuum injected into the capillary. The first three plugs (A, B, and C) were individually electrophoresed for 1.5 min at 10 kV and terminated. The fourth sample (D) was injected and electrophoresed at 30 kV for 5.0 min. The sample plugs eluted A to D (Fig. 3). Measurement of the change in electrophoretic mobility using Eq. 1 due to complexation resulted in the values for binding constants given in Table 1. Correlation coefficients (r) and standard deviation (s) for the average K_b are also given in Table 1. Binding constants were obtained for three charged arylsulfonamides. The values for the binding constants agree well with previous estimates by ACE [16,24,33]. Fig. 2 shows a representative series of electropherograms of CAB in buffer containing various concentrations of **1**. Upon the addition of increasing concentrations of **1** in the running buffer the four CAB peaks shift to the right. The complexation between **1** and CAB resulted in an increased negative charge and the complex is detected later than the uncomplexed form. Peak

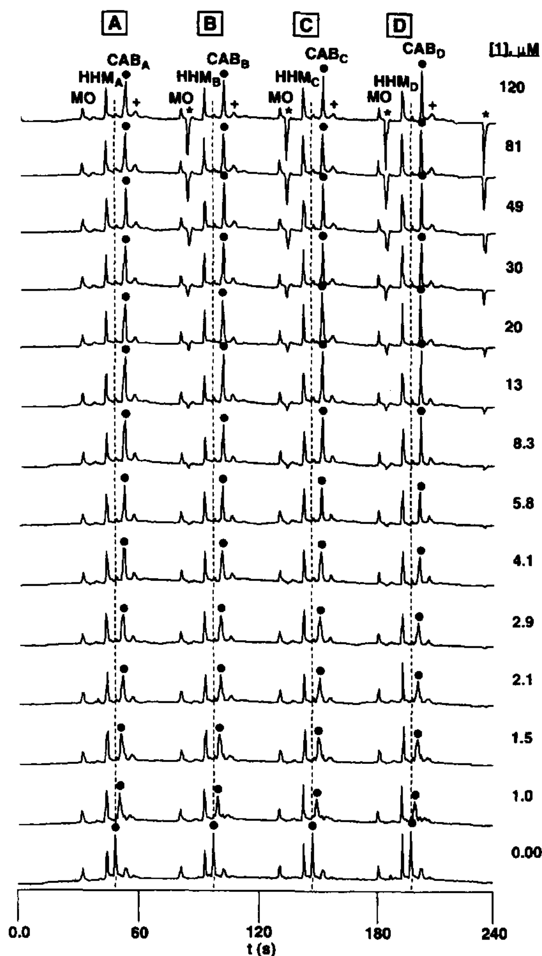

Fig. 3. Representative set of electropherograms of carbonic anhydrase B (CAB) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **1** using the multiple-plug binding assay. The total analysis time in each experiment was 5.0 min at 30 kV (current: 5.2 μA) using a 60.5 cm (inlet to detector), 50 μm I.D. open, uncoated quartz capillary. Horse heart myoglobin (HHM) and mesityl oxide (MO) were used as internal standards. The subscripts A–D following CAB, and HHM refer to plugs A–D, respectively. The inverted peaks (*) and the peaks denoted by (+) are discussed in the text.

broadening was observed at intermediate concentrations. This is generally caused by the retardation of migrating molecules due to their frequent interactions with the ligand in the region of intermediate status [20]. The CAB peaks become sharper at the saturating concentrations of the ligand. The neutral marker, mesityl oxide (MO), as a component in the sample mixture, retains a constant migration time for all four sample plugs. The inverted peaks (*) resulted from the dilution of **1** present in the electrophoresis buffer. CAA (+), a protein having binding constants very similar to CAB, gives values of K_b by the multiple-plug binding assay that are indistinguishable from CAB. Fig. 4 shows Scatchard plots of the data for the four sample plugs of CAB. An increased correlation between ligand concentration and change in electrophoretic mobility $\Delta\mu$ is observed from sample injection A to D. This is seen in higher correlation coefficients for CAB_C and CAB_D than for CAB_A and CAB_B (Table 1). This general trend is observed for all three arylsulfonamide ligands.

Fig. 5 shows the changes in electrophoretic mobilities of CAB and HHM for the four sample plugs on increasing the concentration of **1**. The horizontal line for HHM indicates that **1** has no affinity for HHM. An increased correlation between the electrophoretic mobility μ of CAB and the log of the concentration of **1** is observed from injection A to D. In other words, data acquired at short migration times give poorer correlations between ligand concentration and CAB peak mobilities than those obtained at longer migration times. Sample injections C and D yield binding constants more in agreement with those obtained by other ACE methods than do sample injections A and B.

In the second type of experiment (E_2), four plugs of sample were injected into the capillary. The first three plugs (A, B, and C) were individually electrophoresed for 0.90 min (**1** and **2**; 0.85 for **3**) at 30 kV and terminated. The fourth sample (D) was injected and electrophoresed for 5.0 min. A similar series of electropherograms as that observed for E_1 was obtained. The values for the binding constants agree well with E_1 and with previous estimates by ACE [16,24,33]. This proves that a variable electrophoresis voltage (in this case, artificially created by running at 10 kV in the first experiment, and 30 kV in the second) does not hinder the analysis and use of the multiple-plug binding assay. Binding constants were not obtained for all sample plugs. The large randomness of the data, particularly for plug A, did not permit analysis of this data set. One explanation

Table 1
Experimental values of binding constants K_b (10^6 M^{-1}) of ligands 1–3 and carbonic anhydrase B obtained by the multiple-plug binding assay

Ligand	Experiment	K_b (correlation coefficient r) for plug				Average K_b (standard deviation s)
		A	B	C	D	
1	E_1	0.68 (0.964)	0.79 (0.993)	0.75 (0.993)	0.74 (0.998)	0.74[a] (±0.04)
1	E_2	–[b]	0.53 (0.849)	0.62 (0.972)	0.66 (0.988)	0.60 (±0.05)
2	E_1	–[b]	0.78 (0.788)	1.19 (0.963)	1.05 (0.976)	0.99[c] (±0.20)
3	E_1	–[b]	–[b]	0.90 (0.881)	0.98 (0.986)	0.72[d] (±0.04)
3	E_2	0.99 (0.837)	0.83 (0.979)	0.88 (0.992)	0.72 (0.979)	0.84 (±0.10)

[a] Previous estimate [16]: K_b=0.60×10^6 M^{-1}.
[b] Not measured.
[c] Previous estimate [24]: K_b=0.80×10^6 M^{-1}.
[d] Previous estimate [33]: K_b=0.50×10^6 M^{-1}.

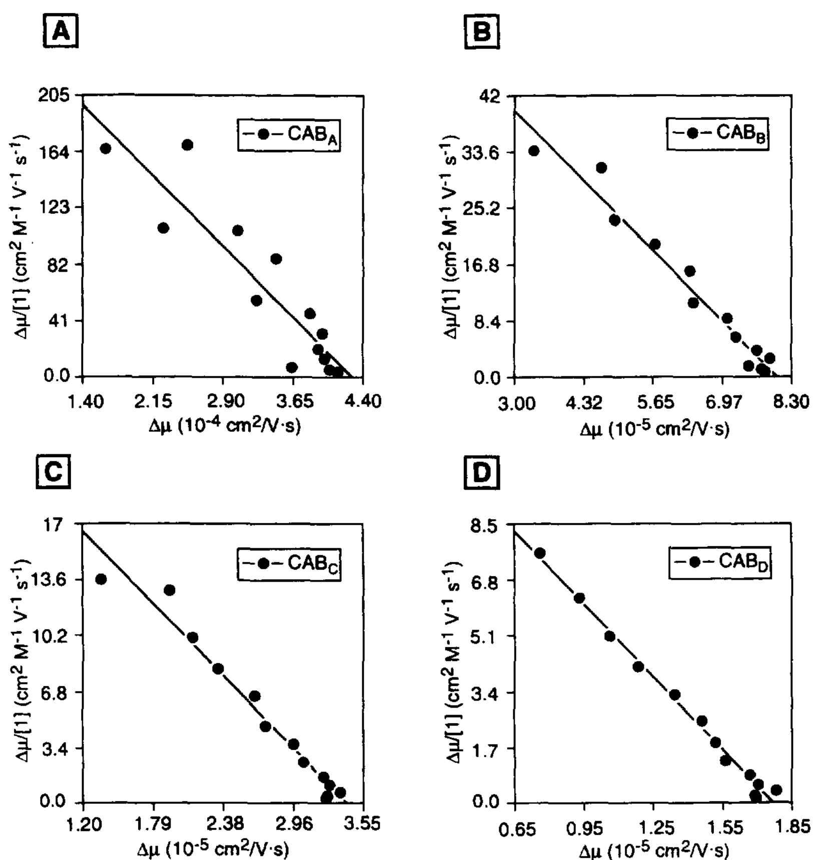

Fig. 4. Scatchard plots A–D of the data for carbonic anhydrase B according to Eq. 1.

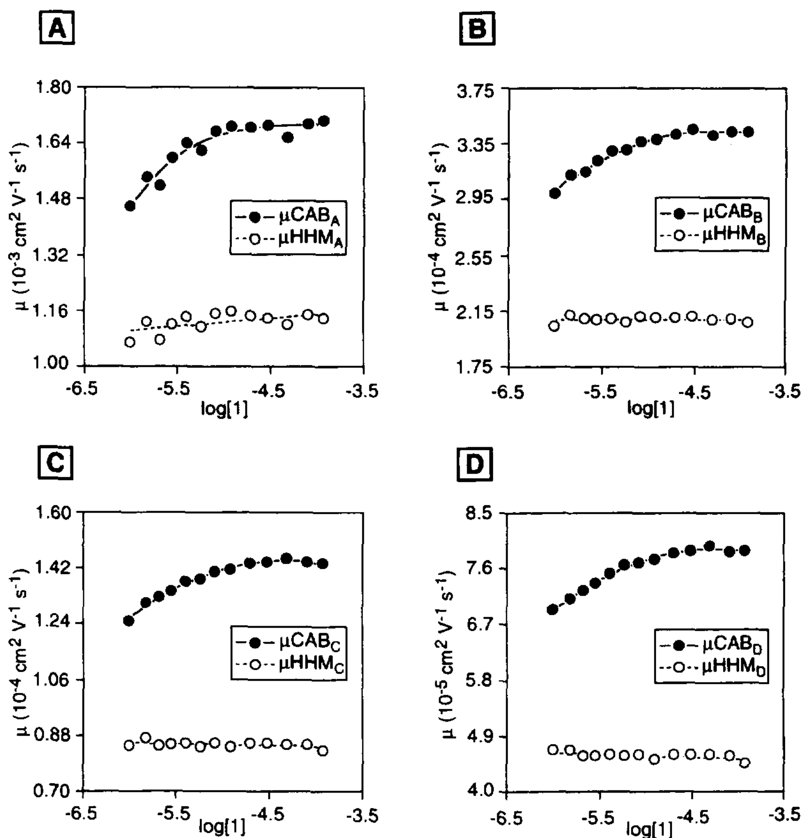

Fig. 5. Change in the electrophoretic mobilities of CAB and HHM on increasing the concentration of **1** for sample plug mixtures A–D.

is that the capillary wall had not reached equilibrium with the ligand in the running buffer thereby causing a heterogeneous ligand–protein interaction upon electrophoresis. Plug A is made diffuse by subsequent sample injections B, C and D. A more uniform interaction, as is observed in conventional ACE experiments, was eventually achieved for plugs B–D permitting analysis of the data. This effect is not limited to the multiple-plug binding assay but is also observed in conventional ACE techniques.

In the third type of experiment (E_3), one plug of sample was injected into the capillary and electrophoresed. Four repetitions were conducted and for each series of repetitions at increasing concentrations of ligand **1** a binding constant was obtained. This is the conventional form of ACE. In the present study, a minimum of twice the time is required to complete a binding study by this form of ACE as that observed in a multiple-plug binding assay. The average binding constant obtained was 0.76×10^6 M^{-1}. This agrees well with the average binding constants (0.74 and 0.60×10^6 M^{-1} for repetitions three and four, respectively; 0.64 and 0.77×10^{-6} M^{-1} for repetitions three and four, respectively, for ligand 2) obtained by the multiple-plug binding assay.

We used CAB as a model system for several reasons: (i) it does not absorb to the walls of uncoated capillaries; (ii) we have data describing its electrophoretic behavior in other circumstances; (iii) it is commercially available and inexpensive; (iv) ligands for it can be easily synthesized; (v) many ligands bind to it with values of K_b between 10^5 and 10^9 M^{-1}.

Similar types of experiments (E_2 and E_3) were

conducted with vancomycin and the dipeptide N-acetyl-D-Ala-D-Ala. Fig. 6 shows a representative series of electropherograms of vancomycin in buffer containing various concentrations of **4**. The interaction between **4** and vancomycin is weaker than CAB to arylsulfonamides and higher concentrations of ligand are required. These high concentrations of ligand induce changes in electroosmotic flow and sample peaks elute at increasing migration times with increasing amounts of ligand in the buffer. The inverted peaks (*) resulted from the dilution of **4** present in the electrophoresis buffer. Fig. 7 shows Scatchard plots of the data for the four sample plugs of vancomycin. Analysis by Eq. 1 yielded an average binding constant of 5.6×10^3 M^{-1} (Table 2). A conventional ACE experiment yielded an average binding constant of 5.2×10^3 M^{-1}. These values agree well with a previous ACE study which determined binding constants of 8.7 and 4.8×10^3 M^{-1} for the same dipeptide at pH 7.1 by two different methods and with those obtained from other assays for structurally similar compounds [20,34–36].

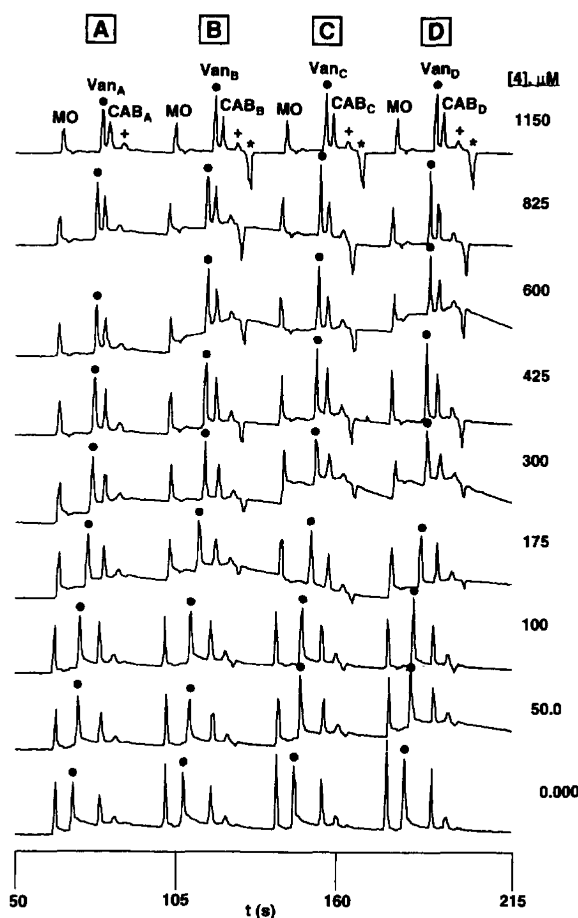

Fig. 6. Representative set of electropherograms of vancomycin (Van) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **4** using the multiple-plug binding assay. The total analysis time in each experiment was 4.0 min at 30 kV (current: 5.2 μA) using a 60.5 cm (inlet to detector), 50 μm I.D. open, uncoated quartz capillary. Carbonic anhydrase B (CAB) and mesityl oxide (MO) were used as internal standards. The subscripts A–D following Van, and CAB refer to plugs A–D, respectively. The inverted peaks (*) and the peaks denoted as (+) are discussed in the text.

We used vancomycin as a model system for several reasons: (i) it has been extensively studied using other techniques; (ii) there is a body of information on binding constants available for comparison with those estimated by ACE and the multiple-plug binding assay; (iii) there has been considerable interest in recent years in vancomycin owing to its novel mechanism of bacterial resistance.

We have preliminary data suggesting no limit in the number of binding constant values for the same interaction that may be obtained by the multiple-plug binding assay, although careful consideration in length of capillary and electrophoresis conditions are important. The most serious shortcoming of the technique at present is the requirement that the negative peak, caused by dilution of the ligand in the electrophoresis buffer, does not appear in a part of the electropherogram where it interferes with the migration and analysis of the protein peak in question.

4. Conclusion

Multiple binding constants between ligands and proteins may be estimated by a multiple-plug binding assay. We have shown this by the use of two model systems: CAB and arylsulfonamide ligands, and vancomycin and the dipeptide N-acetyl-D-Ala-D-Ala. The binding constants obtained by this procedure agree well with those obtained by other assay methods and by other ACE techniques. The multiple-plug binding assay has several advantages as a method for determining binding constants over other

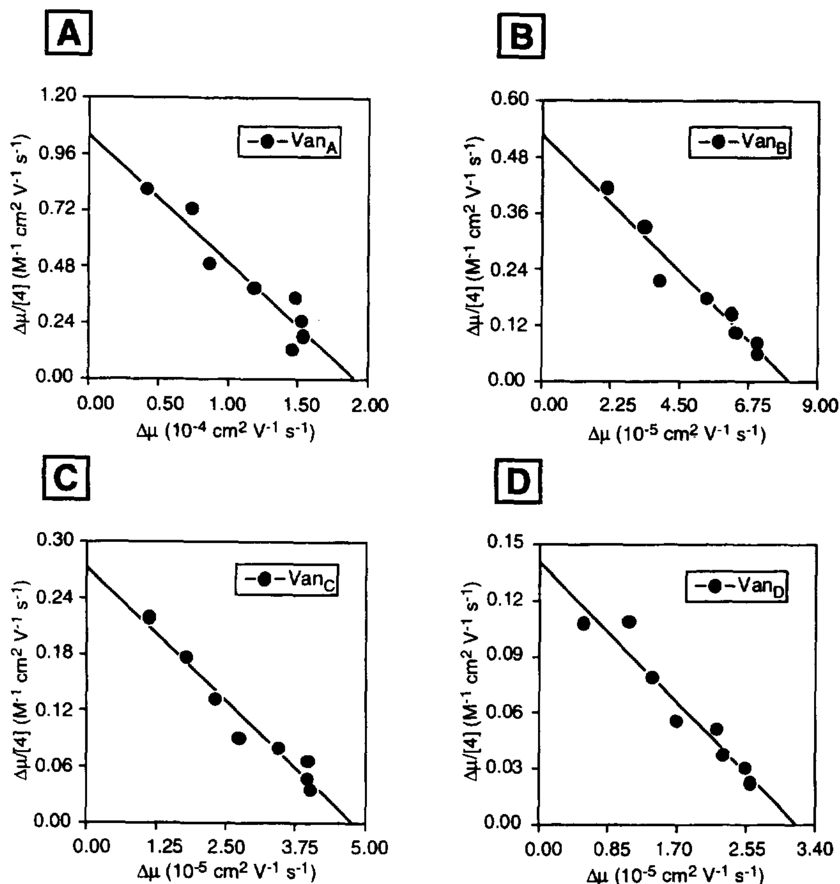

Fig. 7. Scatchard plots A–D of the data for vancomycin according to Eq. 1.

methods and conventional ACE techniques. First, it is expedient: much faster than previous forms of ACE. Second, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient. Third, the values of binding constants obtained from a number of sample plugs agree with conventional ACE techniques.

Table 2
Experimental values of binding constants K_b (10^3 M^{-1}) of ligand **4** and vancomycin obtained by the multiple-plug binding assay

Ligand	Experiment	K_b (correlation coefficient r) for plug				Average K_b (standard deviation s)
		A	B	C	D	
4	E_2	5.5 (0.978)	6.6 (0.989)	5.7 (0.990)	4.5 (0.984)	5.6[a] (±0.7)

[a] Previous estimates: K_b=8.7 and 4.8×10^3 M^{-1} obtained by the two ACE methods of Chu et al. [4]; K_b=16×10^3 M^{-1} obtained from the data of Popieniek and Pratt using a fluorescence-based assay in 100 mM phosphate buffer (pH 7.0) [34]; K_b=20×10^3 M^{-1} obtained from the data of Nieto and Perkins using a UV-difference binding assay in 20 mM citrate (pH 5.1) [35]; K_b=18×10^3 M^{-1} obtained from the data of Bugg et al. using a UV-difference binding assay in 20 mM citrate (pH 5.1) [36].

Acknowledgments

The authors gratefully acknowledge financial support for this research by a grant from the Research Corporation (Grant CC3957).

References

[1] N.H.H. Heegaard and F.A. Robey, Am. Lab., (1991) 28T.
[2] J.E. Bell and E.T. Bell, Proteins and Enzymes, Prentice-Hall, Englewood Cliffs, NJ, 1988.
[3] C.A. Monnig and R.T. Kennedy, Anal. Chem., 66 (1994) 280R.
[4] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck and G.M. Whitesides, J. Med. Chem., 35 (1992) 2915.
[5] L.Z. Avila, Y.-H. Chu, E.C. Blossey and G.M. Whitesides, J. Med. Chem., 36 (1993) 126.
[6] F.A. Gomez, J.K. Chen, A. Tanaka, S.L. Schreiber and G.M. Whitesides, J. Org. Chem., 59 (1994) 2885.
[7] Y.-H. Chu, W.J. Lees, A. Stassinopoulos and C.T. Walsh, Biochemistry, 33 (1994) 10616.
[8] K. Shimura and B.L. Karger, Anal. Chem., 66 (1994) 9.
[9] P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, H.H. Sittampalam and E.C. Rickard, Anal. Chem., 61 (1989) 1186.
[10] N.H.H. Heegaard and F.A. Robey, J. Lig. Chromatogr., 16 (1993) 1923.
[11] S. Busch, J.C. Kraak and H. Poppe, J. Chromatogr., 635 (1993) 119.
[12] L. Valtcheva, J. Mohammad, G. Pettersson and S. Hjerten, J. Chromatogr., 638 (1993) 263.
[13] R. Vespaleck, V. Sustacek and P. Bocek, J. Chromatogr., 638 (1993) 255.
[14] S. Busch, J.C. Kraak and H. Poppe, J. Chromatogr., 608 (1993) 257.
[15] G.E. Barker, P. Russo and R.A. Hartwick, Anal. Chem., 64 (1992) 3024.
[16] F.A. Gomez, L.Z. Avila, Y.-H. Chu and G.M. Whitesides, Anal. Chem., 66 (1994) 1785.
[17] L.Z. Avila, Y.-H. Chu, E.C. Blossey and G.M. Whitesides, J. Med. Chem., 36 (1993) 126.
[18] R. Kuhn, R. Frei and M. Christen, Anal. Biochem., 218 (1994) 131.
[19] S. Honda, A. Taga, K. Suzuki, S. Suzuki and K. Kakehi, J. Chromatogr., 597 (1992) 377.
[20] J. Liu, K.J. Volk, M.S. Lee, M. Pucci and S. Handwerger, Anal. Chem., 66 (1984) 2412.
[21] S. Handwerger, M. Pucci, K.J. Volk, J. Liu and M.S. Lee, J. Bacteriol., 176 (1994) 260.
[22] D.M. Goodall, Biochem. Soc. Trans., 21 (1993) 125.
[23] J.L. Carpenter, P. Camilleri, D. Dhanak and D. Goodall, J. Chem. Soc. Chem. Commun., (1992) 804.
[24] Y.-H. Chu and G.M. Whitesides, J. Org. Chem., 57 (1992) 3524.
[25] Y.-H. Chu, L.Z. Avila, H.A. Biebuyck and G.M. Whitesides, J. Org. Chem., 58 (1992) 648.
[26] N.H.H. Heegaard and F.A. Robey, Anal. Chem., 64 (1993) 2479.
[27] R. Biehler, and A. Jacobs, Capillary Electrophoresis Technical Information A-1727, Beckman Instruments, 1993.
[28] S.A.C. Wren and R.C. Rowe, J. Chromatogr., 603 (1992) 235.
[29] M. Mammen, F.A. Gomez and G.M. Whitesides, Anal. Chem., in press.
[30] K. Takeo, in A. Chrambach, M.J. Dunn and B.J. Radola (Editors), Advances in Electrophoresis, Vol. 1, VCH Publishers, New York, 1987, p. 229.
[31] R.A. Copeland, Methods for Protein Analysis, Chapman and Hall, New York, 1994.
[32] A.T. Andrews, Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications, Oxford University Press, Oxford, 1986.
[33] J. Gao, personal communication.
[34] P.H. Popeniak and R.G. Pratt, Anal. Biochem., 165 (1987) 108.
[35] M. Nieto and H.R. Perkins, Biochem. J., 123 (1971) 789.
[36] T.D.H. Bugg, G.D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin and C.T. Walsh, Biochemistry, 30 (1991) 10408.