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Use of mobility ratios to estimate binding constants of ligands to proteins in affinity capillary electrophoresis

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Abstract

This work evaluates the use of mobility ratios (M) to estimate binding constants of proteins to ligands using affinity capillary electrophoresis (ACE). This concept is demonstrated using two model systems: vancomycin (Van) from *Streptomyces orientalis* and carbonic anhydrase B (CAB, EC 4.2.1.1). A plot of change in M (ΔM) over the concentration of ligand [L] versus ΔM yields a more useful representation of the Scatchard plot in capillary electrophoresis (CE) than traditional plots of the change in mobility $\Delta\mu$ over [L] versus $\Delta\mu$ in a wide set of circumstances, especially when comparing electropherograms obtained in the presence of substantial variations in electroosmotic flow. Altering the voltage and/or capillary length of the CE system produced only small variations in M, but much larger changes in the more standard measures of migration used by the μ form of analysis. The use of M in the Scatchard analysis offers a new approach to estimating binding constants of ligands to proteins using ACE.

Keywords: Mobility ratios; Binding constants; Ligands; Proteins; Vancomycin; Carbonic anhydrase

1. Introduction

Capillary electrophoresis (CE) is a versatile microanalytical technique that has gained much attention, particularly from those working with biologically active molecules. CE differentiates charged species on the basis of mobility under the influence of an applied electric field gradient. The characterization of specific interactions is the focus of much biochemical research [1]. There are a number of methods to measure binding parameters for noncovalent interactions [2]. Affinity capillary electrophoresis (ACE) is a new technique that has been shown to be an efficient and accurate tool in studying biomolecular noncovalent interactions and determining binding and dissociation constants of formed complexes [3–30]. For example, Mammen et al. [27] have shown that ACE can be used to determine the two dissociation constants of the complex between an antibody and charged *N*-dinitrophenyl ligands. Kwak and Gomez [26] have demonstrated the use of ACE to study the binding of adamantane carboxylic acids to cyclodextrins using indirect detection. Finally, Chu et al. [6] have used ACE to determine binding stoichiometries of protein–ligand interactions.

Analysis via ACE requires the measurement of the electrophoretic mobility, μ, of a species which is directly related to the net charge and inversely related to its hydrodynamic drag. In this form of analysis changes in the electrophoretic mobility μ_P of a protein (P) on complexation with a ligand (L)

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present in the buffer can be correlated to the binding constant K_b [13,31–33]. 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).

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

Detection time and mobility of a species are related by Eq. (2):

$$\Delta\mu_{P,L} = l_c l_d/V[(1/t_{ref} - 1/t_P) - (1/t_{ref,L} - 1/t_{P,L})] \quad (2)$$

Here, l_c (cm) is the total length of the capillary, l_d (cm) is the length of the capillary from the sample or inlet end of the capillary to the detector, V is the voltage across the capillary, $t_{P,L}$ and $t_{ref,L}$ are the measured migration times of the protein peak and reference ion at the concentration of ligand [L], respectively, and t_P and t_{ref} are the measured migration time of the protein peak and reference ion at [L]=0, respectively.

In practice, however, there are limitations to this form of analysis. In situations where electroosmotic flow (EOF) is variable, the μ form of analysis is not always permissible. EOF is represented by the migration time of a neutral species and can be affected by a number of parameters, including temperature, pH, ionic strength and applied voltage. The variance in EOF during a given experiment is important in ACE studies since the estimation of binding constants is dependent on electrophoretic mobilities. Yang et al. [34] and Bose et al. [35] recently examined the use of mobility ratios (M) as a means for providing a more reliable description of analyte migration in CE. It was theoretically demonstrated that M should be independent of such factors as voltage and solvent viscosity. In this work, M for each injected solute was defined as in Eq. (3) when using a neutral marker as the reference:

$$M = \mu_{net}/\mu_{eo} \quad (3)$$

$$= (\mu + \mu_{eo})/\mu_{eo} \quad (4)$$

In Eqs. (3) and (4), μ_{eo} is the mobility due to EOF, μ is the inherent mobility of the solute and μ_{net} is the net mobility measured for the solute. A given μ can be defined as shown below:

$$\mu = l_c l_d/Vt \quad (5)$$

When Eq. (5) is substituted into Eq. (3), the following result for M is obtained:

$$M = t_{eo}/t_p + 1 \quad (6)$$

Here, t_{eo} and t_p are the measured migration times of a reference peak and the protein peak, respectively.

We wish to extend the use of the M form of analysis to ACE. Analysis that can be performed using M is thus independent of the capillary length and voltage; the expression depends solely on the migration time of the receptor relative to a neutral marker. A Scatchard plot can be obtained via Eq. (7):

$$\Delta M_{P,L}/[L] = K_b\Delta M_{P,L}^{max} - K_b\Delta M_{P,L} \quad (7)$$

Here $\Delta M_{P,L}$ is the magnitude of the change in the migration ratio as a function of the concentration of ligand. Eq. (7) allows for the estimation of binding constants on a relative time scale, rather than an absolute time scale. Variances in voltage and/or capillary length that may effect the migration time of a species do not alter M since either are not required in the estimation of M. Eq. (7) does not contain a concentration term of the sample. Hence, binding constants can be principally measured at any protein concentration which is still detectable.

This work will demonstrate the use of M to estimate binding constants of proteins to ligands in ACE. This concept is described using two model systems: vancomycin from *Streptomyces orientalis* and carbonic anhydrase B (CAB, EC, 4.2.1.1). We illustrate the general utility of this form of analysis in ACE by examining the effect variances in voltage and capillary length have on the estimation of binding constants.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade. Vancomycin from *Streptomyces orientalis*, N-Ac–D-Ala–D-Ala

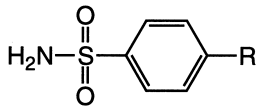
(**1**), horse heart myoglobin, carbonic anhydrase B (CAB, EC 4.2.1.1, containing CAA and CAB isozymes, from bovine erythrocytes), and 4-carboxybenzenesulfonamide (**3**) (see Fig. 1) were purchased from Sigma (St. Louis, MO, USA) and used without further purification. Mesityl oxide was purchased from Calbiochem (San Diego, CA, USA). Compound **2** (see Fig. 1) was a gift of J. Gao (Harvard University, Cambridge, MA, USA). Stock solutions of vancomycin (1 mg/ml), bovine carbonic anhydrase B (1 mg/ml), and horse heart myoglobin (1 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 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 50.5 or 60.5 cm from inlet to detector×50 μm, I.D., 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, 10–30 kV; current, 4.0–6.0 μA; detection, 200 nm; temperature, 25±1°C.

2.3. Procedures

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 Tris–gly buffer and appropriate concentrations of the N-acetyl–D-Ala–D-Ala ligand (0–1150 μ*M*). For CAB, 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 μ*M*).



2 R = $CH_2NHC(O)(CH_2)_4CO_2^-$

3 R = CO_2^-

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

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 technique utilizes μ as the basis for analysis and assumes small variations in EOF. In the event of more drastic changes in EOF, analysis by μ is difficult and another form of analysis is required. Our goal was to demonstrate the use of *M* as the basis for analysis in ACE thereby circumventing the need for a stable EOF. We examined the use of *M* as the basis for ACE analysis using two model systems: (i) the binding of vancomycin to N-Ac-D-Ala D-Ala, and (ii) the binding of CAB to two arylsulfonamides. We demonstrate this by varying (i) voltage, (ii) capillary length and (iii) voltage and capillary length simultaneously.

3.1. Effect of changes in the operating voltage on ACE analysis

In a first series of experiments the interaction of vancomycin with N-Ac–D-Ala–D-Ala was investigated. Extensive studies on molecular recognition between vancomycin and various small peptides with D-Ala–D-Ala terminus and structurally modified variants have been conducted by other techniques [17]. It is known that the three amide linkages and the free terminal carboxyl group are essential for vancomycin binding. In our initial studies a plug of vancomycin (Van), CAB and mesityl oxide (MO) were vacuum injected into the capillary and electrophoresed at 30 kV. In this experiment the concentration of **1** was successively increased from 0 to 1150 μ*M*. Similar ACE studies were subsequently conducted at both 10 and 20 kV. Constant temperature and capillary length conditions were used during these studies.

Fig. 2 shows a representative series of elec-

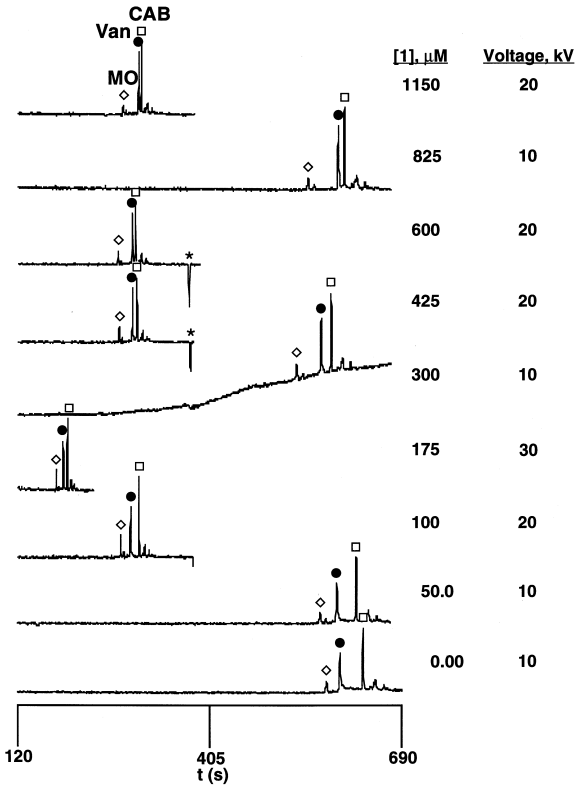


Fig. 2. Representative set of electropherograms of vancomycin (Van) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **1**. Total analysis time in each experiment ranged from 4.0 to 11.5 min and 10–30 kV (current: 4.0–6.0 μA) using a 60.5-cm (inlet to detector), 50-μm I.D. open, uncoated quartz capillary. Carbonic anhydrase B (CAB) and mesityl oxide (MO) were used as internal standards. The inverted peaks (*) are discussed in the text.

tropherograms of Van in buffer containing various concentrations of **1** at varying voltages. Upon addition of increasing concentrations of **1** to the running buffer the Van peak shifts to the right for any given concentration. MO and CAB are used as a neutral and protein marker in the analysis. They do not interact with **1** in the running buffer under the conditions of electrophoresis. All the electropherograms had the same elution pattern but differed significantly in migration times due to changes in the applied voltage. The complexation between **1** and Van resulted in an increasing negative charge and the complex is detected later than the uncomplexed form. Very little peak broadening was observed at the 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. The inverted peaks (*) resulted from the dilution of **1** present in the electrophoresis buffer. In this experiment each different concentration of ligand was run four times. The relative standard deviation (R.S.D.) for the values in K_b were ±4%. These types of fluctuations may be the result of changes within the capillary over time, such as ligand and/or protein adsorption to the capillary wall or variations in temperature and current [34]. Fig. 3 is a Scatchard plot of the data according to Eq. (4) for **1**. The data is reproducible. Little scattering of the data points is observed.

Table 1 shows the binding constants obtained by Eqs. (7) and (1) under conditions of mixed voltage (entry 1) and at the three individual voltage conditions (entries 2–5). Under conditions of variable voltage the measurement of K_b is possible using Eq. (7). Using Eq. (7), only random variations in the measurement of K_b and, hence, M were observed. This type of behavior was expected since the measurement of K_b using Eq. (7) is independent of voltage. We were unable to estimate the values of a binding constant using Eq. (1) (entry 1); the Scatchard plot was dramatically nonlinear. Using data obtained under constant operating voltages, Eq. (7) yields values for K_b comparable to those found using Eq. (1). This proves that Eq. (7) effectively compen-

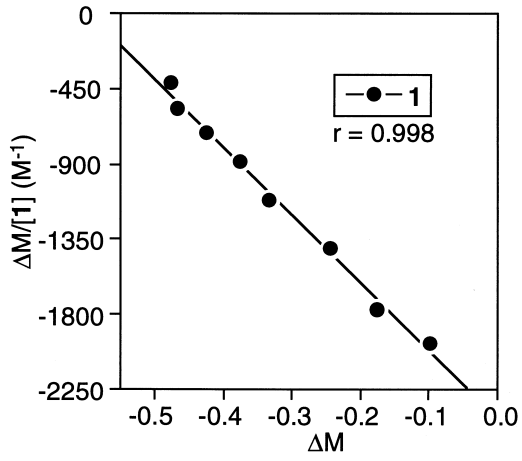


Fig. 3. Scatchard plot of the data for vancomycin according to Eq. (7).

Table 1
Experimental values of binding constants K_b (10^3 M^{-1}) of ligand **1** and vancomycin obtained by Eqs. (1) and (7)

Entry	Voltage (kV)	Capillary length (cm)	K_b	
			Eq. (1)	Eq. (7)
1	10, 20, 30	67	[a]	4.1
2	10	67	3.8	3.9
3	20	57	6.1	6.3
4	20	67	4.5	4.1
5	30	67	3.3	4.5
6	20	57, 67	[a]	7.9
7	10, 20, 30	57, 67	[a]	5.8

[a] Unable to be measured.

sates for changes in EOF. These values agree well with previous ACE studies which determined binding constants of 8.7, 4.8, and $5.6 \cdot 10^3$ M^{-1} for the same dipeptide at pH 7.1 and 8.3 respectively, by two different methods and with those obtained from other assays for structurally similar compounds [4,30,36–38].

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, (iii) there has been considerable interest in recent years in vancomycin owing to its novel mechanism of bacterial resistance.

A similar series of experiments were conducted using CAB and the arylsulfonamide **2**. In this case a plug of MO, CAB and horse heart myoglobin (HHM) were injected into the capillary and the sample electrophoresed at 30 kV. HHM does not interact with **2** in the running buffer. The concentration of **2** was successively increased from 0 to 120 μ*M*. Ligand **2** and CAB form a complex which is more negative than the native protein. Hence, the complex is detected later than the uncomplexed form. Similar ACE studies were conducted at both 20 and 25 kV. Table 2 shows the binding constants obtained using Eqs. (7) and (1) for both the mixed voltage (entry 1) and individual voltage conditions (entries 2–4). Under conditions of variable voltage only the *M* form of analysis allows for the measurement of K_b. Binding constants obtained using Eq. (7) agree well with those obtained from previous ACE studies (0.60 and $0.74 \cdot 10^3$ M^{-1} for **2**) [13,30]. A similar series of experiments was conducted using ligand **3** (entries 7–10). These studies yielded values for K_b similar to that obtained from previous ACE studies (0.80 and $0.99 \cdot 10^6$ M^{-1}) [21,30]. We could

Table 2
Experimental values of binding constants K_b (10^6 M^{-1}) of ligands **2** and **3** and carbonic anhydrase B obtained by Eqs. (1) and (7)

Entry	Ligand	Voltage (kV)	Capillary length (cm)	K_b	
				Eq. (1)	Eq. (7)
1	2	20, 25, 30	67	[a]	0.92
2	2	20	67	0.90	0.89
3	2	25	67	0.76	0.82
4	2	30	67	[a]	0.79
5	2	20	57, 67	[a]	0.89
6	2	20	57, 67	[a]	0.91
7	3	20, 25, 30	67	[a]	0.72
8	3	20	67	2.0	1.3
9	3	25	67	1.0	0.90
10	3	30	67	1.1	1.1

[a] Unable to be measured.

not evaluate a value for K_b using Eq. (1) (entries 1 and 7).

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}.

3.2. Effect of changes in the capillary length on ACE analysis

In a second series of experiments we examined and compared binding constants obtained using capillaries of different lengths. Using a 67-cm length capillary a similar ACE study was conducted as that mentioned in Section 3.1 using Van. Similar ACE studies were subsequently conducted using a 57-cm length capillary. Fig. 4 shows a representative series of electropherograms of Van in buffer containing various concentrations of **1** at varying voltages. Changing the length of the capillary from 57 to 65 cm increases the migration times of all peaks in the electropherograms. Upon addition of increasing concentrations of **1** in the running buffer the Van peak shifts to the right. The inverted peaks (*) resulted from the dilution of **1** present in the electrophoresis buffer. Fig. 5 is a Scatchard plot of the data according to Eq. (7) for **1**. Increasing the length of the capillary from the injection end to the detector by 10 cm resulted in only a nominal change in K_b.

Table 2 shows the binding constants obtained using both Eqs. (7) and (1) under conditions of mixed capillary length (entry 6). Under conditions of variable capillary length, measurement of K_b is possible by the *M* form of analysis whereas it is not possible using the μ form (entries 5 and 6).

A similar series of experiments were conducted using CAB and the arylsulfonamide **2**. Table 2 shows the binding constants obtained using Eqs. (7) and (1) (entry 5). The binding constant obtained is comparable to that observed by other ACE techniques and other forms of analysis [13,21,30]. Under conditions of variable capillary length only the *M* form of analysis provides for measurement of K_b.

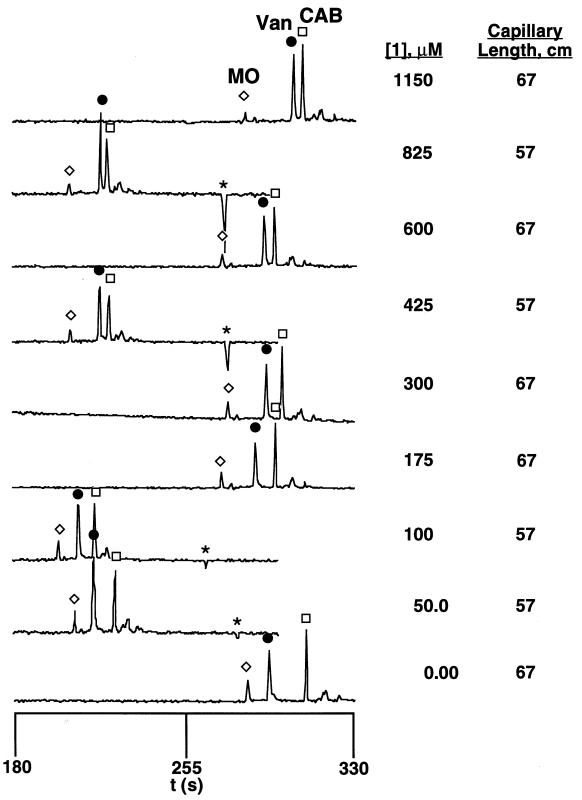


Fig. 4. Representative set of electropherograms of vancomycin (Van) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **1**. Total analysis time in each experiment ranged from 4.5 to 5.5 min and 20 kV (current: 4.9–5.9 μA) using a 50.5 and 60.5-cm (inlet to detector), 50 μm I.D. open, uncoated quartz capillary. Carbonic anhydrase B (CAB) and mesityl oxide (MO) were used as internal standards. The inverted peaks (*) are discussed in the text.

3.3. Effect of changes in both the operating voltage and the capillary length on ACE analysis

We then examined the effect changing both voltage and capillary length had on estimating K_b. We utilized the data obtained from the two previous studies in this experiment. Fig. 6 shows a representative series of electropherograms of Van in buffer containing various concentrations of **1** at both varying voltage and capillary length. The large fluctuation in EOF and migration time of all peaks does not permit analysis by the μ form. The inverted peaks (*) resulted from the dilution of **1** present in the electrophoresis buffer. Fig. 7 is a Scatchard plot of

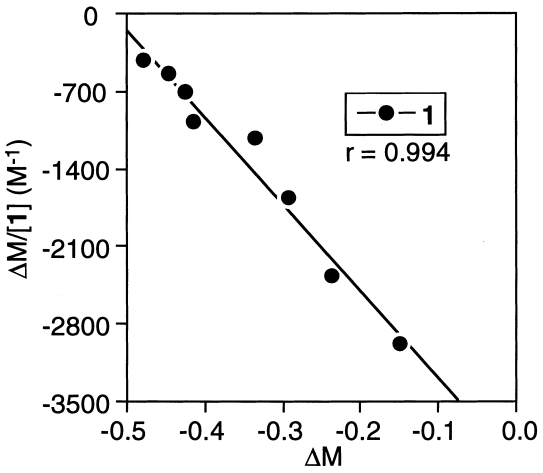


Fig. 5. Scatchard plot of the data for vancomycin according to Eq. (7).

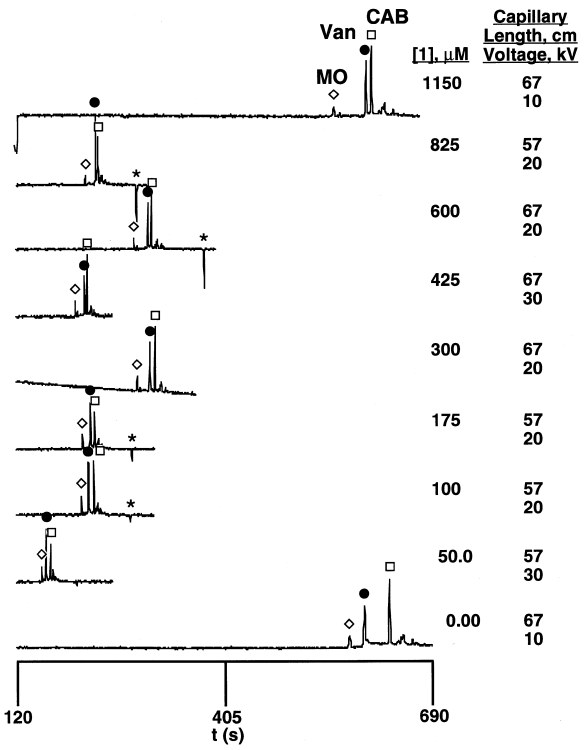


Fig. 6. Representative set of electropherograms of vancomycin (Van) in 0.192 *M* glycine–0.025 *M* Tris buffer (pH 8.3) containing various concentrations of **1**. Total analysis time in each experiment ranged from 4.0 to 11.5 min and 20–30 kV (current: 4.0–6.0 μA) using a 50.5 and 60.5 cm (inlet to detector), 50 μm I.D. open, uncoated quartz capillary. Carbonic anhydrase B (CAB) and mesityl oxide (MO) were used as internal standards. The inverted peaks (*) are discussed in the text.

the data according to Eq. (7) for **1**. Little scattering of the data points is observed. Table 2 shows the binding constants obtained using the two different forms of analysis (entry 7).

A similar series of experiments were conducted using CAB and the arylsulfonamide **2**. Table 2 shows the binding constants obtained using the *M* and μ forms of analysis (entry 6). As can be seen, under conditions of both variable voltage and capillary length, only the *M* form of analysis provides for measurement of K_b.

This form of analysis in ACE has several advantages as a method for determining binding constants over other methods: (i) it does not require a stable EOF, (ii) experiments can be conducted under conditions of variable voltages and/or with capillaries of varying lengths, and (iii) only small quantities of ligand and protein are required. Upon achieving the proper experimental conditions the ability of this form of analysis to quantitate reversible binding interactions is unchallenged by any other present method.

4. Conclusion

Binding constants in ACE may be estimated using the mobility ratio *M* as the basis for the analysis even in the event of large fluctuations in EOF. We have shown this by the use of two model systems: vancomycin and the dipeptide *N*-acetyl–D-Ala–D-Ala and CAB and arylsulfonamide ligands. In these experiments binding constants were estimated using data from individual ACE experiments where voltage and capillary lengths had been modified. The binding constants obtained by this form of analysis agree well with those obtained by other assay techniques. Because *M* is independent of capillary length and voltage, variations in EOF induced by these two parameters do not interfere with the estimation of K_b contrary to the traditional form of analysis which requires an accurate measurement of μ. Mobility ratios offer an alternative method of analysis of binding constants in ACE. The use of this technique

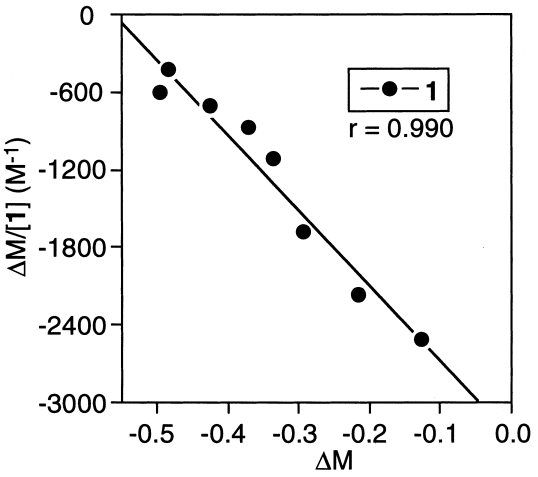


Fig. 7. Scatchard plot of the data for vancomycin according to Eq. (7).

provides for greater use of ACE in studying biomolecular noncovalent interactions.

Acknowledgements

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