Anal. Chem. 1994, *66*, 1785–1791

Determination of Binding Constants of Ligands to Proteins by Affinity Capillary Electrophoresis: Compensation for Electroosmotic Flow[1]

Frank A. Gomez,[2] Luis Z. Avila, Yen-Ho Chu, and George M. Whitesides*

Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

This paper describes the estimation of binding constants (K_b) between carbonic anhydrase B (CAB, EC 4.2.1.1, from bovine erythrocytes) and charged benzenesulfonamides by affinity capillary electrophoresis (ACE) under conditions in which the migration time is affected by changes in electroosmotic flow and by nonspecific interactions accompanying changes in the concentration of ligand. Comparisons of values of migration times of the protein of interest, and of "noninteracting" marker proteins, with those of a neutral internal standard provide the basis for corrections for variable electroosmotic flow; these corrections make possible the estimation of K_b and its uncertainty even in the presence of substantial variations in electroosmotic flow.

This paper describes a method of estimating binding constants (K_b) between carbonic anhydrase B (CAB, EC 4.2.1.1, from bovine erythrocytes)[3] and charged ligands by affinity capillary electrophoresis (ACE)[4–9] under conditions in which migration times (t_m) are affected by changes in electroosmotic flow and by other characteristics of the experiment that reflect changes in the concentration of the ligand in the buffer. We illustrate this method by comparative analyses of binding of CAB to both positively and negatively charged ligands. CAB is a useful model protein for four reasons: (i) we have data describing its electrophoretic behavior in other circumstances;[4,5] (ii) it can be used with uncoated capillaries;[4] (iii) ligands for it can be easily synthesized with analytically useful variation in their structures; (iv) it is commercially available and inexpensive. The analysis examines several of the factors that influence t_m; these factors include the effects of ligand binding to the protein, of ligand interaction with the capillary wall,[10] and of ligand interaction with nonbinding proteins.

Description of the Principle of ACE. Affinity capillary electrophoresis (ACE) relates changes in the electrophoretic mobility μ_P of a protein (P) on complexation with a ligand (L) present in the buffer to the binding constant K_b.[4–7,11–15] Analysis of the magnitude of the change in mobility, $\Delta\mu_P$, as a function of the concentration [L] of ligand yields K_b. The value of μ_P ($cm^2\ V^{-1}\ s^{-1}$) of a protein is approximately related to its mass M and net charge Z by a relationship of the form of eq 1, and that for the ligand (with mass m and charge z), by eq 2.[11–14] In these equations, the constants C and L relate the charge and mass to the mobility for that class of compounds. Other functional forms have also been used to relate mass and charge to electrophoretic mobility;[11–13] we *assume* the forms

(1) This work was supported by the National Institutes of Health (Grant GM 30367) and the National Science Foundation under the Engineering Research Initiative to the M.I.T. Biotechnology Processing Engineering Center (Cooperative Agreement CDR-88-03014).

(2) Damon Runyon-Walter Winchell Cancer Research Fund Postdoctoral Fellow, 1991–1994.

(3) Botre, F.; Gros, G.; Storey, B. T. *Carbonic Anhydrases: From Biochemistry and Genetics to Physiology and Clinical Medicine*; VCH Publishers: New York, 1991. Dodgson, S. J.; Tashian, R. E.; Gros, G.; Carter, N. D. *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*; Plenum Press: New York, 1991.

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

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

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

(7) Chu, Y.-H.; Avila, L. Z.; Biebuyck, H. A.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 648–652.

(8) Honda, S.; Suzuki, K.; Suzuki, S.; Kakehl, K. *J. Chromatogr.* **1992**, *597*, 377–382.

(9) Carpenter, J. L.; Camilleri, P.; Dhanak, D.; Goodall, D. *J. Chem. Soc., Chem. Commun.* **1992**, 804–806.

(10) Kuhr, W. G.; Monnig, C. A. *Anal. Chem.* **1992**, *64*, 389R–407R. Mazzeo, J. R.; Krull, I. S. *Biotechniques* **1991**, *10*, 638–645. Jandik, P.; Jones, W. R.; Weston, A.; Brown, P. R. *LC-GC* **1991**, *9* (9), 634–645. Novotny, M. V.; Cobb, K. A.; Liu, J. *Electrophoresis* **1990**, *11*, 735–749.

(11) Compton, B. J.; O'Grady, E. A. *Anal. Chem.* **1991**, *63*, 2597–2602.

(12) Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. *Anal. Biochem.* **1991**, *197*, 197–207.

(13) Grossman, P. D.; Colburn, J. C.; Lauer, H. H. *Anal. Biochem.* **1989**, *179*, 28–33.

(14) Deyl, Z.; Rohlicek, V.; Adam, M. *J. Chromatogr.* **1989**, *480*, 371–378.

(15) Xu, Yan, *Anal. Chem.* **1993**, *65*, 425R–433R.

0003-2700/94/0366-1785$04.50/0
© 1994 American Chemical Society

$$\mu_P \simeq CZ/(M^{2/3}) \quad (1)$$

$$\mu_L \simeq Lz/(m^{2/3}) \quad (2)$$

of eqs 1 and 2, but the exact form is not crucial for our discussions, since we are interested primarily in relative changes in values of μ_P and in their dependence on [L]. We assume the corresponding relation for the protein–ligand complex, P·L, to be described by eq 3; that is, the protein and ligand

$$\mu_{P,L}^{max} \simeq C(Z + z)/(M + m)^{2/3} \quad (3)$$

preserve charge and mass on complexation, and the hydrodynamic characteristics of the complex are sufficiently similar to those of uncomplexed protein that the same constant C applies.

Complexation of protein with ligand results in a change in mobility $\Delta\mu_{P,L} = \mu_{P,L} - \mu_P$, where μ_P is the mobility in the absence of ligand and $\mu_{P,L}$ is the mobility at ligand concentration [L]. An observed difference Δt_L between the migration time (t_L) of a protein at a concentration of ligand [L] and the migration time $t_{L=0}$ at [L] = 0 can, however, also be influenced by factors other than binding of ligand at the active site of the protein. Electroosmotic flow is a particularly important contributor to t_L.[10,13] When the change in electroosmotic flow is negligible over the concentration range used in the analysis, Δt_L is proportional to $\Delta\mu_L$, and eq 4 can be used to estimate

$$\Delta t_L/[L] = K_b\Delta t^{max} - K_b\Delta t_L \quad (4)$$

the binding constant.[4] Experiments of this type are easily recognized as those where the mobilities of the neutral and noninteracting protein markers remained unchanged on increasing the concentration of ligand in the buffer.

During a series of runs in an ACE experiment, the migration times of neutral marker and noninteracting proteins do not, however, necessarily remain constant. A number of phenomena may contribute to these changes in migration times. In particular, a positively charged ligand present at high concentrations in the buffer may interact with the negatively charged wall of the capillary[16,17] and change the velocity of electroosmotic (EO) flow.[16] Positively charged ligands normally have a bigger influence on EO flow than do negatively charged ones. Changes in the viscosity or thermal conductivity of the buffer with changes in the concentration of ligand can also cause Δt_L and $\Delta\mu_L$ to be non-zero for the neutral marker species and cause the proportionality between Δt_L and $\Delta\mu_L$ to become nonlinear.

Method: Correction for Electroosmotic Flow. In this paper, we test the hypothesis that it is possible to correct values of Δt_L empirically for changes in electroosmotic flow by observing the neutral internal standard and that, with this correction, it is possible to infer and analyze the contributions from biospecific interactions even in the presence of variable EO flow. The analysis of EO flow is based on considerations of velocities (v) of movement of species in the capillary under the influence of bulk flow and of the electric field gradient.

The analysis starts with eq 5. In this equation, v_{EO} is the net (measured) velocity of migration (cm s^{-1}) of a neutral marker(in all of this work we use mesityl oxide, MO) under

$$v_{EO} = l_d/t_{EO} \quad (5)$$

given experimental conditions and is used as a measure of electroosmotic flow; l_d (cm) is the length of the capillary from the "sample" or "inlet" end of the capillary to the detector, and t_{EO} (s) is the measured migration time of the neutral marker. Equation 5 assumes that the velocity of the MO provides an accurate measure to electroosmotic flow.[13]

The mobility of the protein of interest in the sample in the absence of ligand ([L] = 0), μ_P (cm^2 V^{-1} s^{-1}), is given by eq 6. Here, V is the voltage across the capillary, l_c (cm) is the

$$\mu_P = l_c(v_P - v_{EO})/V \quad (6)$$

total length of the capillary, and v_P is the net (measured) migration velocity of the protein. Combination of eqs 5 and 6 gives eq 7; here t_P is the measured migration time of the

$$\mu_P = l_c l_d(1/t_P - 1/t_{EO})/V \quad (7)$$

protein peak. The change in mobility of the protein as a function of the concentration of added ligand $\Delta\mu_{P,L}$ is given by eq 8; $t_{P,L}$ and $t_{EO,L}$ are the measured migration times of

$$\Delta\mu_{P,L} = \mu_{P,L} - \mu_P = l_c l_d/V[(1/t_{P,L} - 1/t_{EO,L}) - (1/t_P - 1/t_{EO})] \quad (8)$$

the sample peak and neutral marker at the concentration of ligand [L], respectively. The values of $\Delta\mu_{P,L}$ obtained using eq 8 over a range of concentrations of L may then be used for Scatchard analysis (eq 9). When carrying out the analysis in

$$\Delta\mu_{P,L}/[L] = K_b\Delta\mu_{P,L}^{max} - K_b\Delta\mu_{P,L} \quad (9)$$

this form, both K_b and $\Delta\mu_{P,L}^{max}$ are obtained from the analysis; $\Delta\mu_{P,L}^{max}$ is the electrophoretic mobility of the protein when saturated with the ligand (that is, when its active site is completely occupied by ligand). In the case where the electroosmotic flow is not altered by the addition of the ligand(s) in the buffer, eq 8 simplifies to eq 10. Here, Δt is

$$\Delta\mu_{P,L} \sim l_c l_d\Delta t/Vt^2 \quad (10)$$

the difference between $t_{P,L}$ and t_P and t is the average of $t_{P,L}$ and t_P. A useful measure of the fractional change in the experimentally determined mobility $\delta\mu_{P,L}^{max}$ is given by eq 11.

$$\delta\mu_{P,L}^{max} = \Delta\mu_{P,L}^{max}/\mu_P \quad (11)$$

(16) Towns, J. K.; Regnier, F. E. *Anal. Chem.* **1992**, *64*, 2473–2478.
(17) Towns, J. K.; Regnier, F. E. *Anal. Chem.* **1991**, *63*, 1132–1138.

Estimates of Changes in Mobility on Complexation. It is useful to have an approximate method of *estimating* the values of the *maximum* change in mobility $\Delta\mu_{P,L}^{max}$ (eqs 12 and 13)

$$\Delta\mu_{P,L}^{max} = \mu_{P,L}^{max} - \mu_P \tag{12}$$

$$= C\frac{(Z+z)}{(M+m)^{2/3}} - C\frac{Z}{M^{2/3}} \tag{13}$$

$$= \left[\left(1+\frac{z}{Z}\right)\left(\frac{M}{M+m}\right)^{2/3} - 1\right]\mu_P \tag{14}$$

and the *fractional* change in mobility $\delta\mu_{P,L}^{max}$ (eq 15) that can occur on complexation, based on the masses and charges of the protein and ligand. Substituting $\Delta\mu_{P,L}^{max}$ from eq 14 into eq 11 yields eq 15. Straightforward algebra simplifies eq

$$\delta\mu_{P,L}^{max} = \left(1+\frac{z}{Z}\right)\left(\frac{M}{M+m}\right)^{2/3} - 1 \tag{15}$$

$$= \left(1+\left(\frac{C}{L}\right)\left(\frac{m}{M}\right)^{2/3}\left(\frac{\mu_L}{\mu_P}\right)\right)\left(\frac{M}{M+m}\right)^{2/3} - 1 \tag{16}$$

$$\simeq \frac{C}{L}\left(\frac{m}{M}\right)^{2/3}\left(\frac{t_{eo}-t_L}{t_L}\right)\left(\frac{t_{eo}-t_P}{t_P}\right)^{-1} \tag{17}$$

$$\simeq \frac{C}{L}\left(\frac{m}{M}\right)^{2/3}\frac{\mu_L}{\mu_P} \tag{18}$$

13 to eq 14 and permits eq 15 also to be written as eq 16. After substitution for μ_L and μ_P, eq 16 becomes eq 17. If $M \gg m$, then eq 16 becomes eq 18. This last equation is useful if C, L, μ_L, and μ_P can all be obtained or estimated experimentally.

EXPERIMENTAL SECTION

Materials. All chemicals were analytical grade. Dimethyl aminoterephthalate, 4-(3-aminopropyl)morpholine, 1-amino-4-methylpiperazine, dicyclohexylcarbodiimide, *N*-hydroxybenzotriazole hydrate, and methyl iodide were purchased from Aldrich. 4-Carboxybenzenesulfonamide was purchased from Sigma. Dimethylformamide (Fisher) was kept over molecular sieves (4 Å). Compound **2** was available from studies to be published.[18] Compound **11** and *N*-hydroxysuccinimidyl 4-sulfamoylbenzenecarboxylate were synthesized on the basis of literature procedures.[4,19]

Carbonic anhydrase B (CAB, EC 4.2.1.1, from bovine erythrocytes) was purchased from Sigma. Horse heart myoglobin was purchased from U.S. Biochemical Corp. Mesityl oxide was purchased from Eastman Organic Chemical (Rochester, NY). Stock solutions (1 mg/mL) of bovine carbonic anhydrase B and horse heart myoglobin were each prepared by dissolving the lyophilized protein in buffer (192 mM glycine–25 mM Tris; pH 8.3).

Equipment. The capillary electrophoresis (CE) system used in this study was an Isco Model 3140 (Lincoln, NE). The capillary tubing (Polymicro Technologies, Inc., Phoenix, AZ) was of uncoated fused silica with an internal diameter of 50 μM, a total length of 79 cm, and a length from inlet to detector of 50 cm. The conditions used in CE were as follows: voltage, 30 kV (current, 9 μA); detection, 200 nm; temperature, 27 ± 2 °C.

Melting points were uncorrected. Thin layer chromatography was carried out on EM Science silica gel 60-F254 glass-backed plates (0.25-mm layer thickness). Column chromatography was performed on EM Science silica gel 60 (230–400 mesh) or Fluka C_{18} reversed-phase silica gel (Ronkonkoma, NY). Mass spectra (fast atom bombardment) were measured on a Vacuum Generator Model VG 7070E spectrometer. All reactions were carried out in oven-dried glassware under an argon atmosphere.

Procedures. A sample (8 nL) of solution containing 0.08 mg/mL of bovine carbonic anhydrase B, 0.1 mg/mL of horse heart myoglobin, and 0.2 mg/mL of mesityl oxide in buffer was introduced into the capillary by vacuum injection. The electrophoresis was carried out using an electrophoresis buffer and appropriate concentrations of the arylsulfonamide ligand (0–170 μM, depending on L).

5-[[6-[[[4-(Aminosulfonyl)phenyl]methyl]amino]-1,6-dioxohexyl]amino]-*N,N'*-bis[3-(4-morpholinyl)propyl]-1,3-benzenedicarboxamide (3). To a solution of diacid **2**[18] (1.5 g, 3.0 mmol) in DMF (30 mL) were added *N*-hydroxybenzotriazole hydrate (1.0 g, 6.7 mmol) and dicyclohexylcarbodiimide (1.4 g, 6.7 mmol) at room temperature. After 30 min, a solution of 4-(3-aminopropyl)morpholine (0.9 mL, 6.1 mmol) in DMF (3 mL) was added in drops with stirring and the solution was allowed to stir overnight. The solution was filtered and concentrated in vacuo. Chromatography on silica gel (ethyl acetate:hexane, 5:1) afforded **3** as a white solid (0.6 g, 26%), mp 88 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1 H, NH), 8.62 (t, 2 H, NH, J = 5.4 Hz), 8.49 (t, 1 H, NH, J = 5.8 Hz), 8.17 (s, 2 H, aryl CH), 7.94 (s, 1 H, aryl CH), 7.76 (d, 2 H, aryl CH, J = 8.3 Hz), 7.40 (d, 2 H, aryl CH, J = 8.4 Hz), 7.34 (s, 2 H, NH_2), 4.31 (d, 2 H, CH_2N), 3.54 (m, 8 H, CH_2), 3.29 (q, 4 H, CH_2, J = 6.3 Hz), 2.31 (m, 14 H, CH_2), 1.66 (qt, 4 H, CH_2, J = 7.0 Hz), 1.59 (s, 4 H, CH_2); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 172.5, 171.8, 166.3, 144.0, 143.1, 142.8, 135.8, 127.6, 125.9, 123.8, 123.1, 66.3, 56.3, 53.4, 41.9, 38.2, 36.4, 35.4, 24.9, 25.2, 25.0; HRMS (FAB) m/e 730.3582 $(M + H)^+$, calcd for $C_{35}H_{52}N_7SO_8$ 730.3598.

4,4'-[[5-[[6-[[[4-(Aminosulfonyl)phenyl]methyl]amino]-1,6-dioxohexyl]amino]-1,3-phenylene]bis[(carbonylimino)-3,1-propanediyl]]bis[4-methylmorpholinium] Diiodide (4). To a solution of **3** (101 mg, 0.14 mmol) in DMF (3 mL) was added methyl iodide (70 μL, 1.1 mmol). The solution was stirred for 2 h and concentrated in vacuo. Recrystallization from ethanol afforded **4** as a yellow solid (87 mg, 62%), dec 130 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1 H, NH), 8.66 (t, 2 H, NH), 8.41 (t, 1 H, NH), 8.19 (s, 2 H, aryl CH), 8.00 (s, 1 H, aryl CH), 7.74 (d, 2 H, aryl CH, J = 6.3 Hz), 7.40 (d, 2 H, aryl CH, J = 6.2 Hz), 7.29 (s, 2 H, NH_2), 4.30 (d, 2 H, CH_2, J = 4.4 Hz), 3.92 (s, 8 H, CH_2), 3.53 (s, 4 H, CH_2), 3.43 (s, 4 H, CH_2), 3.33 (s, 14 H, CH_2), 3.14 (s, 6 H, CH_3), 2.35 (s, 2 H, CH_2), 2.18 (s, 2 H, CH_2), 1.98 (s, 4 H, CH_2), 1.58 (s, 4 H, CH_2); ^{13}C NMR (100.6 MHz, DMSO-d_6); HRMS (positive ion FAB) m/e 886.3022 $(M - I)^+$, calcd for $C_{37}H_{57}N_7SO_8$ 886.3036.

(18) Gao, J.; Avila, L. Z.; Kim, B.; Whitesides, G. M. Unpublished data.
(19) Jain, A.; Huang, S. G.; Whitesides, G. M. *J. Am. Chem. Soc.*, submitted.

Table 1. Randomly Assigned Concentrations of Potassium Sulfate for Experiments I–III[a]

	[1], μM					
expt	0.00	0.50	1.0	3.0	12.0	67.0
I	6	3	6	3	3	1
II	3	6	1	6	6	1
III	3	1	6	3	6	1

[a] 1, 3, and 6 represent K_2SO_4 concentrations of 1, 3, and 6 mM, respectively.

N-[[4-(Aminosulfonyl)phenyl]methyl]-N'-[3-(4-morpholinyl)-propyl]hexanediamide (5). To a solution of acid **1**[4] (1.1 g, 3.1 mmol) in CH_2Cl_2 (30 mL) were added *N*-hydroxybenzotriazole hydrate (0.5 g, 3.9 mmol) and dicyclohexylcarbodiimide (0.8 g, 3.9 mmol) at room temperature. After 30 min, a solution of 4-(3-aminopropyl)morpholine (0.5 mL, 3.5 mmol) in CH_2Cl_2 (25 mL) was added in drops with stirring and the solution was allowed to stir overnight. The solution was filtered and concentrated in vacuo. Chromatography on silica gel (ethyl acetate:methanol:hexane, 5:5:2) afforded **5** as a white solid (0.6 g, 41%), mp 40 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.47 (t, 1 H, NH, J = 5.5 Hz), 7.83 (t, 1 H, NH, J = 5.3 Hz), 7.75 (d, 2 H, aryl CH, J = 8.2 Hz), 7.39 (d, 2 H, aryl CH, J = 8.2 Hz), 7.33 (s, 2 H, NH_2), 4.30 (d, 2 H, NCH_2, J = 5.9 Hz), 3.54 (t, 4 H, OCH_2, J = 4.5 Hz), 3.04 (q, 2 H, CH_2, J = 6.5 Hz), 2.30 (s, 4 H, CH_2N), 2.24 (t, 2 H, CH_2, J = 7.2 Hz), 2.14 (t, 2 H, CH_2, J = 6.4 Hz), 2.04 (t, 2 H, CH_2, J = 6.4 Hz), 1.52 (m, 2 H, CH_2), 1.47 (s, 4 H, CH_2); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 172.2, 171.9, 143.9, 127.4, 125.7, 66.2, 55.9, 53.3, 41.7, 36.8, 35.3, 35.1, 26.1, 25.1, 25.0; HRMS (FAB) m/e 441.2178 $(M + H)^+$, calcd for $C_{20}H_{33}N_4SO_5$ 441.2171.

4-[3-[[6-[[[4-(Aminosulfonyl)phenyl]methyl]amino]-1,6-dioxohexyl]amino]propyl]-4-methylmorpholinium Iodide (6). To a solution of **5** (0.6 g, 1.4 mmol) in DMF (3 mL) was added methyl iodide (0.3 mL, 4.1 mmol). The solution was stirred for 2 h and concentrated in vacuo. Chromatography on reversed-phase silica gel (acetonitrile:water, 1:1) afforded **5** as a white solid (0.6 g, 70%), dec >185 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.43 (t, 1 H, NH, J = 5.9 Hz), 7.92 (t, 1 H, NH, J = 5.6 Hz), 7.74 (d, 2 H, aryl CH, J = 8.3 Hz), 7.39 (d, 2 H, aryl CH, J = 8.2 Hz), 7.31 (s, 2 H, NH_2), 4.29 (d, 2 H, CH_2, J = 5.9 Hz), 3.90 (m, 4 H, CH_2), 3.41 (m, 8 H, CH_2), 3.12 (s, 3 H, CH_3), 2.15 (t, 2 H, CH_2, J =5.1 Hz), 2.08 (t, 2 H, CH_2, J = 5.5 Hz), 1.82 (m, 2 H, CH_2), 1.48 (s, 4 H, CH_2); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 172.3, 172.2, 143.8, 142.4, 127.4, 125.6, 62.0, 59.8, 59.0, 46.1, 35.4, 35.2, 35.0, 24.8, 21.5; HRMS (positive ion FAB) m/e 455.2351 $(M - I)^+$, calcd for $C_{21}H_{35}N_4SO_5$ 455.2328.

4-(Aminosulfonyl)-N-(4-methyl-1-piperazinyl)benzamide (7). To a solution of acid **9** (5.0 g, 24.9 mmol) in DMF (80 mL) was added dicyclohexylcarbodiimide (0.8 g, 29.2 mmol) at room temperature. After 30 min, a solution of 1-amino-4-methylpiperazine (3.1 mL, 26.1 mmol) in CH_2Cl_2 (5 mL) was added in drops with stirring and the solution was allowed to stir for 2 days. The solution was filtered and concentrated in vacuo. Chromatography on silica gel (ethyl acetate: methanol, 4:1) afforded **7** as a white solid (1.0 g, 13%), mp 273 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.93 (s, 1 H, NH), 7.23 (m, 4 H, aryl CH), 2.20 (t, 4 H, CH_2N, J = 4.6 Hz), 1.82 (s, 4 H, CH_2N), 1.49 (s, 3 H, CH_3); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 163.2, 146.3, 137.0, 128.0, 125.6, 54.4, 53.7; HRMS (FAB) m/e 299.1182 $(M + H)^+$, calcd for $C_{12}H_{19}N_4SO_3$ 299.1178.

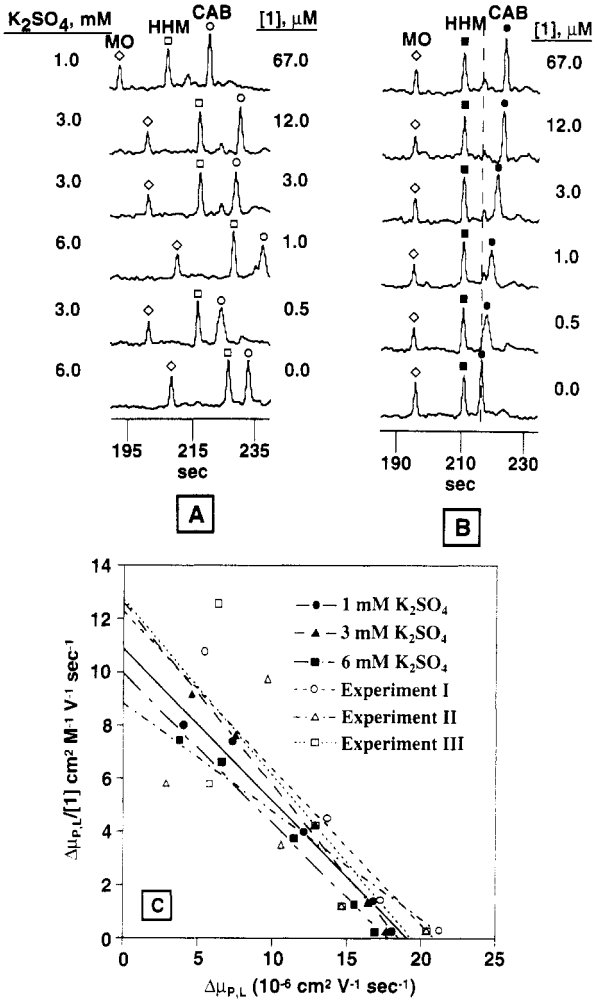


Figure 1. Affinity capillary electrophoresis (ACE) of bovine carbonic anhydrase B (CAB) in 0.192 M glycine–0.025 M Tris buffer (pH = 8.3) containing various concentrations of **1**. The total analysis time in each experiment was 6.0 min at 30 kV using a 50-cm (inlet to detector), 50-μm open, uncoated quartz capillary. Horse heart myoglobin (HHM) and mesityl oxide (MO) were used as internal standards. (A) Electropherograms for experiment I using increasing concentrations of ligand in the buffer and random concentrations of buffer additive K_2SO_4. (B) A representative set of electropherograms at 1 mM K_2SO_4. (C) Scatchard plot of the data according to eq 9.

4-[[4-(Aminosulfonyl)benzoyl]amino]-1,1-dimethylpiperazinium Iodide (8). To a solution of **7** (0.5 g, 1.5 mmol) in DMF (10 mL) at 75 °C was added methyl iodide (0.4 mL, 6.0 mmol). The solution was stirred for 2 h and concentrated in vacuo. Recrystallization from water afforded **7** as a white solid (0.1 g, 16%), dec >280 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.89 (s, 1 H, NH), 7.93 (m, 4 H, aryl CH), 3.60 (s, 4 H, NH_2), 3.43 (s, 4 H, CH_2), 3.24 (s, 6 H, CH_3); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 172.3, 172.2, 143.8, 142.4, 127.4, 125.6, 62.0, 59.8, 59.0, 46.1, 35.4, 35.2, 24.8, 21.5; HRMS (positive ion FAB) m/e 313.1314 $(M)^+$, calcd for $C_{13}H_{21}N_4SO_3$ 313.1334.

4-(Aminosulfonyl)-N-[3-(dimethylamino)propyl]benzamide (9). To a solution of *N*-hydroxysuccinimidyl 4-sul-

Table 2. Experimental Values of Binding Constants K_b (10^6 M^{-1}) and $\Delta\mu_{P,L}^{max}$ (10^{-5} cm^2 V^{-1} s^{-1}) of 1 and Carbonic Anhydrase B Obtained by ACE with Addition of Potassium Sulfate to Induce Changes in Electroosmotic Flow

	eq 8[a,b]		eq 10[a,c]	
$[K_2SO_4]$, mM	K_b	$\Delta\mu_{P,L}^{max}$	K_b	$\Delta\mu_{P,L}^{max}$
1	0.57	−1.9	0.61	−2.1
3	0.68	−1.9	0.63	−2.1
6	0.56	−1.8	0.57	−1.6
I[d]	0.59	−2.1	0.44	−3.1
II[d]	0.40	−2.2	0.72	−0.53
III[d]	0.66	−1.9	0.56	−0.53

[a] $\Delta K_b = K_b^{max} - K_b^{min}$ for the 1, 3, and 6 mM K_2SO_4 sets and experiments I–III. [b] ΔK_b(1, 3, and 6 mM K_2SO_4) = 0.12 × 10^6 M^{-1}; ΔK_b(I–III) = 0.26 × 10^6 M^{-1}. [c] ΔK_b(1, 3, and 6 mM K_2SO_4) = 0.06 × 10^6 M^{-1}; ΔK_b(I–III) = 0.28 × 10^6 M^{-1}. [d] The value of $[K_2SO_4]$ was changed during the sequence of runs at different values of ligand required to generate the data for a Scatchard analysis. Table 1 gives details.

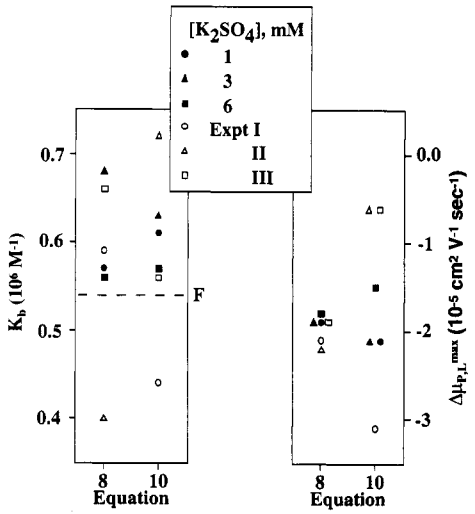


Figure 2. Values of binding constants K_b and $\Delta\mu_{P,L}^{max}$ of **1** and CAB with addition of potassium sulfate to induce changes in electroosmotic flow and the value obtained by a fluorescence assay (*F*).

famoylbenzenecarboxylate (0.6 g, 2.2 mmol) in acetone (10 mL) was added a solution of 3-(dimethylamino)propylamine (0.3 mL, 2.2 mmol) in acetone (5 mL), and the solution was allowed to stir overnight. The solution was concentrated in vacuo. Chromatography on silica gel (ethyl acetate:methanol: hexane, 5:5:2) afforded **9** as a white solid (0.5 g, 72%), mp 65 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.73 (t, 1 H, NH, J = 5.3 Hz), 8.00 (d, 2 H, aryl CH, J = 8.4 Hz), 7.91 (d, 2 H, aryl, J = 8.5 Hz), 7.50 (s, 1 H, NH_2), 3.30 (q, 2 H, CH_2, J = 6.5 Hz), 2.27 (t, 2 H, CH_2, J = 7.1 Hz), 1.67 (q, 2 H, CH_2, J = 7.0 Hz), 2.13 (s, 6 H, CH_3); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 165.2, 146.2, 137.6, 127.8, 125.7, 56.9, 45.1, 38.0, 26.9; HRMS (FAB) m/e 286.1236 $(M + H)^+$, calcd for $C_{12}H_{19}N_3SO_3$ 286.1225.

3-[[4-(Aminosulfonyl)benzoyl]amino]-*N,N,N*-trimethyl-1-propanaminium Iodide (10). To a solution of **9** (115 mg, 0.4 mmol) in methanol (10 mL) at room temperature was added methyl iodide (0.4 mL, 6.0 mmol). The solution was stirred for 5 h and concentrated in vacuo. Recrystallization from ethanol afforded **10** as a white solid (79 mg, 46%), mp 175 °C: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.76 (t, 1 H, NH, J = 5.2 Hz), 8.00 (d, 2 H, aryl CH, J = 7.6 Hz), 7.89 (d, 2 H, aryl CH, J = 7.6 Hz), 7.49 (s, 2 H, NH_2), 3.36 (s, 4 H,

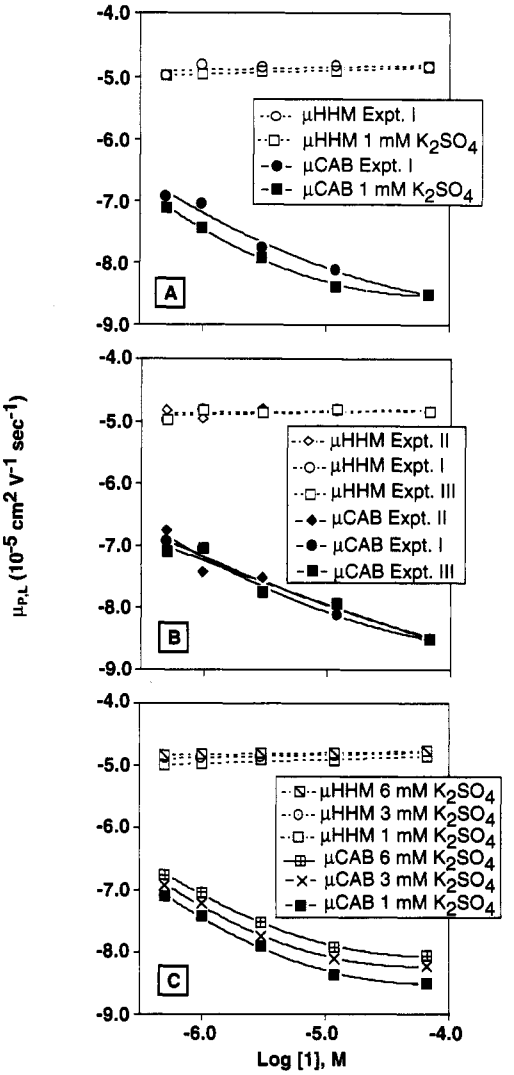
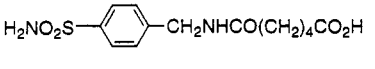


Figure 3. (A) Change in the electrophoretic mobilities of CAB and horse heart myoglobin (HHM) on increasing the concentration of **1** for experiment I and experiment at 1 mM K_2SO_4. (B) Change in electrophoretic mobilities of CAB and horse heart myoglobin (HHM) on increasing the concentration of **1** for experiments I–III. (C) Effect of increasing the concentration of K_2SO_4 on the electrophoretic mobilities of CAB and horse heart myoglobin (HHM) on increasing the concentration of **1**.

CH_2), 3.06 (s, 9 H, CH_3), 1.96 (m, 2 H, CH_2); ^{13}C NMR (100.6 MHz, DMSO-d_6) δ 165.4, 146.3, 137.1, 127.9, 125.5, 63.5, 52.3, 36.3, 22.8; HRMS (positive ion FAB) m/e 300.1391 $(M)^+$, calcd for $C_{13}H_{22}N_3SO_3$ 300.1382.

RESULTS AND DISCUSSION

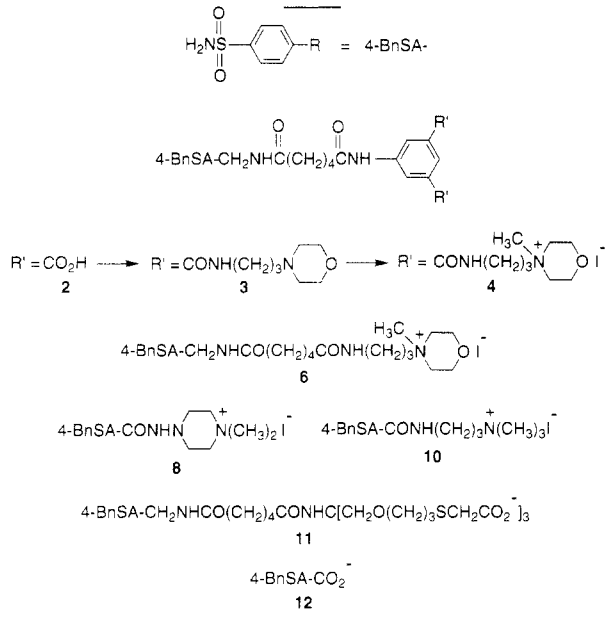
We tested eq 8 using two types of experiments. In the first, we conducted a study of the binding of **1**[3,4] to carbonic



1

anhydrase B (CAB) in which we intentionally caused the electroosmotic flow to vary by changing the ionic composition of the buffer. In this set of experiments, we made up three sets of solutions containing **1**; each solution in a set contained an increasing concentration of **1** and randomly assigned

Scheme 1. Synthesis of Cationic Benzenesulfonamide (BnSA) Ligand 4 and Other Charged Compounds Used in This Study.



concentrations of potassium sulfate (1, 3 or 6 mM) to induce random changes in the electroosmotic flow (Table 1). The pH of the buffer (8.3) was sufficiently high that the $-CO_2H$ group of **1** was essentially completely ionized. Figure 1A presents the electropherograms from experiment I, showing the changes in the migration time of the CAB peak with changing concentration of charged ligand **1** in the electrophoresis buffer. Figure 1B presents the electropherograms obtained at 1 mM K_2SO_4. Figure 1C is a Scatchard plot of the data according to eq 9 using eq 8 to estimate changes in electrophoretic mobility.

Measurement of the change in electrophoretic mobility (eq 8) due to complexation resulted in the values for binding constants given in Table 2. Figure 2 compares the constants for binding (K_b) of **1** to CAB using eqs 8 and 10 and also gives the value of K_b obtained by an assay based on fluorescence.[4] This figure also compares values of $\Delta\mu_{P,L}{}^{max}$ obtained using eqs 8 and 10. K_b appears to be relatively insensitive to the approximation involved in going from eq 8 to eq 10; $\Delta\mu_{P,L}{}^{max}$ appears to be more sensitive, with eq 8 giving much more tightly clustered values. The values of K_b agree with that obtained by a competitive fluorescence assay ($0.52 \times 10^6\ M^{-1}$).[4] Using eq 4, we earlier estimated the value of the binding constant for **1** and CAB by ACE to be $0.48 \times 10^6\ M^{-1}$.[4]

Figure 3A shows the effect of K_2SO_4 on the electrophoretic mobilities of CAB and horse heart myoglobin (HHM) on increasing the concentration of **1** for the concentrations of K_2SO_4 used in experiment I (Table 1) and at a constant concentration of K_2SO_4 of 1 mM. Figure 3B,C is a summary of the effect of K_2SO_4 on the electrophoretic mobilities of CAB and HHM for experiments I–III and at constant salt concentration, respectively. On increasing the concentration of K_2SO_4, the electrophoretic mobility of CAB becomes more negative (Figure 3C). HHM shows a similar trend but to a lesser extent. The nearly horizontal line for HHM indicates that **1** has no affinity for HHM.

We also examined the binding of cationic ligand **4** (Scheme 1) to CAB. We examined **4** because it changed electroosmotic flow more than negatively charged and neutral species. Scheme 1 also shows other charged ligands examined in this study.

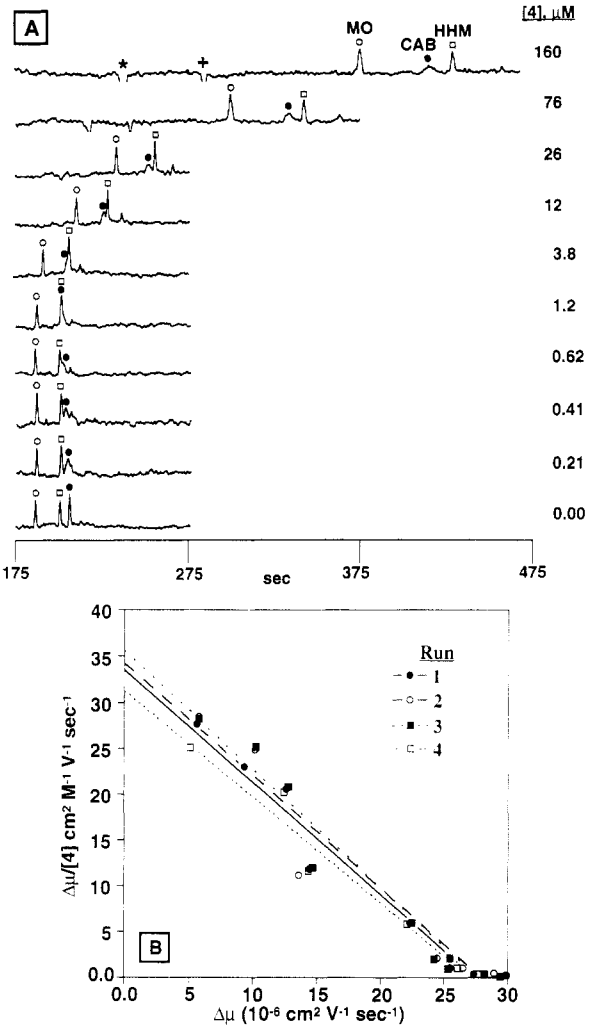


Figure 4. Affinity capillary electrophoresis (ACE) of bovine carbonic anhydrase B (CAB) in 0.192 M glycine–0.025 M Tris buffer (pH = 8.3) containing various concentrations of **4**. The total analysis time in each experiment was 6.0 min (10.0 min for the final data point) at 30 kV using a 50-cm (inlet to detector), 50-μm open, uncoated quartz capillary. Horse heart myoglobin (HHM) and mesityl oxide (MO) were used as internal standards. Four replicates were run for this experiment and are labeled as runs 1–4 in the legend. (A) Representative set of electropherograms using increasing concentrations of ligand in the buffer. (B) Scatchard plot of the data according to eq 9. The inverted peaks (* and +) are discussed in the text.

Figure 4A shows a representative series of electropherograms of CAB in buffer containing various concentrations of **4**.[20] Four electrophoresis runs were conducted at each concentration of **4**. Horse heart myoglobin (HHM) and mesityl oxide (MO) were used as internal standards. Figure 4B is a Scatchard plot of the data according to eq 9. The initial inverted peak (*) resulted from the dilution of **4** present in the electrophoresis buffer at the point of injection. The second inverted peak (+) may be due to the mono-*N*-methylated morpholino species as an impurity. This species was not observed by NMR. Pronounced changes in elec-

(20) We are able to resolve the peaks for horse heart myoglobin (HHM) and carbonic anhydrase B (CAB) prior to the transfer of data from the capillary electrophoresis system to the computer.

(21) Krebs, H. A. *Biochem. J.* **1948**, *43*, 525–528.

Table 3. Values of Binding Constants K_b (10^6 M^{-1}), $\Delta\mu_{P,L}^{max}$ (10^{-5} cm^2 V^{-1} s^{-1}), $\delta\mu_{P,L}$, μ_L (10^{-4} cm^2 V^{-1} s^{-1}), and L (10^{-19} cm^2 $Da^{2/3}$ V^{-1} s^{-1}) of Ligands and Carbonic Anhydrase B Measured by Affinity Capillary Electrophoresis (ACE)[a]

		eq 8		eq 10	eq 8	eq 15	eq 18		
ligand	z	K_b	$\Delta\mu_{P,L}^{max}$	$\Delta\mu_{P,L}^{max}$	$\delta\mu_{P,L}^{max}$	$\delta\mu_{P,L}^{max}$	$\delta\mu_{P,L}^{max}$	L	μ_L
1	−1	0.60	−1.9	−2.1	0.28	0.29	0.30	12	−1.9
4	+2	2.0	2.5	32	−0.49	−0.61	*b*	*b*	*b*
6	+1	1.2	1.7	1.1	−0.27	−0.31	−0.30	12	1.4
8	+1	2.0	2.5	3.8	−0.31	−0.31	−0.30	10	1.5
10	+1	1.1	1.9	6.0	−0.28	−0.31	−0.30	11	1.6
11[c]	−3	1.7	−6.0	−4.6	0.82	0.87	0.91	13	−3.2
12[d]	−1	0.80	−1.8	−2.6	0.28	0.30	0.30	13	−2.7

[a] For carbonic anhydrase B, we estimate $C = 27$, where C is 10^{-19} cm^2 $Da^{2/3}$ V^{-1} s^{-1}. [b] Not measured. [c] Previous estimate:[4] $K_b = 0.22 \times 10^6$ M^{-1}. This was estimated by the use of eq 4. [d] Previous estimate:[21] $K_b = 5.0 \times 10^6$ M^{-1}.

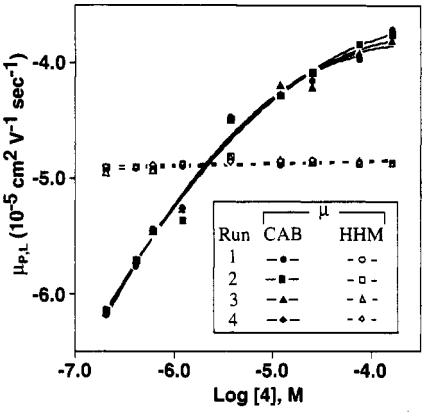


Figure 5. Change in the electrophoretic mobilities of CAB and HHM on increasing the concentration of **4**. Four replicates were run for this experiment and are labeled as 1–4 in the legend.

troosmotic flow are observed at high concentrations of **4** in the buffer. The binding constant obtained was 1.24×10^6 M^{-1}. We were *unable* to estimate the values of a binding constant using eq 10: the Scatchard plot was dramatically nonlinear.

Figure 5 shows the changes in electrophoresis mobilities of CAB and HHM on increasing the concentration of **4**. The approximately horizontal line for HHM indicates that **4** has no affinity for HHM and that analysis using eq 7 effectively compensates for changes in EO flow. Figure 5 also validates the use of a neutral internal standard to correct for variable rates of electroosmotic flow.

Comparison of Observed and Predicted Values of $\Delta\mu_{P,L}$. Table 3 shows the values for binding constants $\Delta\mu_{P,L}^{max}$, $\delta\mu_{P,L}^{max}$, and μ_L obtained by eq 8 for several inhibitors of CAB, values for C and L obtained by eqs 1 and 2, values of $\Delta\mu_{P,L}^{max}$ estimated experimentally using the approximate eq 10, and the values for $\delta\mu_{P,L}^{max}$ estimated on the basis of the charges and masses of protein and ligand using eqs 16 and 19.

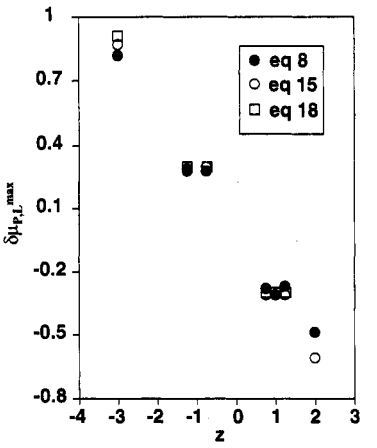


Figure 6. Plot of the change in electrophoretic mobility of the protein ($\delta\mu_{P,L}^{max}$ from eq 8 (●), eq 15 (○), and eq 18, □) vs the charge z of the ligand.

The use of eq 10 realizes values for $\Delta\mu_P^{max}$ over a broader range, albeit of the same magnitude, as those obtained using eq 8. This larger deviation from the estimated value for $\Delta\mu_P^{max}$ is due to the inability of eq 10 to correct for variable electroosmotic flow.

The experimental values obtained for $\Delta\mu_{P,L}^{max}$ using eq 8 are internally consistent; they agree remarkably well with those estimated from charge and mass using eq 15 and from eq 18. A plot of the values of $\delta\mu_{P,L}^{max}$ as a function of charge (Figure 6) shows the expected proportionality. This agreement supports the value of eq 9 for the determination of K_b and $\Delta\mu_{P,L}^{max}$.

CONCLUSIONS

Binding constants between CAB and charged benzenesulfonamides may be estimated by ACE under conditions in which the migration time is affected by changes in electroosmotic flow resulting from changes in the concentration of ligand in the buffer and from other characteristics of the experiment. By measuring the rate of migration of a neutral internal standard, it is possible to correct for the variable rate of electroosmotic flow empirically with sufficient accuracy to give accurate values of K_b. The ability to *estimate* the change in mobility $\delta\mu_{P,L}^{max}$ on the basis of charges and masses of protein and ligands provides a useful check on the experimental results.

ACKNOWLEDGMENT

F.A.G. thanks Jinming Gao for the procedure for the preparation of compound **2**.

Received for review September 24, 1993. Accepted March 8, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, April 15, 1994.