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### Use of Affinity Capillary Electrophoresis To Measure Binding Constants of Ligands to Proteins<sup>1</sup>

This paper outlines the use of affinity capillary electrophoresis<sup>2,3</sup> (ACE) as a technique for measuring binding constants of proteins for ligands. We illustrate this use with a model system comprising carbonic anhydrase B (CAB, EC 4.2.1.1, from bovine erythrocytes) and 4-alkylbenzenesulfonamides.<sup>4</sup> The principle of the method is illustrated schematically in eq 1.



Mass	M	m	M + m
Net Charge	Z	± z	Z ± z
Electrophoretic Mobility $\mu$	$-Z / M^{2/3}$		$-(Z \pm z) / (M + m)^{2/3}$

The electrophoretic mobility  $\mu$  of a protein is related to its mass ( $M$ ) and net charge ( $Z$ ) by a relationship of the approximate form  $\mu \sim Z/M^{2/3}$ .<sup>5</sup> If the protein binds a charged ligand of relatively small mass, the change in  $\mu$  due to the change in mass [from  $M^{2/3}$  to  $(M + m)^{2/3}$ ] is small relative to the change in  $\mu$  due to the change in charge (from  $Z$  to  $Z \pm z$ ). Thus, the protein-ligand complex will migrate at a different rate than the uncomplexed protein.<sup>6</sup> By measuring migration times ( $t$ ) as a function of the concentration of charged ligand present in the buffer, it is possible to estimate  $K_b$ . These measurements are best carried out by measuring changes in  $t$  relative to another protein having a similar value of migration time

- (1) This research was supported by the NSF through the M.I.T. Biotechnology Processing Engineering Center (Cooperative Agreement CDR-88-03014) and by the NIH (GM 39589 and GM 30367).
- (2) Reviews of capillary electrophoresis of proteins: Mazzeo, J. R.; Krull, I. S. Coated Capillaries and Additives for the Separation of Proteins by Capillary Zone Electrophoresis and Capillary Isoelectric Focusing. *BioTechniques* 1991, 10, 638-645. Novotny, M. V.; Cobb, K. A.; Liu, J. Recent Advances in Capillary Electrophoresis of Proteins, Peptides, and Amino Acids. *Electrophoresis* 1990, 11, 735-749.
- (3) Reviews of affinity electrophoresis (AE): Takeo, K. Affinity Electrophoresis. In *Advances in Electrophoresis*; Chrambach, A., Dunn, M. J., Radola, B. J., Eds.; VCH Publishers: New York, 1987; Vol. 1, pp 229-279. Shimura, K. Progress in Affinity Electrophoresis. *J. Chromatogr.* 1990, 510, 251-270. Horejsi, V.; Ticha, M. Qualitative and Quantitative Applications of Affinity Electrophoresis for the Study of Protein-Ligand Interactions: A Review. *J. Chromatogr.* 1986, 376, 49-67.
- (4) Dodgson, S. J.; Tashian, R. E.; Gros, G.; Carter, N. D. *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*; Plenum Press: New York, 1991.
- (5) This expression is approximate. Other functional forms have been suggested: Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. Correlation of Electrophoretic Mobilities From Capillary Electrophoresis With Physicochemical Properties of Proteins and Peptides. *Anal. Biochem.* 1991, 197, 197-207. Grossman, P. D.; Colburn, J. C.; Lauer, H. H. A Semiempirical Model for the Electrophoretic Mobilities of Peptides in Free-Solution Capillary Electrophoresis. *Anal. Biochem.* 1989, 179, 28-33. Deyl, Z.; Rohlicek, V.; Adam, M. Separation of Collagens by Capillary Electrophoresis. *J. Chromatogr.* 1989, 480, 371-378. Offord, R. E. Electrophoretic Mobilities of Peptides on Paper and Their Use in the Determination of Amide Groups. *Nature* 1966, 211, 591-593.
- (6) If  $\mu_b$  and  $t_b$  are the values of electrophoretic mobility and migration time of the protein-ligand complex, and  $\mu_0$  and  $t_0$  are these values for free protein, eq 1 allows an estimate of the change in mobility or migration time for given values of  $Z$  and  $z$ .

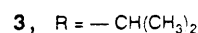
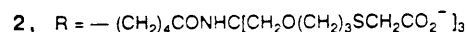
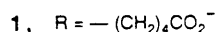
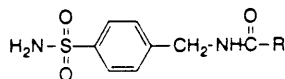
$$\begin{aligned} (\mu_b - \mu_0) / \mu_0 &= \\ (t_b - t_0) / t_0 &\approx [(z/Z) + 1][M/(M + m)]^{2/3} - 1 \approx z/Z \end{aligned} \quad (i)$$

that does not bind the ligand. Equation 2 gives a convenient form for Scatchard analysis:

$$(\delta\Delta t / \delta\Delta t_{\max})(1/[L]) = K_b - K_b(\delta\Delta t / \delta\Delta t_{\max}) \quad (2)$$

here  $\Delta t_{[L]}$  is the difference between the migration time of the protein of interest and the reference protein at concentration  $[L]$  of the charged ligand,  $\delta\Delta t_{[L]} = \Delta t_{[L]} - \Delta t_{[L]=0}$ , and  $\delta\Delta t_{\max}$  is the value of  $\delta\Delta t_{[L]}$  at saturating concentrations of  $L$ .

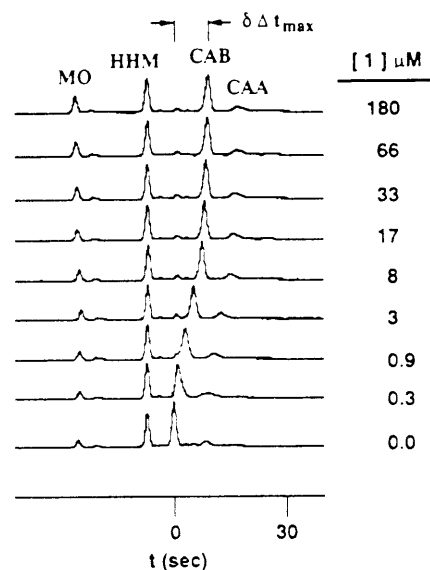
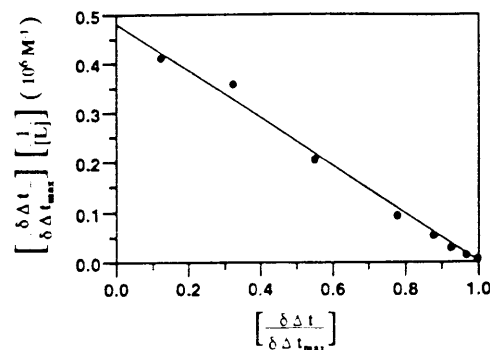
We used CAB as a model protein with which to demonstrate this method and the aryl sulfonamides 1 and 2



as affinity ligands.<sup>4</sup> The samples of CAB also contained carbonic anhydrase A (CAA). We selected CAB for three reasons: it shows little adsorption on the walls of uncoated capillaries,<sup>7</sup> and is therefore a particularly convenient protein with which to work; it accepts a wide structural range of benzenesulfonamides as inhibitors; it is readily available and structurally well-characterized.

Figure 1 shows a representative series of electropherograms of CAB in buffer containing various concentrations of 1 and the Scatchard plot (eq 2) derived from these data. The appearance times<sup>8</sup> of horse heart myoglobin (HHM) and mesityl oxide (MO), used as internal standards in these experiments, were independent of the concentration of 1 and 2. Binding constants estimated by ACE ( $K_b = 0.48 \times 10^6 M^{-1}$  for 1,  $0.22 \times 10^6 M^{-1}$  for 2) agree well with those obtained from a competitive fluorescence-based assay<sup>9</sup> ( $0.51 \times 10^6 M^{-1}$  for 1,  $0.14 \times 10^6 M^{-1}$  for 2). CAA, a protein having binding constants very similar to those of CAB,<sup>4</sup> gives values of  $K_b$  estimated by ACE that are indistinguishable from those of CAB (Figure 1). The electrically neutral 3 used as the control ligand did not change the electrophoretic mobilities of CAB and CAA.<sup>10</sup>

Similar studies with proteins other than CAB have established that ACE is, in principle, a general method (although, in practice, it is limited to the subset of proteins that do not adsorb on the wall of the capillary). The mobility of calmodulin (from bovine testes) varies with the



**Figure 1.** Affinity capillary electrophoresis (ACE) of bovine carbonic anhydrase B (CAB) in 0.192 M glycine–0.025 M tris buffer (pH 8.4) containing various concentrations of 1. The total analysis time in each experiment was  $\sim 5.5$  min at 30 kV using a 70-cm (inlet to detector), 50- $\mu$ m open quartz capillary. Horse heart myoglobin (HHM) and mesityl oxide (MO) were used as internal standards. The graph is a Scatchard plot of the data according to eq 2.

concentration of  $Ca^{2+}$ ; analysis of this variation indicates  $K_b = 0.47 \times 10^6 M^{-1}$ ; the literature values from equilibrium dialysis analysis are 0.1–1.0  $\times 10^6 M^{-1}$ .<sup>11</sup> Mobility of glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) varies with the concentration of both  $NADP^+$  and  $NADPH$ ; analysis of these variations gives  $K_b(NADP^+) = 0.03 \times 10^6 M^{-1}$  (lit.<sup>12</sup>  $K_b = 0.33 \times 10^6 M^{-1}$  by fluorescence analysis) and  $K_b(NADPH) = 0.01 \times 10^6 M^{-1}$  (lit.<sup>12</sup>  $K_b = 0.04 \times 10^6 M^{-1}$ ). We have also examined a number of other proteins, including one antibody (MOPC 315). The results indicate that, although adsorption on the wall of uncoated capillary is a potential problem with all proteins, those having a  $pI$  significantly lower than the pH of the buffer are plausible candidates for analysis by

(7) Most proteins adsorb on the walls of uncoated capillaries.<sup>2</sup> This problem of protein adsorption can, in most cases, be solved by using some combination of coated capillaries and zwitterionic buffers. See: Towns, J. K.; Regnier, F. E. Capillary Electrophoretic Separation of Proteins Using Nonionic Surfactant Coatings. *Anal. Chem.* 1991, 63, 1126–1132. Bushey, M. M.; Jorgenson, J. W. Capillary Electrophoresis of Proteins in Buffers Containing High Concentrations of Zwitterionic Salts. *J. Chromatogr.* 1989, 480, 301–310.

(8) In capillary zone electrophoresis, migration times are determined by a combination of electrophoretic migration and electroosmotic flow (EOF). At pH values greater than 6, the EOF is sufficiently high to ensure a net migration toward the cathode for most analytes, regardless of their charge.

(9) The homogeneous assay followed modifications of the procedure of Chen, R.; Kernhan, J. Combination of Bovine Carbonic Anhydrase With a Fluorescent Sulfonamide. *J. Biol. Chem.* 1967, 242, 5813–5823.

(10) The binding constant of 3 to CA was  $0.25 \times 10^6 M^{-1}$ , measured by the fluorescence assay.

(11) Klee, C. B. Interaction of Calmodulin With  $Ca^{++}$  and Target Proteins. In *Calmodulin*, *Molecular Aspects of Cellular Regulation*; Cohen, P., Klee, C. B., Eds.; Elsevier: New York, 1988; pp 35–56. Kajiwara, H. Application of High-Performance Capillary Electrophoresis to the Analysis of Conformation and Interaction of Metal-Binding Proteins. *J. Chromatogr.* 1991, 559, 345–356.

(12) Grove, T. H.; Ishaque, A.; Levy, H. R. Glucose-6-Phosphate Dehydrogenase From *Leuconostoc mesenteroides*: Interaction of the Enzyme With Coenzymes and Coenzyme Analogs. *Arch. Biochem. Biophys.* 1976, 177, 307–316.

ACE using uncoated capillaries.<sup>13</sup>

Affinity capillary electrophoresis has six advantages as a method of determining binding constants. First, it requires only small quantities of protein and ligand: the complete series of experiments in Figure 1 (with five replicates of each experiment) consumed ~22 ng of carbonic anhydrases (CAB + CAA), and 1.3 mg of 1. Second, it does not require high purity for the protein or an accurate value of its concentration, since values of  $K_b$  are based on migration times, not peak areas. Measurement of  $K_b$  can, as a result of the high resolving power of capillary electrophoresis, be carried out on mixtures of proteins. Third, it is applicable simultaneously to several proteins in the same solution (for example, CAA and CAB in Figure 1). Fourth, it does not require the synthesis of radioactive or chromophoric ligands, although (as with 1 and 2) it will

(13) Rapid equilibration of protein between the buffer and the wall of the capillary does not influence the value of  $K_b$  obtained by this method, unless the equilibration is influenced by the concentration of L or  $K_b$  is substantially different for adsorbed and soluble protein.

require the synthesis of a charged analog of a ligand if the ligand is itself electrically neutral. Fifth, it is capable of distinguishing forms of a protein that bind ligand from forms of the same protein that are denatured and do not bind ligand. Sixth, the commercial availability of automated instrumentation, and the high reproducibility of data, make it experimentally convenient.

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**Supplementary Material Available:** Experimental details for the preparation of 1-3 (5 pages). Ordering information is given on any current masthead page.

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