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Affinity Electrophoresis in Multisectional Polyacrylamide Slab Gels Is a Useful and Convenient Technique for Measuring Binding Constants of Aryl Sulfonamides to Bovine Carbonic Anhydrase B

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This paper describes convenient preparations of heterogeneous multisectional polyacrylamide slab gels and the protocols that use these gels to measure protein–ligand binding constants [using bovine carbonic anhydrase B (CAB) as a model system]. Unlike procedures for affinity electrophoresis using tube gels, all binding information concerning protein–ligand interactions was encoded in a single multisectional gel: the procedure involving for measuring binding constants required no postelectrophoresis manipulation of gels. Use of these types of gels improves the accuracy of affinity gel electrophoresis (AGE) by providing reliable internal protein standards. Binding constants measured by AGE agree with those determined in homogeneous solution by spectrophotometric measurements. This technique has been used to investigate the influence of the length of the spacer separating the ligand and the polyacrylamide backbone on the binding constants. Dissociation constants obtained using the affinity gels approach the values measured in free solution, when the spacer is sufficiently long (≥18 Å); affinity ligands having short spacers give high apparent dissociation constants.

INTRODUCTION

The work reported here is directed toward evaluating affinity gel electrophoresis as a technique for use in rational drug design. Rational drug design[3] is based on the promise that by understanding both the general principles underlying drug–receptor interactions and the specific details of structure of a protein of interest it will be possible to *design* molecules that bind tightly at the active site of the protein more rapidly than it would be possible to discover them by screening.[4] An important component of rational drug design is the development of efficient assays for binding.

Affinity gel electrophoresis (AGE) is a well-established technique,[5] but one that, in practice, is seldom used. We believe that it has many characteristics that recommend it for use in measuring binding constants to proteins, particularly in the context of programs in rational drug design. First, AGE is a technique that permits measurement of binding constants using only small quantities of proteins. Using a sensitive protein stain (e.g., silver[6]), less than 0.5 μg of protein is sufficient to obtain a binding constant. Second, AGE does

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not require radioactive or spectrophotometric ligands or substrates. Third, it requires relatively small quantities of affinity ligands. Fourth, it is applicable to the assay of binding constants in mixtures of proteins. It can also be used to identify proteins in mixtures of proteins by their ability to bind to a particular ligand. Fifth, AGE, like most forms of native gel electrophoresis, is potentially applicable to a very wide range of proteins.

Why has affinity gel electrophoresis not been more widely used? First, it places substantial demands on synthesis and is thus inconvenient for many biological laboratories. Both an appropriate affinity ligand, suitably functionalized to be incorporated into the gel, and the gel itself, must be prepared. Further, the gel slabs used are often more readily interpreted if they are compositionally heterogeneous (see below). Preparing these types of gels is more complex than preparing homogeneous slab gels. Second, in order to retain the native conformation of the protein, there is no SDS present during electrophoresis. In the absence of a charged surfactant that associates with the proteins, the electrophoretic mobility of the proteins in the gel is low. AGE consequently takes longer to carry out (typically 12 h, in the case of carbonic anhydrases) than SDS gel electrophoresis (typically 2 h). Finally, fundamental principles underlying the design and interpretation of the experiments are not well-established. How should the affinity ligand be connected to the gel? What is the influence of the spacer-connecting ligand and gel matrix on the measured binding constant? How close is the binding constant measured by AGE to that measured in homogeneous solution?

The objective of the work reported here was to develop several of the technical aspects of AGE in a way that would make it a more useful technique for determining binding constants. Using carbonic anhydrase B (CAB, EC 4.2.1.1, bovine erythrocytes) as a model protein, we have examined the influence of the spacer group linking polymer matrix and affinity ligand on the measured binding constant. We have compared binding constants obtained by AGE with those in relevant homogeneous media. We have also developed convenient preparations of multisectional slab gels. Use of these gels improves the accuracy of AGE by providing reliable internal standards. In short, we have developed the chemical aspects of AGE, optimizing gel manipulation and interpretation.

We chose CAB from bovine erythrocytes as the protein for this study for several reasons. This enzyme is well-characterized,[7] commercially available, inexpensive, and medicinally relevant.[8] The single-crystal X-ray structure determination of carbonic anhydrase has been carried out at 2.0-Å resolution.[7] A broad range of aryl sulfonamides inhibit the enzyme.[7] The binding of aryl sulfonamides to the active site of the enzyme is well-understood:[7,10] the active site is a cavity, approximately 15 Å deep and 15 Å wide, containing a zinc ion coordinated to three histidine residues located at its bottom. The aryl sulfonamide group (in its anionic form, $ArSO_2NH^-$) binds to this zinc ion, with its nitrogen 3 Å from zinc.[10] The dissociation constants of complexes of aryl sulfonamides and CAB range from 10^{-6} to 10^{-9} M.[7]

The application of affinity gel electrophoresis to the measurement of dissociation constants of protein–ligand complexes has been studied previously.[5] The dissociation constant in the gel (K_d^{gel}) is defined by eqs 1–4. K_d^{gel} is the

$$K_d = [P][L]/[PL] \quad (1)$$

$$R_f = r/R_0 = [P]/([P] + [PL]) \quad (2)$$

$$1/R_f = 1 + ([L]/K_d) \quad (3)$$

$$[L]R_f = K_d - K_dR_f \quad (4)$$

value for the concentration of the affinity ligand [L] in the gel at which the sample protein (P) migrates half of the distance that it would in the absence of an affinity ligand in the gel. R_0 and r represent the protein mobility in the absence and the presence of affinity ligand. K_d obtained by this method is an *apparent* dissociation constant (K_d^{gel}) and may not be equal to the dissociation constant (K_d^{sol}) in solution. Because a ligand bound to the polymer gel matrix might not be as accessible to protein as the same ligand free in solution, K_d^{gel} would normally be expected to be larger than K_d^{sol}. Energetically favorable interactions between protein and the matrix or cooperative, multivalent interactions between protein and ligand (and/or gel) might, however, make K_d^{gel} smaller than K_d^{sol}.

EXPERIMENTAL SECTION

Materials. Bovine carbonic anhydrase B (CAB) and bovine carbonic anhydrase (CA), consisting of a mixture of A and B isozymes, and bovine pancreatic trypsin inhibitor (BPTI) were obtained from Sigma. Materials for the syntheses of affinity ligands **1**–**19** were available from Aldrich, Fluka, Bachem Bioscience, or Sigma. Di-*tert*-butyl iminodicarbonate was prepared according to the method of Grehn and Ragnarsson.[11] Affinity electrophoresis (AE) was carried out with a SE 250 Mighty Small II slab gel electrophoresis apparatus (Hoefer Scientific Instruments). Polyacrylamide (average molecular weight 12 000) was available from Polyscience. Hemagglutinin from influenza virus (strain X-31) was a gift of N. K. Sauter and Professor D. C. Wiley (Department of Biochemistry and Molecular Biology, Harvard University). The protein sample of bovine blood, obtained from the local slaughterhouse, was prepared according to a modified method of Lindskog.[12] The plasma was separated from the red cells by centrifugation for 20 min at 2500*g*. The erythrocytes were lysed by addition of distilled water to the washed red cells, and the insoluble cell particulates were removed by centrifugation at 8000*g* for 30 min. The centrifuged hemolysate and the plasma were recombined.

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Scheme II. Polyacrylamide gels (15% T;[13] gel size, 8 × 7 cm) were prepared according to the standard protocol of Margolis et al.[14] in tris–borate buffer at pH 8.5 (without EDTA). Acrylamide monomer solutions (2.0 mL) containing known concentrations (0–300 μM) of affinity ligands were polymerized in the presence of ammonium persulfate (12 μL; 10%, w/v) and *N,N,N′,N′*-tetramethylethylenediamine (TEMED, 2 μL). For each sectional affinity gel, the polymerization was carried out at room temperature for 20 min. The gel cassette was disassembled. This multisectional gel containing various concentrations of affinity ligands was soaked in tris–borate buffer at pH 8.5 (2 × 500 mL) for a total time of 24 h. The gel expanded consistently 1.165 times in its linear dimensions and became 9.5% T after the change of volume of the gel was taken into account. The soaked gel was reassembled between glass plates and rotated by 90°. A stacking gel (3 or 5% T) containing no affinity ligand was polymerized on top of the affinity gel. Protein samples (0.1 μg for silver detection and 1.1 μg for Coomassie Blue staining) were applied to the gels and run at 45 V for 13 h. Migration distances were measured after staining gels with silver or Coomassie Blue.[6]

For the competitive inhibition experiments, affinity gels were prepared as described above. Gels were soaked first in 90 mM tris–borate buffer (400 mL) for 12 h and then soaked in a second solution of 90 mM tris–borate buffer containing 8.0 μM soluble ligands (400 mL) for another 12 h.

Fluorescence Spectroscopic Determination of K_d of Complexes of CAB and the Ligands 17–19. Fluorescence experiments were carried out following a modified procedure of Chen and Kernohan[15,16] using a Perkin-Elmer Model MPF-4 spectrofluorometer. We used this competitive fluorescence-based assay to determine binding strengths of CAB with soluble ligands **17–19** (eqs 5–8): titration of CAB with a fluorescent inhibitor

$$K_d^L = [P][L]/[PL] \quad (5)$$

$$K_d^l = [P][l]/[Pl] \quad (6)$$

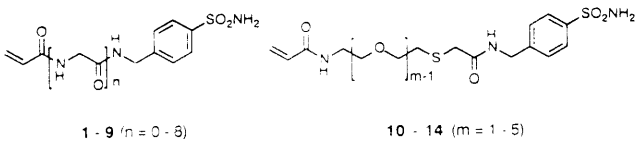
$$[P]_{total} = [P] + [PL] + [Pl] \quad (7)$$

$$[Pl]/([l][P]_{total}) = (1/K_d^l) - (1/K_d^l)(([PL] + [Pl])/[P]_{total}) \quad (8)$$

5-(dimethylamino)-1-naphthalenesulfonamide (dansylamide, DNSA),[16] followed by competition for the binding site between DNSA and ligands **17–19**. Figure 4 shows representative data. L and l represent the DNSA and the soluble ligands **17–19**. K_d^L is the dissociation constant of the DNSA–CAB complex in solution, and K_d^l is the dissociation constant (K_d^{sol}) of the soluble ligand–CAB complex in solution.

RESULTS AND DISUSSION

Synthesis of Affinity Ligands. Two types of spacer groups (oligoglycine and oligo(ethylene glycol)) were used for this study (**1–9**; **10–14**). We chose oligoglycyl peptides as one set of spacers because they are rigid, uncharged, conformationally extended,[17] and commercially available, and oligoethylene glycols as the other set of spacers because they are flexible, uncharged, conformationally random coil,[18] readily soluble in aqueous buffer, biocompatible,[19] and commercially available.



1 - 9 (n = 0 - 8) 10 - 14 (m = 1 - 5)

We used two synthetic routes to prepare affinity ligands **1–9** (Scheme IA,B). For the short, readily water soluble affinity ligands **2** and **3**, syntheses were carried out in organic solvents with moderate yields (overall yield: 74%, **2**; 45%, **3**) (Scheme IA). Synthesis of ligand **1** containing no glycine spacer was accomplished by reaction of *p*-(aminomethyl)-benzenesulfonamide with acryloyl chloride in DMF (91% yield). Affinity ligands **4–9** were prepared in two steps (Scheme IB): acylation of oligoglycine with acryloyl chloride under Schotten–Baumann conditions, and coupling with a sulfonamide group using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) in water (overall yield: 87%, **4**; 79%, **5**; 96%, **6**; 67%, **7**; 75%, 8; 70%, **9**). Compounds **1–9** are solids that can be recrystallized from water.

The synthesis of ligands **10–14** from the aryl sulfonamide was also straightforward, although it involved several steps

(13) % T and % C is used here, as defined by Hjerten. % T is the total concentration of monomer in grams per liter—the number of grams of monomer added to 100 mL of water—and % C is the corresponding concentration of the cross-linker *N,N*-methylenebisacrylamide: Hjerten, S. *Arch. Biochim. Biophys.* **1962**, *Supplement 1*, 147–151.

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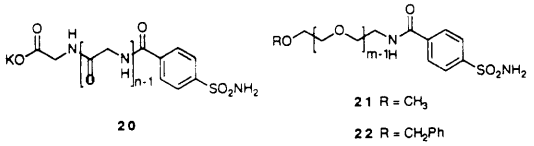

20

21 R = CH_3

22 R = CH_2Ph

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(17) According to the X-ray studies, polyglycine exists in two crystalline forms known as **I** (**PGI**, β form) and **II** (**PGII**, helical form). In the case of oligoglycines, studies by IR, Raman, and vibrational spectra analysis suggested that glycine through pentaglycine is in the **PGI**-like structure whereas the transition from the **PGI**-like structure to the **PGII**-like one starts at the hexaglycine and a complex helix is formed at the dodecaglycine: Dwivedi, A. M.; Gupta, V. D. *Chem. Phys. Lett.* **1971**, *8*, 220–222. Smith, M.; Walton, A. G.; Koening, J. L. *Biopolymers* **1969**, *8*, 29–43. Gupta, V. D.; Gupta, M. K.; Nath, K. *Biopolymers* **1975**, *14*, 1987–1990. Randhawa, H. S.; Rao, C. N. R. *J. Cryst. Mol. Struct.* **1973**, *3*, 309–321. X-ray crystal structure analysis of di- and triglycines confirmed their extended *trans*-planar conformation: Narasinga Rao, S.; Parthasarathy, R. *Acta Crystallogr.* 1973, *B29*, 2379–2388. Srikrishnan, T.; Winiewicz, N.; Parthasarathy, R. *Int. J. Pept. Protein Res.* **1982**, *19*, 103–113. Recent studies on vibrational spectral analysis of *N*-acylglycine oligomers showed that only a conformation similar to **PGII** exists in the solid state and in aqueous solution for these molecules, and the long acyl groups further induce (promote) a **PGII**-like structure in peptide skeletons: Okabayashi, H.; Ohshima, K.; Etori, H.; Taga, K.; Yoshida, T.; Nishio, E. *J. Phys. Chem.* **1989**, *93*, 6638–6642. Okabayashi, H.; Oshima, K.; Etori, H.; Debnath, R.; Taga, K.; Yoshida, T.; Nishio, E. *J. Chem. Soc., Faraday Trans.* **1990**, *86*, 1561–1567.

(18) Results from spectroscopic studies such as NMR (Matsuzaki, K.; Ito, H. *J. Polym. Sci., Part B: Polym. Phys.* **1974**, *12*, 2507–2520. Okada, T. *J. Polym. Sci., Part A: Polym. Chem.* **1979**, *17*, 155–162), Raman (Bartlett, J. R.; Cooney, R. P. *J. Chem. Soc., Faraday Trans. 1* **1986**, *82*, 597–605), and X-ray structural analysis (Tadokoro, H.; Chatani, Y.; Yoshihara, T.; Tahara, S.; Murahashi, S. *Makromol. Chem.* **1964**, *73*, 109–127) showed that in the solid state the *trans, trans, gauche* conformation for the poly(ethylene glycol) (PEG) segment $CH_2OCH_2CH_2$ is the preferred structure corresponding to a helical arrangement of the chain with a repeat distance of seven monomer units comprising two helical turns. It was reported that the conformation of PEG in aqueous solution retains to a large degree the *trans, trans, gauche* sequence characteristic of crystalline PEG: Liu, K.-J.; Parsons, J. L. *Macromolecules* **1969**, *2*, 529–533. Liu, K.-J.; Ullman, R. *J. Chem. Phys.* **1968**, *48*, 1158–1168. Liu, K.-J.; Anderson, J. E. *Macromolecules* **1969**, *2*, 235–237. Koenig, J. L.; Angood, A. C. *J. Polym. Sci., Part B: Polym. Phys.* **1970**, *8*, 1787–1796. In the liquid state, the internal rotation angles (*trans* and *gauche*) about the CH_2–CH_2 and the CH_2–O bonds of the molecular chain of ethylene glycol oligomers (monomer to heptamer) are, however, randomly distributed: Machida, K.; Miyazawa, T. *Spectrochim. Acta* **1964**, *20*, 1865–1873. Uchida, T.; Kurita, Y.; Koizumi, N.; Kubo, M. *J. Polym. Sci.* **1956**, *21*, 313–322.

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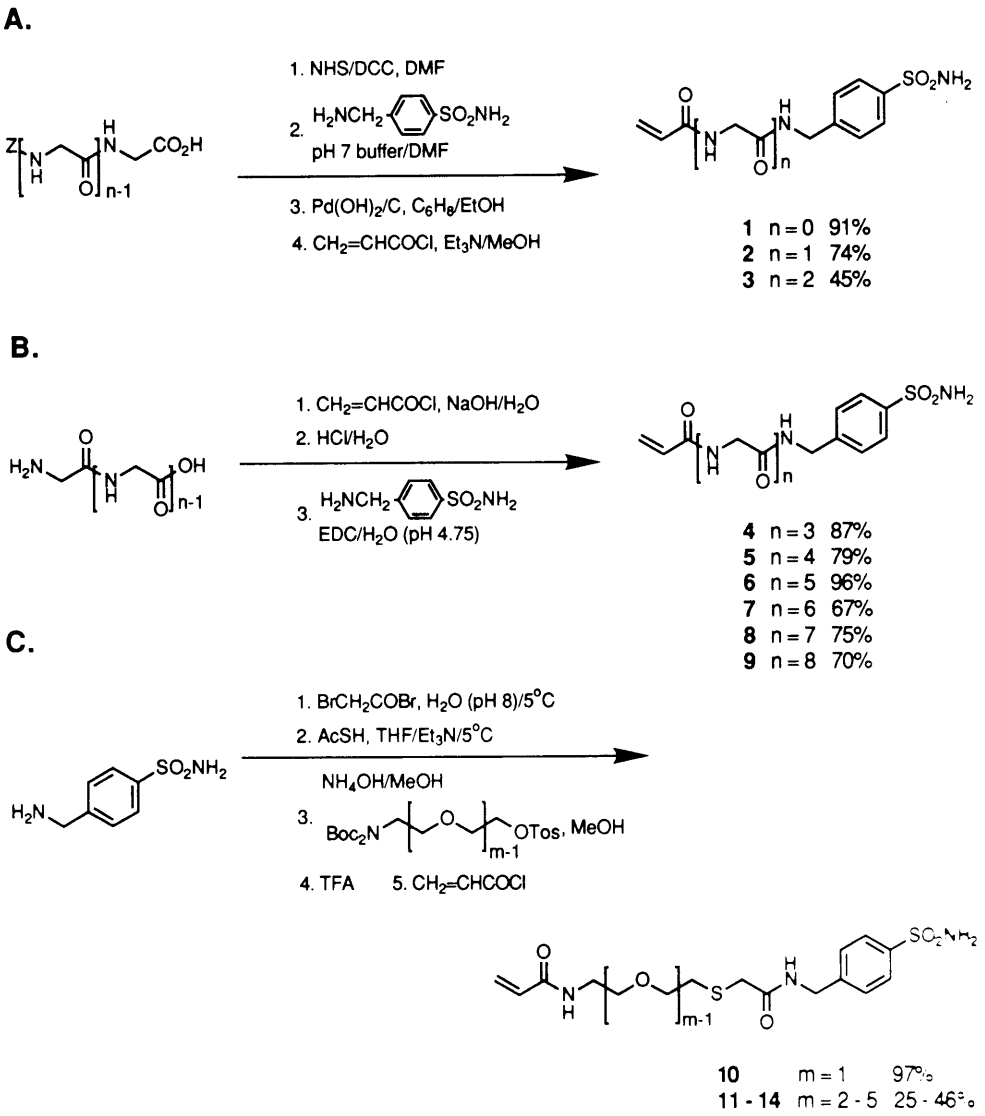
Scheme I. Synthesis of the Oligoglycine-Spaced Affinity Ligands 1–9 and the Oligo(ethylene glycol)-Spaced Affinity Ligands 10–14



(Scheme IC): acylation of *p*-(aminomethyl)benzenesulfonamide by 2-bromoacetyl bromide under Schotten–Baumann conditions, S-alkylation of the resulting 2-bromoacetamide by thioacetic acid, introduction of the monoprotected amine moiety on oligo(ethylene glycol), S_N2 displacement of the tosylate by the thiolate, amine deprotection, and introduction of an acryloyl group. The steps involve commercially available reagents, mild reaction conditions, easy separations, and good yields (25–46% overall). For the synthesis of **10** containing a monoethylene glycol spacer, we have also used a synthesis involving reaction of *N*-bromoacetyl *p*-(aminomethyl)benzenesulfonamide with cysteamine (2-aminoethanethiol) and acryloyl chloride in one pot (97% overall yield). **10** is a solid and **11–14** are viscous liquids.

Preparation of Gel Slabs. We described a convenient procedure for preparing multisectional slab gels. Most prior work on AGE has used disk gels (tube gels).[5] After completion of electrophoresis, the stained gel rods were placed together and aligned before they were photographed. From an experimental point of view, procedures based on disk gels are inconvenient. The thin gel tubes are fragile and difficult to manipulate. Moreover, reproducible mobilities (and thus binding constants) are not easy to obtain, because it is difficult to align proteins serving as internal standards in tube gels. Even though indistinguishable conditions are used for separate gels in electrophoresis, small but significant differences of mobilities of proteins serving as internal standards are often observed.[5] To circumvent these difficulties, we use heterogeneous slab gels. In this procedure (detailed below), different sections of the same slab contain different concentrations of an immobilized affinity ligand.

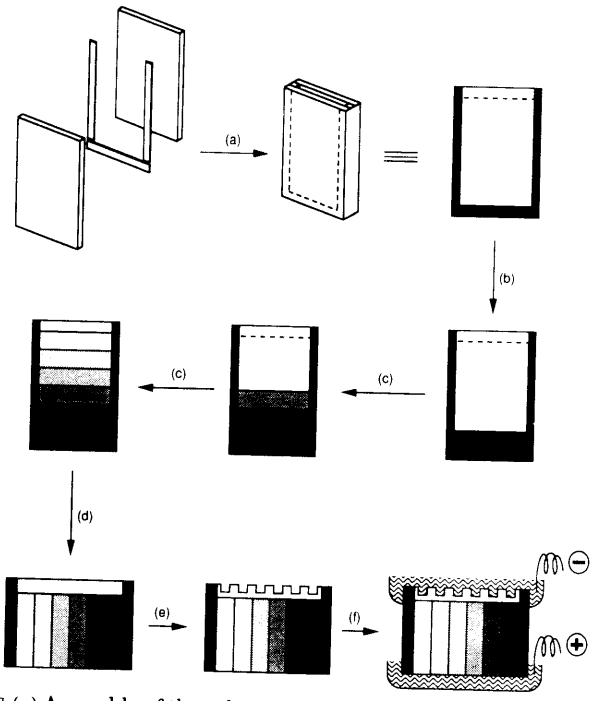
Previous work on AGE has focused predominately on a class of procedures in which the ligands are immobilized by covalent attachment to water-soluble macromolecules (for example, high molecular weight dextran and linear polyacrylamide) or meltable agarose.[5,20] These interpenetrating network systems were used for the affinity gel electrophoresis.

We have covalently immobilized the affinity ligands by copolymerization in a solution containing acrylamide monomer and bis(acrylamide) cross-linker. This procedure enables us to prepare multisectional slab gels, in which different zones of the slab contain different concentrations of the affinity ligand.

Our preparation of the affinity gel slabs is straightforward (Scheme II): monomer solutions containing known concentrations of affinity ligands were copolymerized with acrylamide and bis(acrylamide) layer by layer between two glass plates; this multisectional polyacrylamide slab gel was soaked to remove unpolymerized affinity ligands; the final gel

(20) Horejsi and co-workers have used *O*-allyl glycosides to copolymerize acrylamide and bis(acrylamide) for qualitative analysis of phytohemagglutinins: Horejsi, V.; Kocourek, J. *Biochim. Biophys. Acta* **1974**, *336*, 338–343.

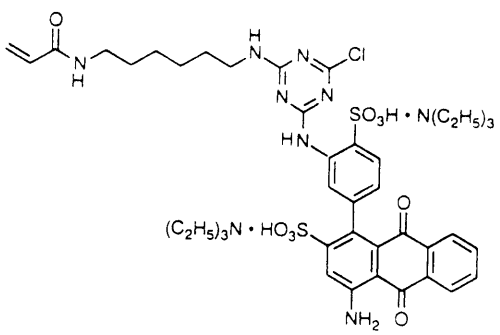
Scheme II. Schematic Illustration of the Procedure for Preparation of Multisectional Slab Gels[a]



[a] (a) Assembly of the gel cassette; (b) addition of the monomer solution containing affinity ligands to the cassette by a syringe, and gel polymerization; (c) continuation of preparation of affinity gels having various concentrations of affinity ligands; (d) disassembly of the affinity gel from glass plates, soaking of the gel in buffer, and reassembly of the soaked affinity gel between glass plates in such a way that this multisectional gel slab is rotated by 90°; (e) preparation of the stacking gel, containing no affinity ligands, on top of the affinity gel; (f) application of protein samples into each well and gel electrophoresis.

assembly was rotated by 90°; finally, a stacking gel containing no affinity ligands was prepared on top of the affinity gel. The exact procedures and formulations used are described in the Experimental Section. This type of gel has a number of advantages for AGE:[21] all useful binding results are obtained from a single multisectional gel slab; the preparation is simple and economical. By comparison with gel rods, the multisectional slab gel is easy to handle; any commercial slab gel apparatus will suffice.

In order to know the concentration of affinity ligand in the gel, it is necessary to know the extent to which the polymerization proceeds to completion. We estimated this number by including a spectrophotometric label in the polymerization. We chose **15** for two reasons. Procion Blue MX-R, the chromophore in **15**, is an inexpensive reactive dye having a convenient absorption maximum at 597 nm. The spacer, 1,6-hexanediamine, separated the large charged dye from the site of polymerization. We examined polyacrylamide gels containing **15** at two concentrations (88 and 528 μM), which covered the range of concentrations of affinity ligands we studied in this paper, in order to estimate the extent of polymerization. We prepared and soaked the gels following the procedures of our affinity gel preparations, then measured the concentrations of **15** spectrometrically, and found that 7.1 and 11.0% of **15** were present in the soaking buffer (see Experimental Section). We used the averaged value 9.0% to

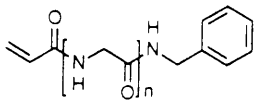


15

correct the concentration of affinity ligands in gels after soaking (i.e., we estimated 91% incorporation of the initial concentrations of **15** into gels). We assumed the same conversion for all of the affinity ligands **1**–**14**. In experiments in which removing unpolymerized affinity monomer from the gel was critical (for example, in systems in which the gel-bound ligand binds significantly less strongly than the same species in solution), **15** may be a useful surrogate for the affinity monomer to follow directly the polymerization and soaking.

Affinity Gel Electrophoresis of Carbonic Anhydrase on Aryl Sulfonamide-Containing Gels. Figure 1A shows the result of a typical affinity gel electropherogram of CAB on the multisectional polyacrylamide gel containing various concentrations of affinity ligands. The experiments were carried out in tris–borate buffer at pH 8.5. The mobility of CAB decreased as the concentration of affinity ligand in the gel increased. Bromelain-released hemagglutinin (HA) of influenza virus X-31 and the bovine pancreatic trypsin inhibitor (BPTI), proteins used as internal standards in these experiments, did not bind to the aryl sulfonamides present in the gels; their mobilities were independent of the concentration of affinity ligands.

A control experiment, replacing the affinity ligand **6** with a similar ligand **16** lacking the crucial sulfonamide group,



16 (n = 5)

showed that nonspecific interaction of an aryl group and the spacer moiety of the affinity ligands with the CAB was negligible compared to the specific interaction of the sulfonamide. At pH 8.5, we observed no retardation for the CAB and BPTI in a two-section gel in which one section of the gel contained a 30 μM concentration of **16** and the other section in this gel was free of **16**.[22] Another control experiment involved electrophoresis at pH 3.5. At this pH, CAB exists in a compact but enzymatically inactive and structurally disordered "molten globule" state.[23] The mobility of this denatured form of CAB was independent of the concentration of **6** in the gel. This experiment established that, as expected, disordering the active site of CAB (and destroying its catalytic activity and, we presume, its ability to bind aryl sulfonamide) eliminated its ability to bind to the gel. The interpretation of the experiment is complicated by the fact that two changes occur in the system on lowering the pH from 8.5 to this value: the protein disorders, and the fraction of aryl sulfonamide

(21) Our method of gel preparation is useful not only for affinity electrophoresis but also for determining the molecular weight of native proteins, using nondenaturing polyacrylamide gel electrophoresis with gels having various percentages of acrylamide in each gel section, and analysis of data using a Ferguson plot: Hames, B. D. In *Gel Electrophoresis of Proteins: a Practical Approach*; Hames, B. D., Rickwood, D., Eds.; IRL Press at Oxford University Press: Oxford, England, 1981; pp 14–15.

(22) Using the exact electrophoretic condition, the same concentration (30 μM) of the affinity ligand **6** having the pentaglycine spacer in the gel significantly retarded CAB (R_f = 0.23) but not BPTI (R_f = 1.0).

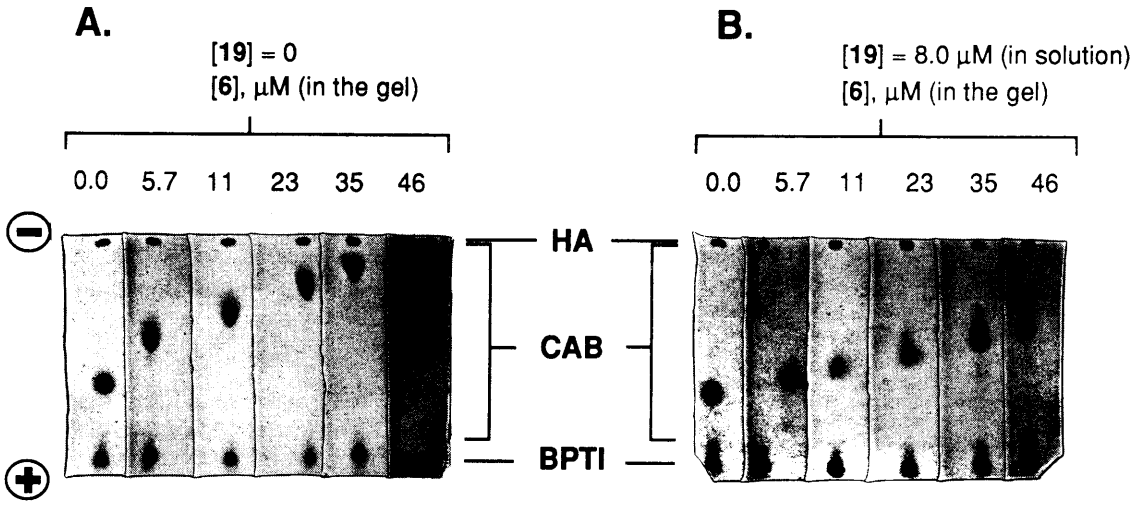


Figure 1. Affinity gel electrophoresis of bovine carbonic anhydrase B (CAB) on multisectional polyacrylamide slab gels containing various concentrations of affinity ligand **6**. Parallel experiments are shown, carried out in the absence (A) and presence (B) of the soluble ligand **19** in the electrophoresis buffer. Bovine pancreatic trypsin inhibitor (BPTI) and bromelain-released hemagglutinin of influenza virus (HA) were used as internal standards. The gel electrophoresis was carried out at constant voltage (45 V) for 13 h in tris–borate buffer (pH 8.5) (with or without added **19**).

present in the anionic form (the form that binds to the zinc ion) decreases by 10^4. Thus, even if the CAB were *not* to disorder, it would bind only very weakly to an aryl sulfonamide at this pH. This experiment is therefore best considered as another demonstration that *non*-specific interactions between protein and aryl sulfonamide (or spacer) are not significant.

Binding of CAB to a gel containing the affinity ligand **6** (incorporating a pentaglycine spacer) was inhibited by the soluble ligand **19** (also having a pentaglycine spacer) in the buffer. Figure 1B illustrates relevant data. Parts A and B of Figure 1 are parallel experiments, carried out with (B) and without (A) **19** included in the buffer. The retardation of the migration of CAB was reduced by the presence of **19**; neither **6** (in the gel) nor **19** (in solution) had any influence on BPTI or HA.

We normally used high-sensitivity silver stains to visualize proteins in the gels in order to keep the ratio [CAB]/[ligand] low.[5] We estimated that the concentration of CAB in each protein zone visualized by silver stain, in the gels, was approximately 0.2 μM, based on the known amounts of protein loaded in the gel (see Experimental Section) and gel volumes occupied by CAB. We did not observe significant differences of CAB mobility (R_f) and K_d^{gel} when results from both silver stains and Coomassie dye stains were compared.

Binding of CAB to Immobilized Sulfonamides in the Gel Is Weak (Values of K_d^{gel} Are High) for Affinity Ligands with Short Spacers. We used Scatchard plots (eq 4) to determine K_d^{gel} from experimental values of R_0, r, and [L]. In order to obtain data giving straight Scatchard plots, it was essential to remove all unpolymerized affinity ligands from the gel before use. We accomplished this removal by soaking the gels twice (for a total of 24 h) in the tris–borate buffer (pH 8.5) used for gel electrophoresis. Unpolymerized affinity ligands remaining in the gel compete with immobilized ligands for binding to CAB. This competition is most

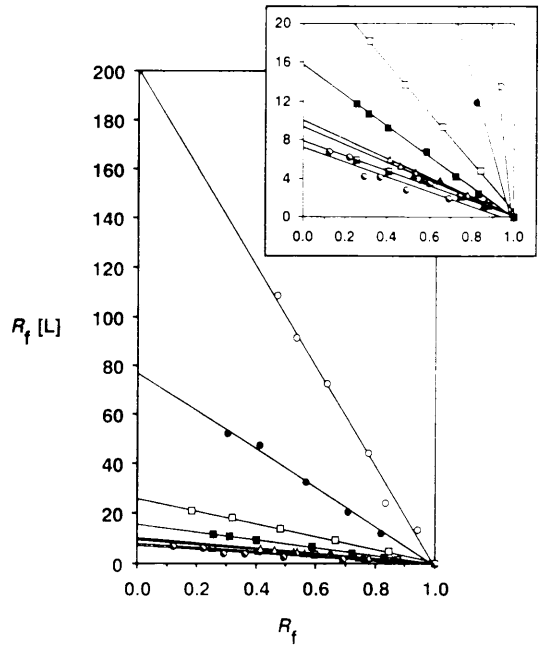


Figure 2. Determination of dissociation constants (K_d^{gel}), using affinity gel electrophoresis, of complexes of bovine carbonic anhydrase B and the affinity ligands **1** (○), **2** (●), **3** (□), **4** (■), **5** (◪), **6** (◐), **7** (△), and **8** (▲) by eq 4. The inset details the region of $R_f[L] \leq 20$ μM.

significant for short affinity ligands. Immobilization of ligands with short spacers decreases their affinity for CAB (see below); in solution, affinity is independent of the length of the attached spacer chain (detailed below).[15]

Figure 2 shows Scatchard plots used to determine K_d^{gel} for affinity ligands **1**–**8**. Gels containing **9** did not give useful data; monomeric **9** was not very soluble under the conditions used in the polymerization, and we did not know its concentration in the gel. For affinity ligands **10**–**14**, we also obtained linear Scatchard plots. We observed significant decrease of binding affinities to CAB for the rigid, glycine-based ligands **1**–**8** and small changes of K_d^{gel} for the flexible, ethylene glycol-based affinity ligands **10**–**14** as the spacer length between ligand and polymer backbone decreased. We estimated the concentrations of immobilized ligands: $[L]_{final} = (0.91/1.58)[L]_{initial}$, where 0.91 was the percentage of

(23) It has been reported that a stable folding intermediate was the predominant form present with 2.0 M guanidine hydrochloride or at pH 3.5 and that this intermediate contained major elements of secondary structure of the native protein, but retained no esterase activity and binding ability for DNSA: Henkens, R. W.; Kitchell, B. B.; Lottich, S. C.; Stain, P. J.; Williams, T. J. *Biochemistry* **1982**, *21*, 5918–5923. Dolgikh, D. A.; Kolomiets, A. P.; Bolotina, I. A.; Ptitsyn, O. B. *FEBS Lett.* **1984**, *165*, 88–92. We observed equally sharp bands of CAB with the same mobilities, both at pH 3.5 and at pH 8.5, on affinity ligand-free and immobilized polyacrylamide gels using 0.2 M glycine as pH 3.5 buffer and 90 mM tris–borate as pH 8.5 buffer.

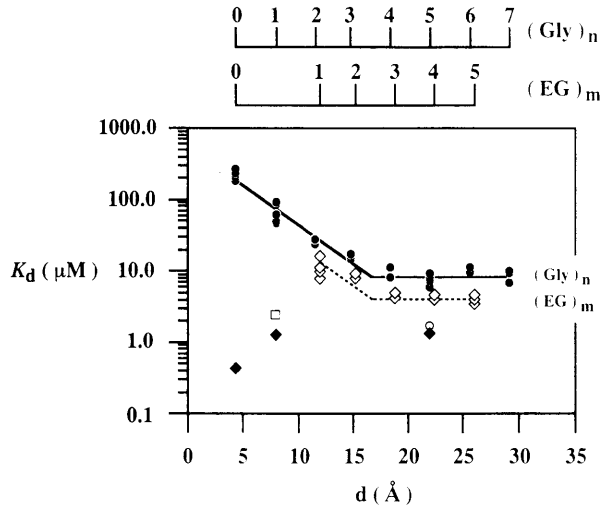
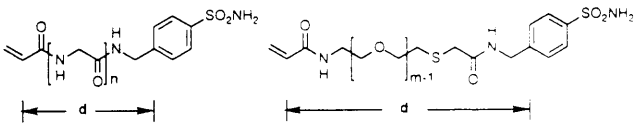


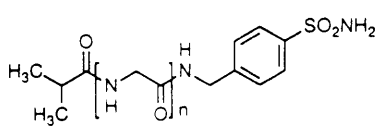
Figure 3. Dissociation constants, K_d^{gel}, of complexes of bovine carbonic anhydrase B obtained using immobilized affinity ligands **1–8** (●) and **10–14** (◇); dissociation constants K_d^{sol} for the soluble ligands **17–19** (◆), with **18** (□) obtained in the presence of soluble polyacrylamide and **19** (○) determined by competition inhibition gel experiment. The horizontal axis is the estimated distance (*d*) between C-4 of the benzenesulfonamide ring and the carbon of the polyacrylamide chain (C-2 of the acrylamide moiety) with the spacer chain assumed to be in the maximally extended conformation.

completion on radical polymerization under our experimental conditions and 1.58 was an experimental factor of gel expansion during soaking in buffer (see Experimental Section). Figure 3 summarize values of K_d^{gel}. The horizontal axis in this plot is the distance (*d*) calculated for the fully extended spacer chain.



The important inference from these data is that K_d^{gel} is constant for linking groups longer than 16 bonds ($Gly_n \geq 4$; $EG_m \geq 3$; spacer length ≥18 Å). Shorter linking groups give values of K_d^{gel} that are higher, an observation we suggest to be due to steric inhibition to binding by the polyacrylamide backbone (see below). The suggestion (Figure 3) that separation of the sulfonamide group from the polymer backbone by 20 Å (based on an assumption that the spacer molecules still retain their fully extended conformation in the binding site of CAB) eliminates steric interferences is consistent with the crystal structure of carbonic anhydrase and with previous work in our laboratory.[15]

Control Experiments: Values of K_d^{sol} for Soluble Ligands. In order to verify that the changes in K_d^{gel} observed in the affinity gels resulted from the differences in accessibility of affinity ligands, we measured dissociation constants for analogous ligands **17–19** in solution, using the buffer employed for electrophoresis, by a competitive fluorescence-based assay.[15,16] These compounds all have similar affinity for CAB in solution: K_d^{sol} (μM) = 1.1 for **18**, 1.3 for **19**, and 0.4 for **17**



17 (n = 0), **18** (n = 1), **19** (n = 5)

(Figure 4). We propose, without definitive evidence, that

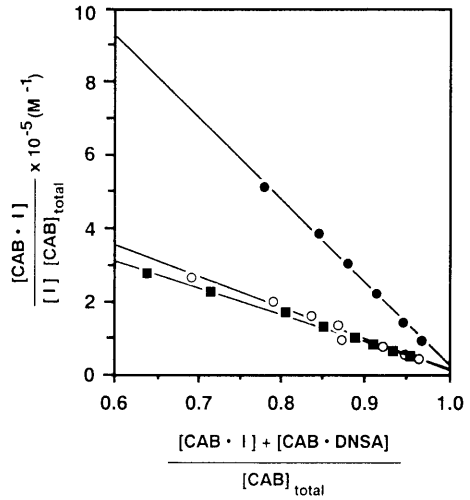


Figure 4. Determination of dissociation constants (K_d^{sol}) of complexes of CAB and the soluble ligands **17** (●), **18** (○), and **19** (■) using a competitive fluorescence-based assay (eq 8).

the enhanced binding of **17** reflects an additional hydrophobic interaction of the lipophilic acyl group in **17** with the hydrophobic core in the active site pocket of CAB.[7,16] Dissociation constants of protein–ligand complexes increased in the presence of soluble polyacrylamide (9.5%, w/v; average molecular weight 12 000): K_d^{sol} (μM) = 2.5 and 1.1 for **18** in the presence and absence of the polymer, respectively.

Affinity Gel Electrophoresis With Soluble Ligands. It is also possible to determine the values of K_d^{sol} for the soluble ligands **17–19** in the gels, by competition with the immobilized affinity ligands. Figure 1B shows representative data. The experimental values of R_f (eq 2) are converted to values of K_d^{sol} using eqs 9 and 10.[5]

$$[L]B_f = K' - K'R_f \quad (9)$$

$$K' \equiv K_d^{gel}(1 + ([I]/K_d^{sol})) \quad (10)$$

The values of K_d^{sol} depend on the values of K_d^{gel} determined independently for the immobilized affinity ligands. The observation that these values of K_d^{sol} (Figure 3) agree well with those determined independently by the fluorescence assay in solution confirms the consistency of all of these values.

Application of AGE to Mixtures of Proteins. Nondenaturing gel electrophoresis has lower resolution than SDS gel electrophoresis,[24] but it is still sufficient to resolve certain mixtures of proteins. AGE should therefore be useful in analyses involving mixtures of proteins, under certain circumstances.

Figure 5 illustrates the use of AGE to identify a protein that binds a ligand. In this illustration, CAB present in hemolyzed bovine blood is readily identified by its retardation in a gel containing a hexaglycine-spaced affinity ligand **7**. To make identification of the retarded CAB bands easier, the AGE experiment was carried out in a slab gel containing four separate regions, including parallel regions incorporating and not incorporating **7**.

(24) Any species with a net charge will migrate when placed in an electric field. In native polyacrylamide gel electrophoresis, the rate of migration of proteins depends on their charge density (the ratio of charge to mass) and shape. Since all polypeptides appear to have a similar shape (a random coil) and carry a constant binding ratio (approximately one molecule of SDS for every two amino acid residues of the chain) when SDS is bound, the electrophoretic mobility of polypeptides in denaturing polyacrylamide gel electrophoresis (SDS–PAGE) is generally proportional to the logarithm of the molecular weight of proteins. See: Creighton, T. E. *Proteins: Structures and Molecular Properties*; W. H. Freeman and Co.: New York, 1984; pp 33–34.

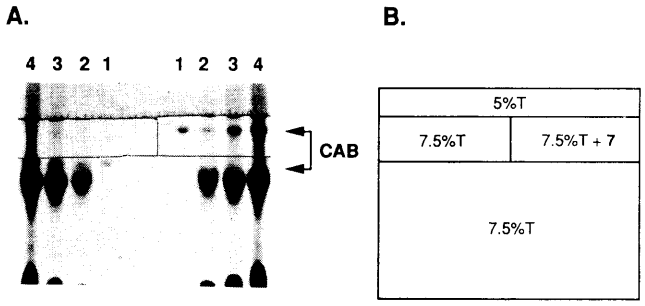


Figure 5. Identification of CAB in bovine blood by a protein band-shift assay based on AGE in a multisectional slab gel. The CAB is present at approximately 0.2% (w/w) of the hemoglobin present in the sample.[28] (A) Lane 1 is the CAB standard. Lanes 2–4 show the binding of CAB to the ligand at increasing concentrations of protein sample from bovine blood. (B) A schematic illustration of the structure of the gel. The sequence used to prepare the slab involved four steps. First, a polyacrylamide gel (7.5% T^{13}), on the bottom, was prepared. Second, the gel assembly was rotated by 90° and a 7.5% T monomer solution containing 21 μM **7** was polymerized. Third, another 7.5% T polyacrylamide gel was formed on the top of the gel containing **7**. Fourth, the gel assembly was rotated back (−90°) and a 5% T polyacrylamide gel was added to act as a stacking gel. Electrophoresis was conducted in 90 mM tris–borate buffer (pH 8.5) at 57 V for 5 h.

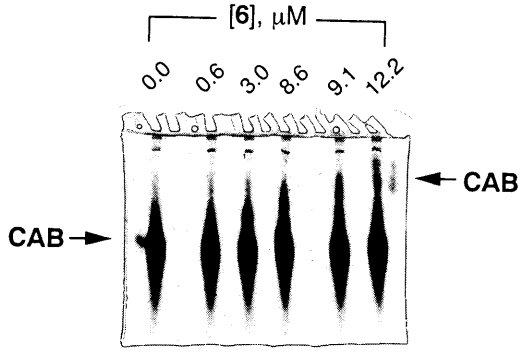


Figure 6. Affinity gel electrophoresis of CAB using bovine blood as the protein sample on the multisectional polyacrylamide gel containing various concentrations of affinity ligand **6**. In addition, the very left and very right lanes contained the commercially purified CAB used as internal standard.

Figure 6 shows the results of an experiment designed to test the ability of AGE to yield values of K_d^{gel} in mixtures of proteins. Data analogous to those in Figure 1 were obtained using an artificial mixture of HA, CAB, and BPTI proteins. The values of K_d^{gel} = 8.2 μM obtained was very similar to that obtained in the absence of the other proteins. Thus, the presence of other proteins during determination of K_d^{gel} does not interfere (at least in this instance).

CONCLUSIONS

Affinity Gel Electrophoresis Is a Useful and Convenient Method for Measuring Binding Constants Involving Proteins, Especially When Only Small Quantities Are Available. AGE is a technique that permits measurement of binding constants using only small quantities of proteins. Sensitive stains for proteins in these gels are reliable, and less than 0.5 μg of protein is sufficient to obtain a binding constant. AGE is a useful analytical technique that can conveniently measure binding constants in mixtures of proteins and screen proteins according to their affinities for various ligands.

Heterogeneous Sectioned Slab Gels Improve the Accuracy of Measurement of Binding Constants in AGE. We have developed a convenient preparation of multisectional polyacrylamide slab gels (Scheme II) and optimized its manipulation to measure binding constants involving proteins.

Figure 7. A plane section through the active site of the carbonic anhydrase with hexaglycine-spaced affinity ligand **7**, covalently attached to a polymer chain, superimposed for scale. The CPK model of the active site of carbonic anhydrase was generated by Jain[15] from the X-ray structural data of Eriksson et al.[9]

Our gel preparations have a number of advantages for AGE. First, all useful binding data are obtained from a single multisectional gel slab. AGE results from these preparations are reproducible. Second, the preparation is straightforward and economical. Third, unlike the commonly used tube gels for AGE, the multisectional gel slabs are easy to handle and any commercial slab gel apparatus will suffice for their preparation and use.

The Structure and Length of the Spacer Linking the Gel Matrix and the Affinity Ligand Influences the Estimated Binding Constant for CAB. Affinity ligands having short spacers give high apparent dissociation constants, probably resulting from the inaccessibility of short ligands immobilized in cross-linked gel matrix and the steric hinderance around the active site of CAB. For both the oligoglycine and the oligo(ethylene glycol) spacers, binding affinities of the ligands to CAB in gels are essentially indistinguishable from the values obtained in free solution, provided the spacer connecting the affinity ligand to the polymer backbone is long enough (≥18 Å). Figure 7 shows a plane section through the active site[9] of carbonic anhydrase with the hexaglycine-spaced affinity ligand **7**, covalently attached to a polymer chain, superimposed for scale.

The conformational properties of the spacers also influence the CAB–ligand binding affinities. The conformationally flexible oligo(ethylene glycol)-containing affinity ligands displayed slightly greater affinities to CAB than the conformationally rigid oligoglycine-containing ligands of comparable length, presumably due to unfavorable binding enthalpies of the conformationally constrained oligoglycines. Binding constants measured by AGE agree with those determined in homogeneous solution, provided that artifacts due to linker length and residual monomer in solution are minimized.

Affinity Gel Electrophoresis Is More Generally Applicable, but Less Convenient, Than Affinity Capillary Electrophoresis (ACE) Using Uncoated Capillaries for Measuring Binding Constants of Ligands to Proteins. Recently we have also explored ACE as a sensitive, rapid, and convenient method for determining binding constants of ligands to proteins and low molecular weight receptors.[25,26]

(25) Chu, Y.-H.; Avila, L. Z.; Biebuyck, H. A.; Whitesides, G. M. *J. Med. Chem.* **1992**, *35*, 2915–2917.
(26) Chu, Y.-H.; Whitesides, G. M. *J. Org. Chem.* **1992**, *57*, 3524–3525.

A serious shortcoming of ACE, at present, is its requirement that the protein not adsorb on the wall of the capillary and the resulting requirements to work with modified capillaries and/or to use complex buffer for analyses of most proteins. Since protein electrophoresis in polyacrylamide gel matrix has been extensively studied and well-characterized, and a large number of proteins show sharp bands in nondenaturing polyacrylamide gels, adsorption of proteins onto gel matrix is unlikely to interfere with measurements of binding constants.[27]

In summary, we have developed methods for convenient preparation of multisectional slab gels containing covalently attached affinity ligands. This heterogeneous sectioned gels have several advantages over conventional disk gels. We believe that these techniques should be applicable to a range of investigations of ligand–receptor binding interactions. We recommend affinity gel electrophoresis as a useful technique for use in protein biochemistry and rational drug design.

(27) Andrews, A. T. *Electrophoresis: Theory, Techniques, and Biochemical and Clinical Applications*, 2nd ed.; Oxford University Press: New York, 1986.
(28) Moore, M. J. *Ann. N.Y. Acad. Sci.* **1984**, *429*, 277–279.

ACKNOWLEDGMENT

We thank Ahamindra Jain for his helpful comments about the work, assistance with the fluorescence assay of carbonic anhydrase, and Figure 7, and Rajeeva Singh for his illuminating discussions on the synthesis of the oligo(ethylene glycol)-spaced affinity ligands.

SUPPLEMENTARY MATERIAL AVAILABLE

Syntheses and spectroscopic data of ligands **1–19**, measurement of unimmobilized ligands in polyacrylamide gels, and a table of dissociation constants (K_d) of ligands **1–14** and **17–19** to bovine carbonic anhydrase (17 pages). Ordering information is given on any current masthead page.

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