Using Affinity Capillary Electrophoresis To Identify the Peptide in a Peptide Library that Binds Most Tightly to Vancomycin[1]

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This paper describes a procedure, based on competitive binding, for identifying tight-binding ligand(s) for a receptor in mixtures of equimolar ligand candidates using affinity capillary electrophoresis (ACE). Vancomycin and a small library of 32 peptides were used as a model system to illustrate the procedure. This procedure should be applicable to peptide mixtures of greater complexity than the one used here, as well as to mixtures of nonpeptidic compounds. Limits and limitations to the procedure are described.

Introduction

We are evaluating affinity capillary electrophoresis (ACE) as an assay technique for use in identifying drug leads.[2,3] Among the most valuable of the strategies now used for discovering lead compounds are those based on screening collections of natural compounds (such as fermentation broths and plant extracts)[4] or libraries of synthetic compounds ("combinatorial library" approaches).[5] These methods require efficient assays.

This paper outlines an experimental procedure, using ACE, for screening small libraries of compounds for their ability to bind to a soluble receptor. It is applicable to two types of problems. First, it is directly applicable to libraries in which constituents are available separately and in which the receptor is not in limiting supply. In this application, the procedure is useful in shortening the total time required for the analysis of binding. Second, it is also applicable, in principle, to guiding a search through a mixture in which isolation of a component of unknown structure is required.

We illustrate this methodology using vancomycin and a small library of peptides. Vancomycin was chosen as a model receptor for its ease of manipulation and because there is a substantial literature available concerning its binding pocket and the peptidyl ligands that bind tightly to it.[6] Here we screened a group of 32 unlabeled peptides for their affinity to vancomycin and correctly identified the tightest binding of these peptides. The methodology used in this search should be applicable to larger libraries and to other receptors.

Capillary electrophoresis (CE) measures the mobility of charged species under the influence of an electric field gradient (typically of magnitude of ~400 V/cm).[7] Capillary zone electrophoresis is a particularly simple technique, capable of rapid, high-resolution separation of compounds from very small volumes of sample (typically, the volume of injected sample is 8 nL). The mechanism of separation by this technique is well-characterized: the surface of the inner wall of the capillary is negatively charged (due to acidic silanol groups) and the boundary layer of the buffer in contact with the inner wall has a net excess of positive charges. When high voltage is applied between the ends of a capillary of fused silica filled with an electrophoresis buffer, electroosmotic flow (EOF) moves the sample along the capillary while maintaining plug-flow geometry. 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. The observed migration time of a species is determined by a combination of its electrophoretic mobility and the velocity of EOF. CE has the advantages of speed, reproducibility, and ease of automation.[7]

We have used affinity capillary electrophoresis (ACE) to measure binding constants of receptors for ligands quantitatively.[2,3] The electrophoretic mobility μ of a species in CE has a relationship to its mass M and net charge Z of the approximate form $\mu \approx Z/M^{2/3}$.[8] If a receptor of high molecular weight (M) binds a charged ligand of relatively small mass (m), the change in μ due to the change in mass [from $M^{2/3}$ to $(M + m)^{2/3}$] is small relative to the change in μ due to the change in charge (from Z to $Z \pm z$). Thus, the receptor–ligand complex will migrate at a different rate than the uncomplexed receptor. We have shown that negatively charged arylsulfonamide ligands, when added to the electrophoresis buffer, increased the electrophoretic mobility of carbonic anhydrase (CA, E.C.4.2.1.1, from bovine erythrocytes);[3] similar effects have been observed with glucose-6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49, from *Leuconostoc mesenteroides*) and NADP(H),[3] calmodulin (from bovine testis) and

(1) This research was supported by the NIH (GM 39589, GM 30367) and by the NSF through the M.I.T. Biotechnology Processing Engineering Center (Cooperative Agreement CDR-88-03014).

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

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

(4) Kuntz, I. D. *Science* **1992**, *257*, 1078–1082. Baumbach, G. A.; Hammond, D. J. *BioPharm* **1992**, *5*, 24–29. Pauwels, R.; Andries, K.; Desmyter, J.; Schols, D.; Kukla, M. J.; Breslin, H. J.; Raeymaeckers, A.; Gelder, J. V.; Woestenborghs, R.; Heykants, J.; Schellekens, K.; Janssen, M. A. C.; Clercq, E. D.; Janssen, P. A. J. *Nature* **1990**, *343*, 470–473. Sandler, M.; Smith, H. J. *Design of Enzyme Inhibitors as Drugs*; Oxford University Press: New York, 1989.

(5) Zuckermann, R. N.; Kerr, J. M.; Siani, M. A.; Banville, S. C.; Santi, D. V. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 4505–4509. Geysen, H.; Meloen, R.; Barteling, S. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3998–4002. Fodor, S.; Read, J.; Pirrung, M.; Stryer, L.; Lu, A. T.; Solas, D. *Science* **1991**, *251*, 767–773. Lam, K.; Salmon, S.; Hersh, E.; Hruby, V.; Kazmiersky, W.; Knapp, R. *Nature (London)* **1991**, *354*, 82–84. Houghton, R.; Pinilla, C.; Blondelie, S.; Appel, J.; Dooley, C.; Cuervo, J. *Nature (London)* **1991**, *354*, 84–86.

(6) Williams, D. H.; Waltho, J. P. *Biochem. Pharmacol.* **1988**, *37*, 133–141.

(7) Frenz, J.; Hancock, W. S. *TIBTECH* **1991**, *9*, 243–250. Novotny, M. V.; Cobb, K. A.; Liu, J. *Electrophoresis* **1990**, *11*, 735–749.

(8) This expression is approximate. Other functional forms have been suggested: Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. *Anal. Biochem.* **1991**, *197*, 197–207. Grossman, P. D.; Colburn, J. C.; Lauer, H. H. *Anal. Biochem.* **1989**, *179*, 28–33. Deyl, Z.; Rohlicek, V.; Adam, M. *J. Chromatogr.* **1989**, *480*, 371–378. Offord, R. E. *Nature* **1966**, *211*, 591–593.

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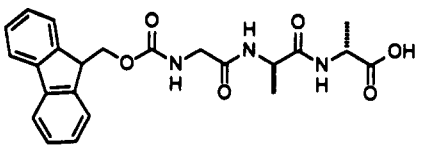
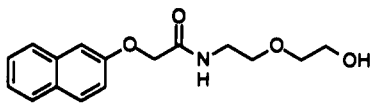
calcium ion,[3] vancomycin and charged peptides,[2,9] and lectins and a charged sugar.[10] Scatchard analysis of the relative electrophoretic mobilities as a function of the concentration of the ligand yields their binding constants.[2,3]

The screening procedure described in this paper was performed with a mixture of peptide ligands and the receptor in the solution phase, under conditions in which the ligands competed for a fixed number of receptor molecules. The protocol used was this: the electrophoresis buffer was made up containing the receptor (in the example used here, vancomycin) and the components of the library to be tested. Using this buffer, the electrophoretic mobility of an easily detected probe ligand L that binds to the receptor was measured. This ligand must have the following characteristics: (1) it must bind to the receptor with a known association constant in the range of interest for compounds in the library being screened; (2) it must be electrically charged, so that it migrates under conditions of electrophoresis; (3) its mobility must be different when it is free in solution and when it is bound to the receptor, so that the extent of its binding can be inferred from its mobility; (4) the rates of its association with and dissociation from the receptor must be fast compared with the time required for the electrophoresis experiment, in order to avoid line broadening;[11] and (5) it must be easily and sensitively detected, against the background attributable to the receptor, components of the library, and other materials present in the buffer. In the experiments described here, we used the probe ligand Fmoc-Gly-D-Ala-D-Ala (L).[2] Uncharged compound M was used to measure the velocity of EOF and to mark the arrival of uncharged species at the detector.



L



M

This method is, thus, based on competition of L, whose electrophoretic mobility is being measured with the other components of the library (present in the buffer) for the receptor (also present in the buffer). When there is no competition for the binding site of vancomycin from components of the library, L migrates at the rate reflecting its partitioning between buffer and the vancomycin binding; when a library component *does* compete with L for vancomycin, the migration time of L shifts toward its value in buffer.

Although both receptor and the library to be screened are components of the buffers, the extreme economy with which CE uses materials permits the method to be practical. A series of 180 electrophoresis runs might require 1 mL of buffer. For a receptor concentration of 1 μM, the amount of receptor needed is 1 nmol (1.5 μg for vancomycin) for the entire series.

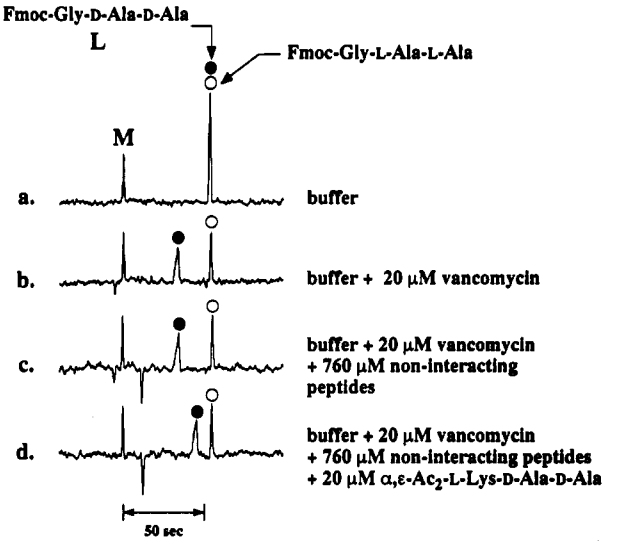


Figure 1. The concentration of vancomycin in the electrophoresis buffer (20 mM phosphate, pH 7.4) affects the electrophoretic mobility of Fmoc-Gly-D-Ala-D-Ala (L, ●) but not Fmoc-Gly-L-Ala-L-Ala (○). The concentration of Fmoc-Gly-D-Ala-D-Ala used was 8 μM. In going from c to d, the change in the mobility of Fmoc-Gly-D-Ala-D-Ala reflected the change (from 20 μM in c to ~10 μM in d) in the concentration of free vancomycin in the electrophoresis buffer because of the presence of a competing ligand α,ε-Ac_2-L-Lys-D-Ala-D-Ala in the buffer used in d. The neutral marker M was used as an internal standard. The negative peak evident in c and d resulted from the dilution of L-Ala-L-Trp present in the electrophoresis buffer at the point of injection. Electropherograms c and d form the basis for the experiments summarized in Figure 2.

Results and Discussion

Demonstration of the Method. We first showed that unlabeled peptides added to the electrophoresis buffer containing vancomycin competed for binding with, and therefore changed the electrophoretic mobility of, a probe ligand L with a high affinity for vancomycin (Figure 1). The binding constant of L to vancomycin is 1.9×10^4 M^{-1}.[2] In a buffer containing vancomycin (Vanc), L migrates with a velocity determined by its partitioning between free ligand and a complex with vancomycin (eq 1).

$$\text{Vanc} + \text{L} \overset{K_b}{\rightleftarrows} \text{Vanc·L} \quad (1)$$

$$\text{Vanc} + \text{A, B, C- -L'- -Z} \overset{K_b'}{\rightleftarrows} \text{Vanc·L'} + \text{A, B, C- -Z} \quad (2)$$

Addition of a library of compounds A, B, C- -Z to the buffer solution perturbs this equilibrium between L and Vanc·L if the library contains a compound L' capable of competing with L for Vanc (eq 2). Changes in the mobility of the easily detected L thus indicate the presence of compounds competing with L for the binding site of Vanc. Figure 1 shows the experimental results for Vanc with a mixture of noninteracting peptides, in the presence and in the absence of a strongly interacting peptide α,ε-Ac_2-L-Lys-D-Ala-D-Ala.

In a general case, it is possible to determine the minimum binding constant that can be detected for a component of

(9) Carpenter, J. L.; Camilleri, P.; Dhanak, D.; Goddall, D. *J. Chem. Soc., Chem. Commun.* **1992**, 804–806.
(10) Honda, S.; Taga, A.; Suzuki, K.; Suzuki, S.; Kakehi, K. *J. Chromatogr.* **1992**, *597*, 377–382.
(11) Avila, L. Z.; Chu, Y.-H.; Blossey, E. C.; Whitesides, G. M. *J. Med. Chem.*, in press.

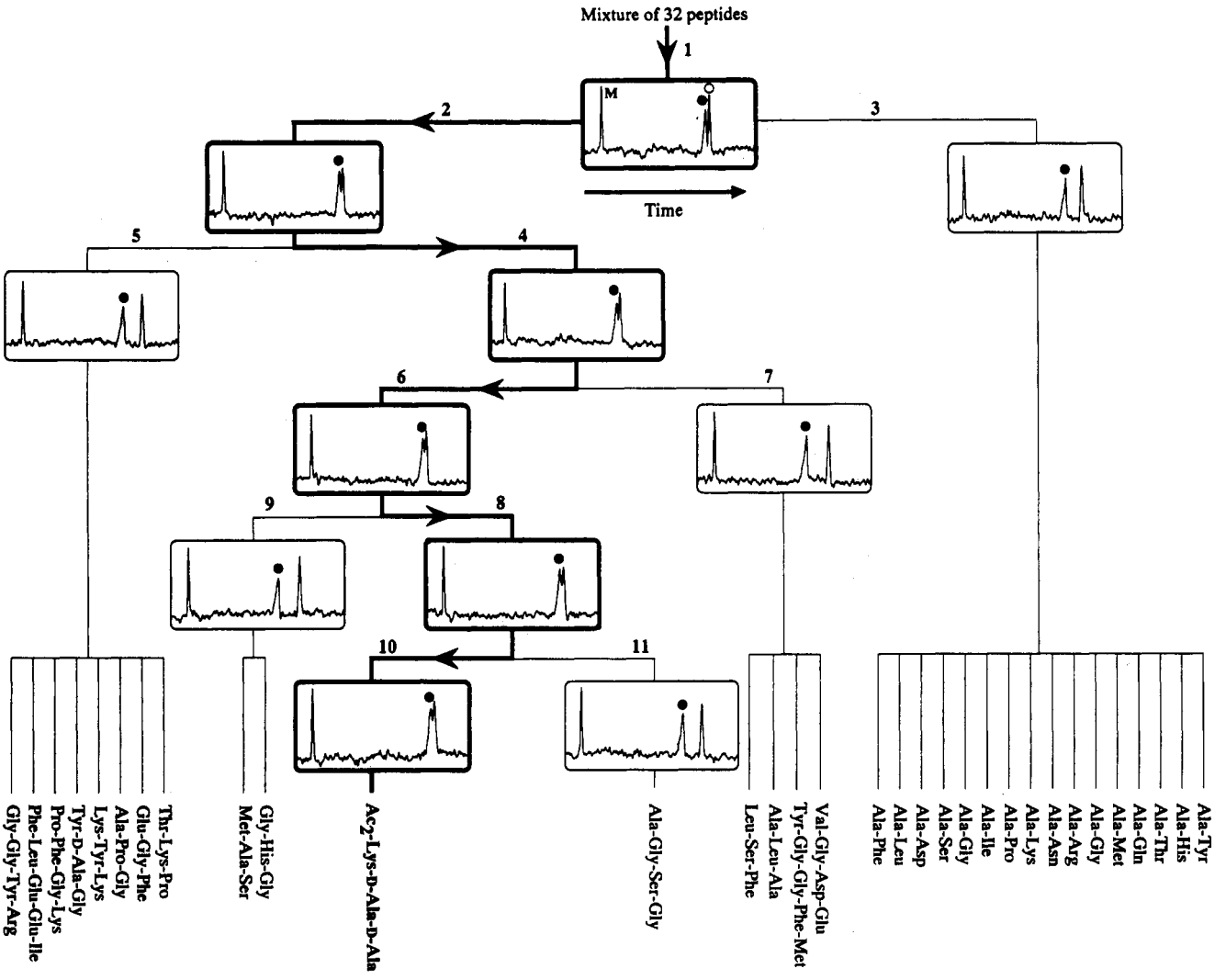


Figure 2. Stepwise elimination of 31 noninteracting peptides from a mixture of 32 peptides and identification of one tight-binding ligand for vancomycin, α,ϵ-Ac_2-L-Lys-D-Ala-D-Ala. Increased migration time of Fmoc-Gly-D-Ala-D-Ala (**L**, peak indicated by ●) in CE signaled the presence of a peptide (or peptides) in the electrophoresis buffer capable of binding to vancomycin (Figure 1). Eleven experiments are required to identify the one tight-binding peptide in the original mixture of 32 peptides and to demonstrate that it is the only one in the mixture.

the library by this method, given the binding constant of **L** with the receptor and the concentration of each of the components in the library (see below).

Experimental Protocol for the Method. We prepared buffers containing vancomycin and known subsets of peptides. The experimental conditions used in screening these peptides were similar to those used in Figure 1, except that the buffers contained the peptides. A solution of electrophoresis buffer (20 mM phosphate; pH 7.4) containing all 32 peptides (20 μM each, 640 μM total) was prepared. Compound **M**,[12] a neutral marker, and Fmoc-Gly-L-Ala-L-Ala were internal standards.

The first cycle of analysis (Figure 2; analysis 1) determined whether *any* of the 32 peptides present in the buffer was capable of competing with the probe ligand Fmoc-Gly-D-Ala-D-Ala ([**L**] = 8 μM) for vancomycin. The observed change in the mobility of **L** indicated that some peptide(s) **L′** was present in the buffer; we then tested new subsets of the peptide library to identify this ligand by elimination.[13]

In the second cycle of analysis, two new solutions were prepared in buffer; one contained half of the members of the peptide library (peptides 1–16, each peptide at 20 μM) and the second the other half (peptides 17–32). The migration time of **L** was determined in both of these buffers (Figure 2; analyses 2 and 3) and compared with that in Figure 1b. The change in the migration time of **L** in the electrophoresis buffer containing peptides 1–16 demonstrated the presence of a peptide **L′** (or peptides) that bound to vancomycin. The observation that the migration time of **L** in the electrophoresis buffer containing peptides 17–32 was indistinguishable from that in a buffer containing no added peptides indicated that none of the peptides 17–32 bound with $K_b > 5 \times 10^4\ M^{-1}$ to vancomycin (see below). A new and smaller set of substrates (peptides

(12) We selected **M** as the neutral marker for two reasons: the naphthyl group provides sensitive detection at 264 nm ($\epsilon = 7 \times 10^3\ M^{-1}\ cm^{-1}$), and the diethylene glycol moiety makes **M** soluble in aqueous buffer.

(13) Since the formation of the complex (Vanc·**L**) is under equilibrium control, we inferred the mobility of the complex from observation of the retention time of the probe ligand with buffers containing different concentrations of vancomycin. A Scatchard analysis of the change in the mobility of the tagged ligand as a function of the concentration of vancomycin in the buffer allowed us to compute the electrophoretic mobility of the complex.

1–16) containing at least one ligand for vancomycin was thus identified.

Two new subsets of peptides—1–8 and 9–16—were prepared and the cycle of analysis repeated (Figure 2; analyses 4 and 5). Repeating this procedure until all of the peptides not interacting with vancomycin were eliminated identified the peptide with the highest affinity for vancomycin. This search confirmed that the peptide α,ϵ-Ac_2-L-Lys-D-Ala-D-Ala ($K_b' = 2.4 \times 10^5$ M^{-1})[2] binds to vancomycin the most tightly of the molecules present in the original library.

Limits to the Method. This methodology of searching for tight-binding ligands obviously benefits from the ability to screen a number of different molecules simultaneously and from the small quantities of materials required in CE. In the experiment summarized in Figure 1, the ratio of the concentration of noninteracting peptides to "inhibitor" peptide was 31:1. Even when this ratio has exceeded 100:1, we have detected similar changes in mobility of **L**. We also found that up to 2 mM of an additional noninteracting peptide could be added to the electrophoresis buffer (20 mM in phosphate; pH 7.4) without causing noticeable shifts in the electrophoretic behavior of a probe peptide injected onto the column. These observations suggest that the limit on the number of peptides screened at one time by ACE is a function of the targeted binding constant, K_b', of the peptide **L**′: the tighter the binding, the larger the number of compounds that can be screened. If a K_b' of 1×10^6 M^{-1} is sought, it should be possible to screen up to 1000 compounds (as an equimolar mixture) simultaneously using the probe ligand **L**; this number is justified in the following section. The theroretical number of compounds (N) that can be screened simultaneously by ACE may be further limited in experimental practice by the effects of high concentration of peptides on the viscosity and electrical properties of the buffer, by the background they contribute to the response of the detector, and by the minimum detectable shift in the retention time of the probe ligand. Higher values of K_b' and K_b should allow for the simultaneous screening of proportionally larger pools of peptides.

The Maximum Number of Ligands that Can Be Screened Simultaneously. The relationship between the binding constants of the probe and target ligands to the receptor (K_b and K_b'), the ease of detection of the probe, and the concentration of the receptor in the buffer can be analyzed explicitly. The electrophoretic mobility of the probe ligand **L** on the column is affected by the concentration of receptor, [R], in the buffer. There are two limits to the time of migration of the probe ligand **L** on the column, depending on [R]. The first is at [R] = 0; the ligand is free in solution, and migrates along the column to the detector in a time t_o. The second is at [R] $\gg K_b^{-1}$; here, the ligand migrates as a complex R·**L**, in a time t_{max}. An intermediate value is the measured time t (between t_o and t_{max}) of migration of **L** is related to the fraction, f, of ligand that is complexed (eq 3); here the total concentration of **L** moieties (as free **L** and as R·**L**) is $[L]_o$. The equilibrium between the ligand, **L**, and its

$$f = [R\cdot L]/[L]_o = (t - t_o)/(t_{max} - t_o) = \Delta t/\Delta t_{max} \quad (3)$$

receptor is given by eq 4:

$$K_b = [R\cdot L]/[R][L] \quad (4)$$

Assuming that the probe ligand, **L**, when injected onto the capillary column at a concentration $[L]_o$, does not alter the concentration $[R]_o$ of receptor present in the electrophoresis buffer, eq 4 is approximated (i.e., $[R]_o \gg [R\cdot L]$) by eq 5:

$$K_b = [R\cdot L]/([R]_o([L]_o - [R\cdot L])). \quad (5)$$

Solving for [R·**L**] in eq 3, and inserting this result in eq 5, gives eq 6:

$$K_b = f/([R]_o(1 - f)) \quad (6)$$

Several experimental parameters must be specified in order to screen a pool of N different ligands, **L**′, efficiently for their ability to bind to a receptor. Specifically, to determine the combination of concentrations of receptor and ligands that maximizes N using affinity capillary electrophoresis, it is necessary to consider three parameters. The first is the total concentration, B, of additives that can be added to the buffer before absolute changes of greater than 1% (caused, for example, by changes in the dielectric strength and viscosity of the buffer) occur in the electrophoretic mobility of **L** (measured by t_o). The value of B is usually ~10% of the concentration of the buffer.[14] The second parameter is the minimum change in the time of migration of the probe ligand, $\delta\Delta t$, that is experimentally significant and can be used in estimating Δf. We assume that the change in the mobility of the probe ligand, **L**, is linearly related to the change in the fraction of the probe ligand, Δf, complexed to its receptor (eq 7). Equation 6 can be rearranged to give the change

$$\delta\Delta t = \Delta f \Delta t_{max} \quad (7)$$

in the concentration of receptor in the buffer, $\Delta[R]_o$, necessary to cause this shift in the migration time of the probe ligand (eq 8):

$$\Delta[R]_o = (1/K_b)((f/(1 - f)) - ((f + \Delta f)/(1 - f - \Delta f))) \quad (8)$$

The third parameter that must be specified is the value of the binding constant K_b' (the "targeted binding constant") that is the minimum binding constant of interest for **L**′ binding to the receptor. The equilibrium between the receptor and **L**′ in the buffer, for a starting concentration of probe ligand $[L']_o$, is given by eq 9. The

$$K_b' = \Delta[R]_o/(([L']_o - \Delta[R]_o)([R]_o - \Delta[R]_o)) \quad (9)$$

concentration $[L']_o$ can be interpreted as the minimum concentration of the ligand **L**′, which binds to the receptor with a binding constant of K_b', that must be present to cause a shift $\delta\Delta t$ in the time of migration of the probe ligand, **L**.

The maximum number of ligands, N, that can thus be added to the buffer is the ratio of B to $[L']_o$ (eq 10):

$$N = B/[L']_o \quad (10)$$

Figure 3 is a plot of the receptor concentration that maximizes N for given values of K_b and K_b'. This plot is obtained by numerical iteration of eq 10 according to the constraints of eqs 3–9.

Uses and Limitations of the Method. The ACE methodology described here provides an efficient approach with which to search for tight-binding ligands in certain

(14) This value of **B** is set empirically by the demands of the specific search and the experimental apparatus. As a rule of thumb, we find that **B** can be set as high as 10% of the concentration of the electrophoresis buffer.

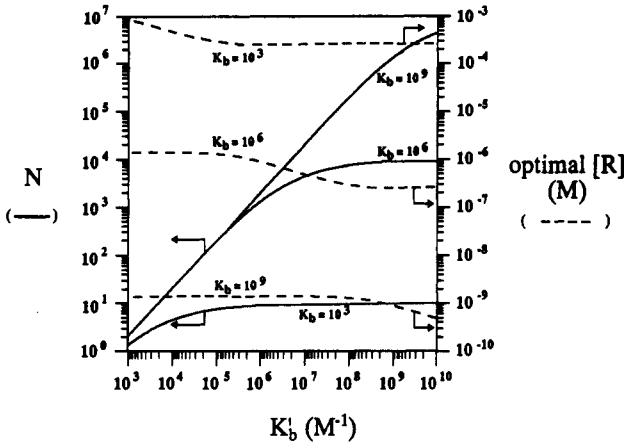


Figure 3. The maximum number, N, of peptides that can be screened simultaneously (as an equimolar mixture) for their ability to bind to a receptor with a binding constant greater than or equal to a value K_b' increases as the targeted binding constant K_b' increases. Equation 10 was iterated numerically, according to the constraints of eqs 3–9, for a given value of K_b' to yield the concentration of the receptor $[R]_o$ (– – –) at which N (—) was at its maximum value. The graphs were generated assuming values of 1×10^3, 1×10^6, and 1×10^9 M^{-1} for K_b of the probe ligand L with the receptor, a value of 5 s for $\delta\Delta t$ and a difference Δt_{max} of 30 s between the migration time of L and the complex R·L.

classes of libraries. The most serious experimental uncertainty in considering it for examination of a new soluble receptor (especially a protein) is adsorption of the receptor to the wall of the capillary. This problem is common with proteins, and in the absence of general methods for preventing adsorption,[15] the application of ACE in conjunction with this search strategy is limited to proteins that do not adsorb under the conditions of the assay.

ACE is useful for searching small libraries of 10^2–10^3 compounds. This class of libraries is an important one in many approaches to the development of drug leads. ACE has five useful characteristics: First, it can screen a number of compounds simultaneously and efficiently eliminate entire sets of compounds that do not contain active members. It is also able to identify and measure binding constants simultaneously for several tight-binding compounds.[2] Second, it does not require derivatization of the members of the library, nor does it require a biological activity: it is a pure binding assay. It *does* require a single, charged, probe ligand (L) with known binding properties. Third, by choosing the chromophore of L correctly, it should be possible to maximize its detectability and independence of interferences from other components of the solution. Fourth, the technique is equally applicable to both peptidyl and non-peptidyl ligands, provided that certain conditions are satisfied (for example, the probe ligand L must be easily detected against the background of the receptor in the buffer and the other members of the library). Fifth, it requires only small quantities of receptors and ligands, and the sequential analyses are easily automated and require only short times (typically minutes).

(15) Most proteins adsorb on the walls of uncoated capillaries. 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. *Anal. Chem.* **1991**, *63*, 1126–1132. Bushey, M. M.; Jorgenson, J. W. *J. Chromatogr.* **1989**, *480*, 301–310.

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Experimental Section

Materials. Vancomycin, from *Streptomyces orientalis*, and α,ϵ-Ac_2-L-Lys-D-Ala-D-Ala were purchased from Fluka and Bachem Bioscience, respectively. Peptides used for this study were obtained from Sigma. Fmoc-Gly-Ala-Ala (D,D and L,L) were synthesized according to the literature procedure.[2] The *N*-hydroxysuccinimide ester of (2-naphthoxy)acetic acid and 2-(2-aminoethoxy)ethanol were obtained from Aldrich.

Synthesis of the Neutral Marker *N*-(2-(2-Hydroxyethoxy)ethyl)-(2-naphthoxy)acetamide (M). To a solution of 2-(2-aminoethoxy)ethanol (67 μL, 0.93 mmol) in CH_2Cl_2 (10 mL) was added the *N*-hydroxysuccinimide ester of (2-naphthoxy)-acetic acid (140 mg, 0.47 mmol) in methylene chloride (10 mL). The reaction was stirred at room temperature for 3 h followed by addition of 10 mL of methylene chloride, washed with water, aqueous HCl, water, aqueous NaOH, water, dried over anhyd $MgSO_4$, and concentrated in vacuo to yield a white solid (101 mg, 75%): mp 68–70 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 8.7 Hz, 2 H, aryl H), 7.73 (d, J = 8.2 Hz, 1 H, aryl H), 7.46 (dt, J = 7.0, 1.0 Hz, 1 H, aryl H), 7.37 (dt, J = 7.0, 1.0 Hz, aryl H), 7.18 (dd, J = 8.9, 2.5 Hz, 1 H, aryl H), 7.13 (d, J = 2.4 Hz, 1 H, aryl H), 7.06 (s, 1 H, N*H*), 4.63 (s, 2 H, OCH_2CO), 3.66 (m, 2 H, NCH_2), 3.57 (m, 4 H, CH_2OCH_2), 3.51 (m, 2 H, CH_2OH), 2.27 (t, J = 5.7 Hz, 1 H, O*H*); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 165.9, 152.5, 131.7, 127.4, 126.9, 125.1, 124.4, 124.2, 121.9, 115.6, 105.0, 69.7, 67.2, 64.8, 59.2, 36.3; HRMS (FAB) *m/e* 312.1186 (M + Na)$^+$, calcd for $C_{16}H_{19}NO_4Na$ 312.1212.

Capillary Electrophoresis. An automated CE system from Isco, Inc (Lincoln, NE; Model 3140) was used for this study. Capillary electrophoresis was carried out using a buffer of 20 mM phosphate (pH 7.4) at a constant voltage of 30 kV (generating a current of approximately 38 μamp) with on-column detection at 264 nm. The capillary (uncoated, 50-μm i.d.), obtained from Polymicro Technologies, Inc. (Phoenix, AZ), was 70 cm in total length (45 cm from injection to detection).

A sample (8 nL) containing Fmoc-Gly-Ala-Ala (D,D and L,L; 8 μM = 64 fmol of each) and the neutral marker M (15 μM) in 20 mM phosphate buffer (pH 7.4) was introduced into the capillary by vacuum injection. The total analysis time in each experiment was ~2.5 min. The composition of the buffers is discussed in the text.