Simultaneous Measurement of Nineteen Binding Constants of Peptides to Vancomycin Using Affinity Capillary Electrophoresis−Mass Spectrometry[§]

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On-line affinity capillary electrophoresis−electrospray ionization-mass spectrometry (ACE−MS) was used for the simultaneous measurement of multiple binding constants of an all-D-tetrapeptide library to the model receptor, vancomycin. Determination of K_d values for the 19 peptides of the form Fmoc-DXYA is demonstrated. The data are compared with the results obtained for individual compounds using ACE−UV, and good correlation between the two detection methods is shown. Simultaneous determination of multiple K_d values by ACE−MS is achieved in one set of experiments, whereas only one K_d value can be obtained by ACE−UV during the same time. ACE−MS measures multiple binding constants in solution in a fast and reliable manner using femtomole amounts of samples.

Introduction

With the advances in combinatorial chemistry for selection of new drug candidates, there is a growing interest in the development of novel methodologies for high-throughput screening of libraries against biological targets, e.g., receptors, enzymes, etc. Measurement of the extent of complex formation between ligands and a target molecule, or determination of binding constants for combinatorial library members, is an important part of this effort. To characterize binding and often functional activity of the compounds of interest fully, quantitative knowledge of binding affinities and inhibition constants is required. A variety of experimental approaches for determination of binding constants rely on measurement of a change in a system property at various concentrations of a ligand in the presence of the receptor.[1,2] Commonly, a binding constant for only a single ligand is determined in one set of experiments.

Affinity capillary electrophoresis (ACE) has been introduced as an attractive tool for determination of the binding constants.[3] This solution-based technique is rapid and requires very small quantities of analytes. For off-rate systems that are fast relative to the migration time of the ligand through the column, ACE relies on the measurement of the mobility change of the ligand upon interaction with the receptor present in the electrophoretic buffer or vice versa.[4] It has been noted that ACE has a potential to measure binding constants for several substances simultaneously, as long as all analytes in the mixture are separated from each other and can be unambiguously identified.[5] For more complex mixtures, identification of specific ligands by UV detection is not possible; however, on-line mass spectrometric detection could be quite powerful since structure identification is achieved.

Recently we have introduced a new methodology, on-line ACE−MS, as a solution-based approach for screening of combinatorial libraries.[6] As a model receptor we used vancomycin, which is known to bind peptides with the C-terminal sequence AA (D,D).[7] Our approach allowed ranking in one run the peptides from libraries of at least 361 components (all D-amino acids) according to their affinity to vancomycin. The study led to the discovery of peptide ligands with the C-terminal consensus sequence FA/YA, which have a greater affinity to vancomycin than the naturally occurring ligand.

To demonstrate the use of ACE−MS as an effective tool for simultaneous measurement of binding affinities in solution for a mixture of compounds, in the present work we carried out the simultaneous measurement of dissociation constants (K_d) for the 19 peptides of the form Fmoc-DXYA, where X is any one of 19 common amino acids except Cys. These measurements demonstrated the effect on binding to vancomycin of the third from the C-terminus D-amino acid in tetrapeptide ligands. When the knowledge of the binding characteristics for a number of library members is needed, ACE−MS is shown to be a powerful solution-based approach that allows determination of multiple binding constants in the same set of experiments. The method is fast and automatable and requires minimal amounts of sample. Another advantage of ACE−MS is that neither the accurate knowledge of the ligand concentrations nor high purity of the receptor are required (assuming the impurities do not exhibit any affinity to the ligands).

Results and Discussion

General principles of ACE used for determination of binding constants have been described.[2,4] In our work, the receptor was added to the running buffer and the mobility of the interacting peptides monitored. Experiments were performed with electroosmotic flow (EOF) (using uncoated capillaries). We chose uncoated capillaries for this work, since the experiments can be performed faster with EOF, than without EOF, espe-

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cially for the higher-affinity peptides. Vancomycin was close to neutral at the experimental pH, and the peptides were negatively charged.[6] The mixture of the peptides was hydrodynamically injected into the capillary and electrophoresed while interacting with the receptor. Since the kinetic off-rates in the present system are fast, the peptides undergo multiple events of association−dissociation with vancomycin during the electrophoretic run. In addition, because vancomycin migrates with the EOF velocity at the experimental pH, the mobility of the complex is faster than that of a free negatively charged ligand. The higher the affinity, the larger the mole fraction of the ligand at any instant that exists in the complexed form, and consequently, the greater the peptide is shifted from its position in the electropherogram when no receptor is in the buffer. The mobility shift of the ligands upon interaction with the receptor can be used to calculate the K_d of the complex:[8]

$$K_d = [R]((\mu_t - \mu_c)/(\mu_0 - \mu_t)) \quad (1)$$

where μ is mobility and subscripts 0 is the free ligand in the absence of the receptor, t is the ligand with receptor present in the running buffer at a concentration [R], and c is the ligand complexed with the receptor.

The mobility of the complex, μ_c, must be measured at saturating receptor concentrations in order to use eq 1 to calculate binding constants. This value often cannot be accurately determined due to experimental limitations (e.g., receptor solubility, change in solution viscosity). However, the value of K_d can be obtained by measuring μ_t as a function of [R] based on several modifications of eq 1.[2] We performed this type of experiment with a small library of peptides in order to determine their K_d values. All peptides were injected simultaneously into the capillary, and electrophoretic mobility of each ligand was measured as a function of vancomycin concentration in the running buffer.

The EOF is generally assumed to remain constant upon increasing the concentration of the receptor or ligand in the buffer. However, the EOF may vary due to changes in the viscosity of the buffer with changes in the concentration of the receptor.[9,10] Therefore, correction for the EOF is necessary for accurate determination of the mobility shift of a peptide. Thus, a marker, which does not interact with the receptor or ligands, was added to the mixture of ligands, transforming eq 1 to:

$$\frac{(\mu'_t - \mu_t^m) + (\mu'_0 - \mu_t^m)}{[R]} = -\frac{1}{K_d}((\mu'_t - \mu_t^m) + (\mu'_0 - \mu_t^m)) + \frac{1}{K_d}((\mu'_c - \mu_c^m) + (\mu'_0 - \mu_0^m)) \quad (2)$$

where m corresponds to the marker, μ'_t and μ'_0 are the apparent mobilities of the ligand with and without the receptor in the running buffer, and μ'_c is the apparent mobility of the complex (all measured with EOF present). If the actual migration time of the ligand (t) is used instead of the apparent mobilities, eq 2 yields the K_d value as $-(1/\text{slope})$ of the line:

$$1/[R] \times [A] \text{ versus } [A] \quad (3)$$

where

$$A = \left(\frac{t_0 - t_t}{t_0 \times t_t} - \frac{t_0^m - t_t^m}{t_0^m \times t_t^m}\right)$$

The analyses were conducted with a library of 19 tetrapeptides of the form Fmoc-DXYA. The Fmoc-modified glycine (Fmoc-G) was utilized as an EOF marker, as it did not interact with vancomycin. Figure 1A, bottom, presents the total ion electropherogram (TIE) of the 19-peptide mixture, with no vancomycin present in the buffer. The peptides migrate in groups, depending on their charge at the experimental pH. This experiment also provided for determination of the library quality. All the peptides were present in the synthesized mixture, and no impurities were detected at the working level of concentrations. The other electropherograms in Figure 1A present CE−MS runs with 2.5, 10, and 50 μM vancomycin in the buffer. Figure 1B shows an example of the extracted ion electropherogram corresponding to the ligand Fmoc-DDYA run at different receptor concentrations in the buffer. The expected decrease in migration time with increasing receptor concentration is evident. The presence of unmarked peaks in the selected ion electropherograms (Figure 1B) was caused by the isotopic contribution from the neighboring peaks of Fmoc-DNYA, -DLYA, and -DIYA. The isotopes of these peptides have large intensities compared to the intensity of DDYA, since the latter peptide is more acidic and has lower ionization efficiency in the positive ion mode. As vancomycin driven by the EOF enters the mass spectrometer, some fraction of the peptide can exist in the complexed form even in the gas phase. In fact, the peak area of the peptide Fmoc-DDYA decreased with increasing concentrations of vancomycin, since only the molecular ion of the free peptide is detected. This phenomenon is observed for all the peptides, as can be seen in Figure 1A. The intensities of the peptides become low with higher concentration of the receptor added to the running buffer. The corresponding Scatchard plot for the peptide Fmoc-DDYA (eq 3) exhibits excellent linearity (correlation coefficient −0.995). Similar results were obtained for the other ligands, and the K_d values of all ligands were thus determined.

The K_d values for all 19 ligands can be measured in the same time frame. The summarized data on dissociation constants for the Fmoc-DXYA peptides from ACE−UV and ACE−MS are given in Table 1 and show good correlation between the two detection methods. The results indicate that the third position only slightly affects the binding to vancomycin; however, the method allows differentiation of even small variations in affinity, as seen in Table 1.

Simultaneous determination of multiple K_d values by ACE−MS can be achieved significantly faster than by ACE−UV experiments, especially if large mixtures of compounds have to be characterized. When binding constants are determined for individual ligands by ACE−UV and a minimum of 10 data points is necessary for Scatchard analysis, it requires 6.5 h to obtain one K_d value, assuming 20 min run time and duplicate experiments (based on the automation option available on CE instruments).

While ranking of ligands is important for rapid qualitative evaluation of relative affinity, determination

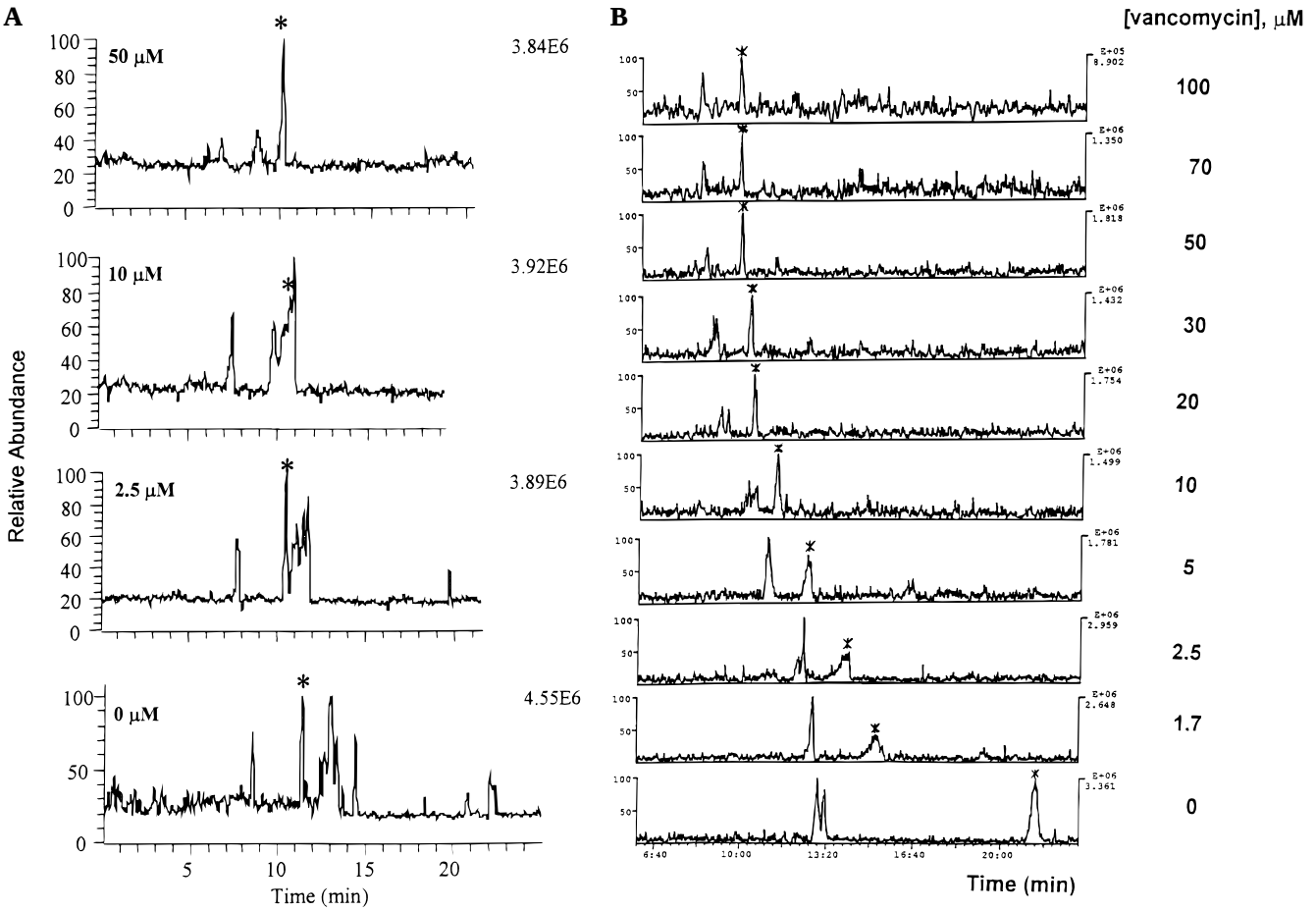

Figure 1. ACE−MS binding experiments with Fmoc-DDYA. A, Total ion electropherogram (TIE), corresponding to the experiments with 0, 2.5, 10, and 50 μM vancomycin in the running buffer; the EOF marker, Fmoc-G, is labeled by an asterisk. B, Extracted ion electropherograms of $(M + Tris + H)^+$, m/z 826 (asterisk). Conditions: uncoated 50 cm capillary, 20 mM Tris/acetate, pH 8.0, 20 kV, 10 s of library sample injection.

Table 1. K_d Values for Fmoc-DXYA Peptides[a]

peptide	K_d, μM	
	ACE−UV	ACE−MS
Fmoc-DLYA	5.6	5.1
Fmoc-DYYA	6.8	7.4
Fmoc-DHYA	7.1	8.2
Fmoc-DMYA	7.1	7.6
Fmoc-DDYA	7.2	6.6
Fmoc-DAYA	7.2	8.2
Fmoc-DFYA	9.6	8.9
Fmoc-DEYA	10	8.5
Fmoc-DNYA	11	12
Fmoc-DVYA	12	10
Fmoc-DRYA	13	11
Fmoc-DKYA	13	12
Fmoc-DSYA	13	14
Fmoc-DWYA	14	12
Fmoc-DQYA	14	15
Fmoc-DPYA	14	16
Fmoc-DGYA	18	21
Fmoc-DIYA	19	21
Fmoc-DTYA	21	24

[a] ACE−UV: capillary 30 cm effective length, 37 cm total length, 20 KV. ACE−MS: conditions same as in Figure 1.

of binding constants is essential for complete quantitative system characterization. Multiple binding constants can be easily measured using ACE−MS even if the migration times of ligands overlap, since the position of each peptide in the electropherogram can be determined by MS. In the present example all the peptides had different molecular weights, except for Fmoc-DQYA, -DKYA (MW 717) and Fmoc-DLYA, -DIYA (MW 702). Fmoc-DQYA and -DKYA had different charges and were thus well-separated from each other. The other two isobaric peptides (Fmoc-DLYA, -DIYA) possessed the same electrophoretic properties and, therefore, could not be distinguished. The dissociation constants still could be determined for both species, since their migration behavior varied as a function of K_d. Dissociation constants were assigned to each isobaric peptide based on the experiment with one of the ligands individually electrophoresed at an arbitrarily chosen vancomycin concentration.

A potential limitation of the simultaneous determination of binding constants by ACE−MS is possible competition between ligands for the receptor at low receptor concentration. However, this would be true only for ligands migrating in the same electrophoretic zone throughout the column. Even in this case, the compound with higher affinity would migrate differently than the weaker ligand due to interactions with the receptor. These analytes will thus also not compete for the receptor molecules during the electrophoretic run. Only the mobility shift for ligands with similar affinity and the same electrophoretic properties would be affected at low receptor concentrations. As an example, we disregarded the measurement at 1.7 μM vancomycin for some of the hydrophobic peptides exhibiting similar

affinity to the receptor. At this low concentration of vancomycin, these peptides practically coeluted, and thus competitive binding to the receptor could have occurred. Indeed, the results at this receptor concentration did not fit the Scatchard plot for other concentrations, and we concluded that the local concentration of the ligands was comparable to the concentration of the receptor, resulting in competitive binding conditions. In our case, the measurements at higher receptor concentrations were sufficient. However, this factor could be an issue for the systems where lower receptor concentrations are necessary for K_d determination, e.g., for complexes with higher binding affinities than in the example presented in this paper. The binding affinity range for study can be increased by use of a more sensitive MS instrument, such as ESI time-of-flight MS, since the ligands could be analyzed at lower concentration levels.

In summary, this work demonstrates that ACE−MS can simultaneously determine binding constants for selected ligands using simple and rapid procedures. This solution-based, low-sample-consumption approach is especially attractive because it provides determination of multiple binding constants in a reliable manner: the values are obtained in the same set of experiments, i.e., all the experimental conditions are identical for the investigated compounds. While simultaneous measurement of binding constants for 19 compounds has been demonstrated, the method can be potentially expanded to libraries containing many more compounds.

Experimental Section

Peptide Library. The synthesis of the 19 peptides of the type Fmoc-DXYA (all D) (Table 1) was performed using 2-chlorotrityl polystyrene resin, containing 0.125 mmol of loaded D-amino acid, employing a standard protocol for solid-phase peptide synthesis described elsewhere.[6] The peptide mixtures were cleaved and released from the resin using a solution of TFA/H_2O (90:10, v/v) at room temperature for 3 h, followed by precipitation with ethyl ether. The peptides were soluble in buffers of pH > 7. The quality of the library (purity of the peptides) has been previously discussed in detail elsewhere.[11]

ACE−MS. The ACE−MS experiments were performed using a Finnigan TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) interfaced with a Finnigan atmospheric pressure ionization (API) source operated in the positive electrospray ionization (+ESI) mode. The instrument was scanned over the expected mass range at a rate of 1.5−2 s/scan. ESI was performed at +4.5 kV with the heated capillary inlet at 150 °C. Uncoated fused-silica gel capillaries were 50 μm i.d., 360 μm o.d., and 50 cm in length. The running buffer was 20 mM Tris in water, adjusted to pH 8.1 with acetic acid, and contained from 0 to 100 μM vancomycin. The liquid sheath was 5 mM Tris−acetate (pH 8.1) in H_2O/MeOH (25:75, v/v) at a flow rate of 1.5 μL/min. Nitrogen was used as a sheath gas at 100−1000 cm^3/min to assist electrospray stability. The injection end of the capillary was held at a positive potential to produce a 400−500 V/cm potential gradient at a current of 5−8 μA. The samples were hydrodynamically injected at a height of 10 cm for 10 s, corresponding to an injection volume of roughly 15 nL.

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