Articles

Screening Derivatized Peptide Libraries for Tight Binding Inhibitors to Carbonic Anhydrase II by Electrospray Ionization-Mass Spectrometry

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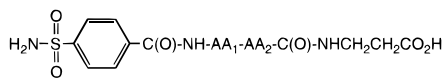
This paper describes the use of electrospray ionization-mass spectrometry (ESI-MS) to screen two libraries of soluble compounds to search for tight binding inhibitors for carbonic anhydrase II (EC 4.2.1.1). The two libraries, $H_2NO_2SC_6H_4C(O)NH\text{-}AA_1\text{-}AA_2\text{-}C(O)NHCH_2CH_2CO_2H$ (**1**), where AA_1 and AA_2 are L-amino acids (library size: 289 compounds) or D-amino acids (256 compounds), were constructed by attaching tripeptides to the carboxyl group of 4-carboxybenzenesulfonamide. Screening of both libraries yielded, as the tightest binding inhibitor, compound **1** ($AA_1 = AA_2 =$ L-Leu; binding constant $K_b = 1.4 \times 10^8\ M^{-1}$). The ability of ESI-MS to estimate simultaneously the relative binding affinities of a protein to soluble ligands in a library, if general, should be useful in drug development.

Introduction

Recently, we reported a method based on electrospray ionization-mass spectrometry (ESI-MS)[1,2] to identify inhibitors that bind tightly to carbonic anhydrase II (CAII; EC 4.2.1.1) and demonstrated this approach by applying it to two small libraries (7 and 18 components) of compounds.[3] The method utilizes ESI to generate ions of intact noncovalent complexes in the gas phase[4−6] and Fourier transform ion cyclotron resonance (FTICR) mass spectrometry[7−10] to identify free tight binding inhibitors after dissociation from the complexes in the FTICR ion trap. The method exploits the unique capabilities of FTICR for *m*/*z*-selective ion accumulation,[11,12] isolation, complex dissociation, and high-resolution product measurements. Our initial communication showed that ESI-MS can be used to estimate relative binding affinities of a mixture of ligands to CAII based on the correlation of relative ion intensities with binding constants (K_b) in solution.[3]

In the current study, we have applied this new ESI-FTICR method to the screening of two larger peptide libraries (**1**, derived from 4-carboxybenzenesulfonamide); these libraries are sufficiently large to be of practical use. We chose CAII as a model protein since



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it is pharmaceutically relevant, inexpensive, and commercially available. The high-resolution X-ray structure of CAII shows that its active site is amphiphilic.[13,14] The ligand libraries were constructed by attaching tripeptides terminating in a common $-NHCH_2CH_2CO_2H$ (β-Ala) group to the carboxyl group at the *para* position of benzenesulfonamide (**1**). We report here the use of ESI-FTICR to identify amino acid residues that maximize the binding affinities by secondary interactions with the active site of CAII. The approach is confirmed by syntheses and evaluations of selected inhibitors in solution. We also discuss four considerations in using this approach to study specific noncovalent complexes: (i) the increased performance possible from the mass spectrometry that results from analyzing free ligands from dissociation of complexes (rather than analyzing intact complexes); (ii) the issue of mass redundancy in the mass spectra of the free ligands; (iii) the basis for the correlation between relative ion intensities in the gas phase and solution binding constants; and (iv) the scope and potential limitations of the method.

Results and Discussion

Syntheses of Derivatized Libraries. We constructed two separate tripeptide libraries, using L- or D-amino acids, by *N*-9-fluorenylmethyloxycarbonyl (Fmoc) chemistry following the split synthesis protocol.[15,16] We first attached a common β-Ala unit to the Wang resin to prevent diketopiperazine formation at the dipeptide step. General coupling procedures utilized 4 equiv of Fmoc-amino acids or 4-carboxybenzenesulfonamide (for the last coupling step), benzotriazol-1-yl tetramethyluronium hexafluorophosphate, and 1-hydroxybenzotriazole mixed with *N*,*N*-diisopropylethylamine in DMF. Fmoc protecting groups were removed with 20% piperidine in DMF for 10 min after each coupling step. The peptides were released from the solid support by stirring the resin in a solution of 90% trifluoroacetic acid, 5% anisole, and 5% water for 3 h. The resin was removed by filtration, and the filtrate was collected and concentrated *in vacuo*. The concentrated filtrate was dissolved

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in water and lyophilized to yield the peptide library as a solid. The solid material was used for screening without further purification. Amino acid analyses showed that each amino acid has the same molar ratio in both libraries, and ESI-MS showed that there were no major contaminants present. We infer from this information that each compound is equally represented in the library.

Observation of Specific Protein−Inhibitor Complexes by ESI-FTICR-MS. The ESI-FTICR instrument offers better sensitivity than conventional mass spectrometry, and milder interface conditions can be used to obtain intact noncovalent complex ions with a good signal-to-noise ratio. A major reason for the difference may be that in the FTICR the complexes are analyzed a minimum of several seconds after trapping, thus allowing a slower and more gentle desolvation, in contrast to the much faster desolvation and ion formation required for conventional mass spectrometers (<milliseconds).

Electrospray ionization of CAII from acidic solutions (pH ~3) produced ion charge states +15 to +26 of CAII (data not shown), from which the molecular weight of CAII can be accurately determined (observed average MW = 28 996.6 ± 0.1 Da using bovine insulin as an internal standard; calculated MW = 28 996.7 Da). In contrast, from neutral or slightly basic solutions (pH 7.0−8.5, 10 mM NH_4OAc or Tris acetate), CAII produces ions with much lower and fewer charge states (±7 to ±10), and having a mass 63.4 Da higher than from acidic solution. The decrease in mass in acidic solution is due to the loss of a Zn(II) from CAII under acid-catalyzed denaturation conditions. When a mixture of CAII and a peptide library was ionized from a 10 mM NH_4OAc solution (pH 7.0), we observed peaks corresponding to the −7 to −9 charge states of the intact protein−ligand complexes (Figure 1A). These complexes were not observed from acidified solutions or when harsher ESI-MS interface conditions were used (by increasing the temperature of the heated capillary inlet used for electrospray desolvation). Additional experiments verified the nature of specific binding of ligands to CAII.[3]

Screening of the Peptide Libraries Using ESI-MS. When a mixture of CAII (2.5 μM) and a peptide library (0.5 μM for each inhibitor; 289 and 256 compounds for the L- and D-libraries, respectively) in a 10 mM NH_4OAc solution (pH 7.0) was analyzed using ESI-FTICR, we observed major peaks in the mass spectra corresponding to intact complexes in the −7 to −9 charge states. The individual protein−ligand complexes cannot be resolved due to the number of different ligands and to the heterogeneity in the isotopic content (Figure 1A). Interestingly, a small fraction (~5%) of the CAII ions are not present as complexes (designated CAII + Zn), indicating that this fraction of the CAII did not bind inhibitor in solution or that the complex dissociated prior to detection.

Collision-induced dissociation of the m/z-isolated −9 charge state complexes primarily produced the −8 charge state of the protein ($[CAII + Zn]^{8-}$) and singly-charged negative ions for nearly all the inhibitors ($[\mathbf{1}]^{1-}$, Figure 1B). When relatively high collision energies were used to ensure complete dissociation of the complexes, we also observed a minor contribution of protein

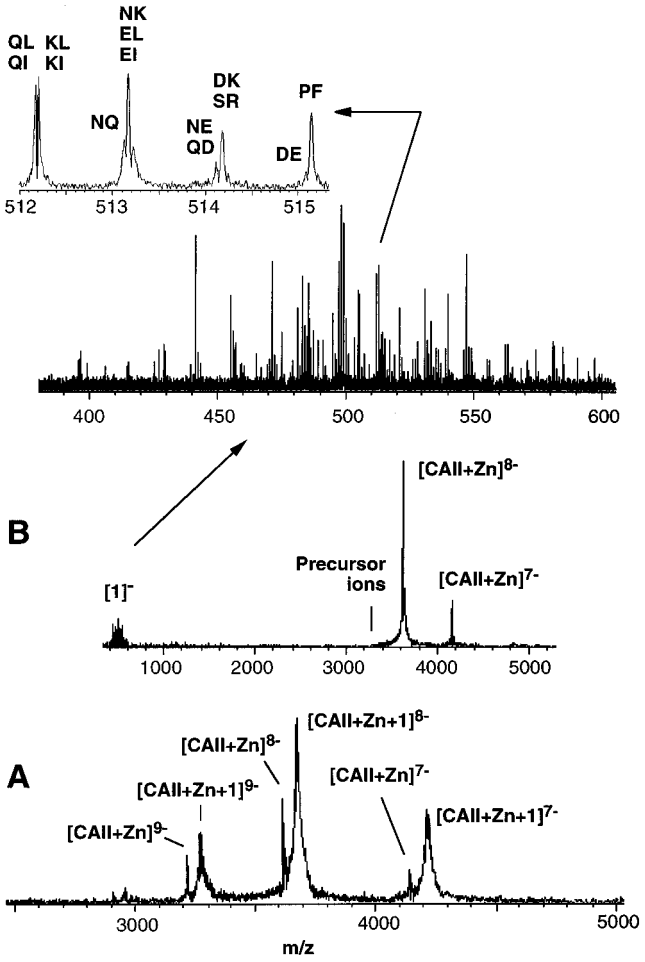

Figure 1. (A) ESI-FTICR mass spectrum from a mixture of the 289-component L-library (0.5 μM each) and CAII (2.5 μM) in 10 mM NH_4OAc (pH 7.0). (B) Collision-induced dissociation (CID) of the isolated complex ions of $[CAII + Zn + \mathbf{1}]^{9-}$. The CID condition was set such that the complex ions were completely dissociated. The insets show expanded views of the singly-charged inhibitors, $[\mathbf{1}]^{-1}$, as the CID product ion and the separation of inhibitor ions with the same nominal masses. The letters in the figure inset denote the amino acid composition of the inhibitor ions.

ions at −7 charge state. Close examination of the low-m/z region of these spectra failed to show any doubly-charged inhibitor ions. Thus, protein ions at −7 charge state are most likely formed through a sequential collision-induced charge exchange (i.e., charge loss) process due to collisions with neutral gases. Further, we did not observe any −9 charge states of CAII + Znions (where the ligands dissociated from the complex as neutral species); therefore, we concluded that the ligands dissociated from the complexes only as singly-charged negative ions. The resulting mass spectra allow the dissociated ligands to be identified based upon their different molecular weights; their ion intensities reflect the relative binding affinities.

Figure 2 shows the dependence of relative ion intensities of the dissociated ligands on the composition of the amino acids for the two libraries. We note that sequence isomers (-AA_x-AA_y- and -AA_y-AA_x-) and structural isomers (e.g., -Leu-Leu- and -Ile-Ile- in the L-library) are not distinguished since their molecular weights are identical.[17] The high resolving power of FTICR enabled separation of compounds with mass difference of ≥0.025 Da. We therefore divided the ion intensities by the

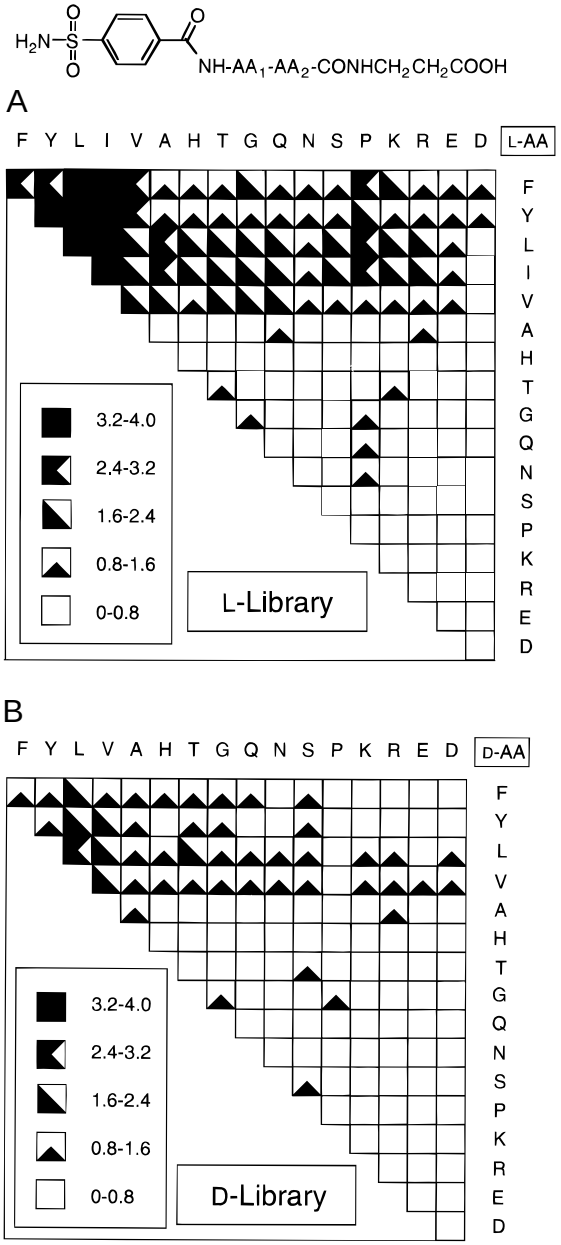

Figure 2. Dependence of relative ion intensities on the composition of amino acids in the peptide library. The structure of the peptide library is shown at the top of the figure. The amino acids (AA_1 and AA_2) are arranged in such a way that their hydrophobicity decreases from left to right and from top to bottom. The relative ion intensity for each ligand was obtained by comparing the ion intensity of this ligand to that of the Gly-Gly compound, which is present in both libraries. The results from L- and D-libraries are plotted on the same scale of relative ion intensity, which is shown in the bottom left corner of each figure: (A) results from the L-library and (B) results from the D-library.

number of possible isomers and compounds with mass difference <0.025 Da to give average values. Thus, the ESI-MS results might be expected to, at most, underestimate the affinity of an inhibitor by a factor of N, the number of possible components in an unresolved peak in the mass spectrum.[18]

Comparison of Binding Affinities of Individual Inhibitors in Solution with Their Relative Ion Intensities. We synthesized seven individual inhibitors from the two libraries that have specific AA_1 and AA_2 to test their binding affinities to CAII in solution using a fluorescence binding assay.[19] In this assay,

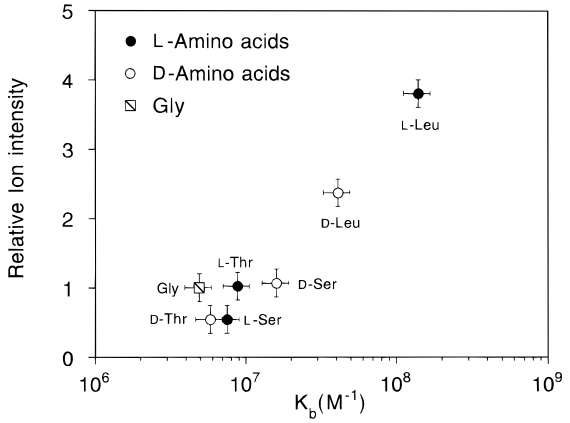

Figure 3. Correlation of relative ion intensities vs K_b in solution for seven peptide inhibitors from L- and D-libraries. We selected inhibitors having identical AA_1 and AA_2, since their ion intensities are free of ambiguities that are caused by sequence isomers. The relative ion intensity for each ligand in both libraries is normalized to that of Gly-Gly, which exists in both libraries. The three-letter codes for amino acids are used to indicate the identity of AA_1 and AA_2. The values of K_b were measured in 20 mM phosphate buffer (pH 7.5) at 37 °C. The uncertainties for the measurement are indicated by the error bars.

affinities of nonfluorescent ligands were measured by competition with dansylamide ($K_b = 4.0 \times 10^6$ M^{-1}; dansylamide fluoresces upon excitation when bound to CAII). The binding constants of the seven inhibitors in solution correlated well with the relative intensities of the ions dissociated from the CAII−inhibitor complexes (Figure 3). The K_b for the tightest binding inhibitor identified by ESI-MS ($AA_1 = AA_2 =$ L-Leu, see Figure 2) was 1.4×10^8 M^{-1}. The K_b of the compound that we tested that bound most weakly ($AA_1 = AA_2 =$ Gly) was 4.9×10^6 M^{-1}. This result provides further support that vapor-phase and solution-phase ordering of binding constants correlate and that the MS data can be used to infer the order of binding affinities in solution.[3] The agreement between vapor and solution data is qualitatively good, but it will be necessary to examine other systems to establish the generality of this type of correlation.

The data in Figure 2 also add support to the hypothesis that the addition of hydrophobic groups at the *para* position of benzenesulfonamide increases binding constants.[20] The chirality of the amino acids appears to influence the binding affinities of the tripeptide inhibitors—the side chains of L-amino acids interact more effectively with the active site of CAII than did D-amino acids in this limited set.

Physical Background of Ordering Relationships in the Screening of Peptide Libraries Using ESI-MS. The results from this work and a previous study[3] establish a correlation between the relative ion intensities and the solution binding constants for a mixture of ligands binding to CAII. We rationalize this correlation using the following physical model.

We assume a 1:1 binding stoichiometry for protein−ligand interactions in this model. When a mixture of ligands (L_i, $i = 1, ..., n$, where $n =$ the total number of ligands) is mixed with a protein in solution (of initial concentration $[P]_o$) and equilibrium is achieved between each ligand and the protein, the concentration of the protein bound with ligand L_i ($[P{\cdot}L_i]$) is determined by

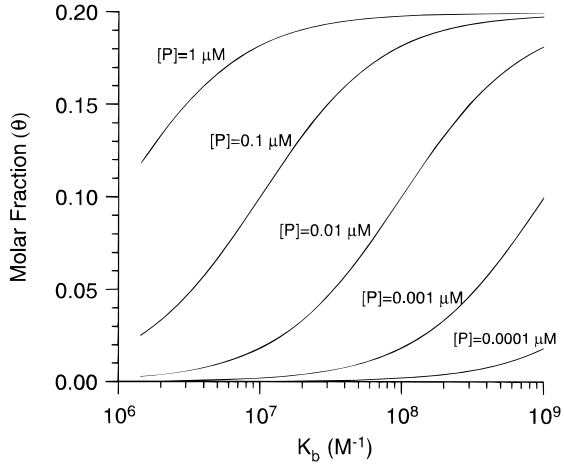

Figure 4. Dependence of molar fraction $\theta = [P{\cdot}L_i]/[P]_o$ on the binding constant K_b and equilibrium concentrations of the protein [P]. The curve was generated based on eq 4 for values of [P] in the range of 0.0001−1 μM and K_b in the range of values observed in this work; $[P]_o = 2.5\ \mu$M and $[L]_o = 0.5\ \mu$M.

the binding constant K_{bi}, the concentration of the free protein ([P]), and the concentration of the free ligand ($[L_i]$) in solution (eq 1). The molar fraction (θ_i) of the protein complexed with ligand L_i is defined as the concentration of $P{\cdot}L_i$ complex over the total concentration of the protein in solution (eq 2). Equation 4 is derived by substituting eq 3 (in eq 3, $[L_i]_o$ is the total concentration of ligand L_i) into eq 2, and it expresses the molar fraction as a function of $[L_i]_o$, $[P]_o$, K_{bi}, and [P].

$$K_{bi} = \frac{[P{\cdot}L_i]}{[P][L_i]},\ i = 1, ..., n \tag{1}$$

$$\theta_i = \frac{[P{\cdot}L_i]}{[P]_o} = \frac{K_{bi}[P][L_i]}{[P]_o} \tag{2}$$

$$[L_i] = [L_i]_o - [PL_i] = [L_i]_o - \theta_i[P]_o \tag{3}$$

$$\theta_i = \frac{[L_i]_o K_{bi}}{[P]_o\left(\frac{1}{[P]} + K_{bi}\right)} \tag{4}$$

When the initial concentration of each ligand is equal, $[L_i]_o = [L_j]_o$, the ratio of molar fraction for two ligands (L_i and L_j) is given by eq 5a:

$$\frac{\theta_i}{\theta_j} = \frac{K_{bi}\left(\frac{1}{[P]} + K_{bj}\right)}{K_{bj}\left(\frac{1}{[P]} + K_{bi}\right)} \tag{5a}$$

$$\approx \frac{K_{bi}}{K_{bj}},\ \left(\frac{1}{[P]} >> K_{bi}, K_{bj}\right) \tag{5b}$$

$$= \frac{1}{2} + \frac{K_{bi}}{2K_{bj}},\ \left(\frac{1}{[P]} = K_{bi}\right) \tag{5c}$$

$$\approx 1,\ \left(\frac{1}{[P]} << K_{bi}, K_{bj}\right) \tag{5d}$$

Figure 4 correlates molar fraction θ with K_b for values of [P] in the range of 0.0001−1 μM and K_b in the range of values observed in this work; values of $[L]_o$ and $[P]_o$ were chosen corresponding to those for the two libraries studied in this work ($[L]_o = 0.5\ \mu$M each and $[P]_o = 2.5\ \mu$M). There is a monotonic correlation between the molar fraction θ and the binding constant K_b when the inverse of the equilibrium concentration of the protein is close to (eq 5c) or higher than (eq 5b) the binding constants of the ligands. Under these conditions, the measurement of molar fraction θ can provide the order of binding affinities of ligands to a protein. The correlation is clearly sensitive to the equilibrium concentration of the protein. In this study, the close resemblance of the experimentally observed correlation between relative ion intensities and solution binding constants and the simulated curve for $[P] = 0.01\ \mu$M should be noted. To obtain *quantitative* information about binding constants, the equilibrium concentration of the protein must be accurately known or the correlation curve (Figure 4) must be calibrated using internal standards.

In this work, we have used ESI-MS to determine the *relative* concentrations of protein−ligand complexes in solution. The basis of this approach is a linear correlation between relative ion intensities (I_{PL_i}) in the gas phase and the molar fractions (θ_i) of these complexes in solution (eq 6, where C_i is a constant that correlates I_{PL_i} with θ_i). Three conditions have to be met in order

$$I_{PL_i} = C_i\theta_i \text{ and } C_1\ ...\ = C_i\ ...\ = C_n = C \tag{6}$$

$$I_{L_i} = C' I_{PL_i} \tag{7}$$

for eq 6 to hold. First, there should be minimal or no dissociation of the complexes during ionization, ion transport, trapping, and detection (since the stability of the complex for each ligand in the gas phase may not follow that in solution). This condition has been shown to be achievable in a number of previous reports using gentle ESI-MS interface conditions[3−6] and is also demonstrated in this work. Second, differences in sensitivity due to ionization, ion transport, and detection of the ligands should also be minimal. Our method assumes similar ionization efficiencies for complexes of different ligands. These complex ions should have very similar formation efficiencies due to the fact that the protein accounts for the bulk of the mass and charge of the complexes, which are the two important factors affecting electrospray ionization. For example, the inhibitors used in this work have two to four acidic groups, while there are 38 acidic groups (including eight Tyr) in CAII. Consistent with this assumption, we observe that the charge states of the CAII−inhibitor complexes are similar to those of the free CAII ions under otherwise similar solution and instrumental conditions (data not shown). Third, the ions from different protein−ligand complexes or ligands (after complete dissociation of the complexes) should be resolved and free of nonspecific formation of adducts from buffer components and metal cations. Due to the high MW of protein, the intact complexes appear at high values of m/z where mass resolution is degraded, and isotopic content of the protein can be problematic for resolution of the complex ions of different ligands. We therefore used the strategy of completely dissociating the complex ions in the gas phase and examining the dissociated ligands instead. Here we assume a quantitative correlation between ion intensities of complexes (I_{PL_i}) and those of free ligands

(I_{L_i}) (eq 7, where C' is a coefficient). In addition to the elimination of the protein isotope contribution and the increased mass spectrometry performance at low-m/z region by detecting free ligands instead of the protein−ligand complexes, this strategy offers the additional advantage of eliminating the effect of possible formation of buffer and cation adducts with the complexes. To ensure faithful representation of the dissociated ligands for the complexes (eq 7), we selected experimental conditions that completely dissociate all the complexes.

Scope and Limitations of the Method. The ESI-FTICR approach we have described generates relative binding affinities for a large number of ligands simultaneously in one relatively short experiment. The sizes of the libraries examined here—289 and 256 members—are relatively small compared to the size of some combinatorial libraries. In principle, screening of larger libraries is possible if the mass differences between the components in the library can be detected by MS and if there are significant differences in binding affinities. The latter concern arises due to the need to have sufficient ligand in the FTICR cell after dissociation for detection (about 50 ions are required for detection with a $S/N \sim 2$). Thus if the solution conditions (e.g., $1/[P] << K_{bi}$) or the ligands (e.g., similar binding affinities) result in many different ligand−protein complexes of comparable abundances, the detached ligands may have insufficient abundances for detection.

We do not yet know how generally applicable this approach will be. The present approach should be most effective for identification of the tight binding ligands, since nonspecific (random) associations induced during, or by, the ESI process may limit the quality of information for weaker binding ligands, particularly in larger libraries. CAII is a small, stable protein that binds a structurally simple class of hydrophobic ligands. Further study of additional well-characterized model systems will be necessary to define the extent to which quantitative information can be derived from this method. In principle, the ESI-FTICR methods should be most useful for determining the relative affinities of a library of compounds to a protein when (i) the binding study can be carried out using volatile buffers such as ammonium acetate, Tris acetate, and ammonium bicarbonate, (ii) the protein sample is moderately pure, (iii) all the library components will be charged after dissociation of complexes in the gas phase (which is likely the case when the library is based on structurally similar compounds having acidic or basic group(s)), and (iv) the library components have different masses.

Conclusions

In summary, this work demonstrates the use of ESI-MS to screen two libraries (289 and 256 components) rapidly for tight binding ligands to CAII. The screening successfully identified a tight binding ligand to CAII with a binding constant of 1.4×10^8 M^{-1}. The method provides a screening tool that is free of the artifacts that may exist in the bead-based or surface screening of libraries. Consistent with our earlier communication, we have found a good correlation between the relative ion intensities of the ligands and their K_b for CAII in solution. Although not hydrated in the MS, the relative concentrations of dehydrated CAII−inhibitor complexes in the gas phase are clearly stable and allow determination of relative binding affinities in solution.

The design of libraries of organic molecules or of collections of natural products in a way that ensures that each component has a different molecular weight can eliminate ambiguities in the identification of ligands and reduce the demands for subsequent dissociation and greater sensitivity in these experiments. Meanwhile, the sensitivity, ease, and high speed of screening by ESI-MS[21] compensate for limitations to the size of the library that can be screened. The fact that relative binding constants can be derived from these experiments for all or most of the members of a library may make it possible to build more broadly representative structure−activity relationships[20] than is possible when only a few of the tightest binding ligands are examined. These unique capabilities of ESI-MS for library screening promises to be useful in drug development.

Experimental Section

Materials and Methods. Carbonic anhydrase II (from bovine erythrocytes; EC 4.2.1.1.) was purchased from Sigma and used without further purification. Fmoc-protected Wang resin was obtained from Novabiochem. Fmoc-protected amino acids were purchased from Bachem California. Benzotriazol-1-yl tetramethyluronium hexafluorophosphate, 1-hydroxybenzotriazole (0.5 M in DMF), and piperidine were obtained from Applied Biosystems. 4-Carboxybenzenesulfonamide was purchased from Aldrich.

Syntheses of Peptide Libraries. The two peptide libraries were synthesized using L- or D-amino acids using standard solid-phase peptide synthesis methods with N-9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. Fmoc-β-Ala was first attached to the Wang resin (200−400 mesh size, 0.6 mmol/g) to prevent diketopiperizine formation at the dipeptide step. A total of 17 natural L-amino acids (omitting Cys, Met, and Trp) or 16 D-amino acids (omitting Cys, Met, Trp, and Ile) were used. For the L-library, the Fmoc protecting groups were first removed with 20% piperidine. The resin was divided into 17 aliquots, and each was allowed to react with a single Fmoc-amino acid. Coupling was initiated by the addition of 4 equiv of benzotriazol-1-yltetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and *N,N*-diisopropylethylamine in DMF. The coupling reaction was driven to completion with a 4-fold molar excess of Fmoc-amino acids and monitored by the standard 2,4,6-trinitrobenzenesulfonic acid (TNBS) test.[22] Subsequently, the aliquots were mixed thoroughly, washed, deprotected with 20% piperidine, washed, and divided into 17 aliquots again for the next cycle of coupling. The resulting library was deprotected with 20% piperidine, washed, and coupled to 4-carboxylbenzenesulfonamide (4-fold excess) using the same initiation procedure described above. The resin was washed with DMF, CH_2Cl_2, and Et_2O extensively and dried *in vacuo*. The derivatized peptide library was cleaved from the solid support by stirring the resin in a solution of 90% trifluoroacetic acid (TFA), 5% anisole, and 5% water for 3 h. The resin was removed by filtration, and the filtrate was collected and concentrated *in vacuo*. The concentrated filtrate was dissolved in water and lyophilized to yield the peptide library as a solid material. The synthesis of the D-library was analogous to that used for the L-library. The amino acid analyses for both libraries showed that each amino acid had the same molar ratio in the peptide library.

Procedures for ESI-MS Experiments. A 7 T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (IonSpec, Irvine, CA) with a modified Analytica (Branford, CT) ESI source was used for the experiments.[10] A home-built microspray emitter optimized for low sample infusion rate (0.2−0.3 μL/min) was used.[23] The mass spectrometer ESI interface consisted of a heated capillary for ion inlet into vacuum and desolvation. The interface capillary was resistively heated at a power of 20 W during these experiments. As reference, the standard power for obtaining protein mass spectra of good quality is 30−35 W; under these conditions, no CAII−inhibitor noncovalent complexes were observed. A

SF_6 gas flow around the ESI source emitter was used to suppress electrical discharges. Time-domain data acquisition was performed with the Odyssey data system (version 4.0; Extrel FTMS, Madison, WI). The ions were injected from the external ESI source, and the m/z range of interest was selectively accumulated in the FTICR cell for 0.5−2 s at ca. 10^{-5} Torr (nitrogen), collisionally cooled, and detected using chirp excitation and broad-band data acquisition.[11,12]

In the CID experiments, the precursor ions were isolated using selective ion accumulation (8 s) in the m/z range of the −9 charge state complex and dissociated with 0.5 s of rf irradiation (using nitrogen collision gas at $\sim 10^{-7}$ Torr). The amplitude of the rf irradiation was adjusted to give complete dissociation of the precursor ions (typically V_{pp} = 10−12 V). The dissociation products, both the relatively low-m/z inhibitors and the higher m/z CAII, were retained in the FTICR cell using broad-band quadrupole excitation and then measured with high resolution and retained for further collisional dissociation studies.[24]

Quantitative Determination of Relative Intensities of Complex Ions. The relative ion intensities of inhibitors were determined from the free inhibitor ions dissociated from the CAII−inhibitor complexes in the gas phase. The m/z values (peak centroid) and intensities of ion in the m/z region 380−610 were transferred to a personal computer, and the data were processed using Microsoft Excel program. First, the m/z values were adjusted to correct for minor deviations due to the use of external m/z calibration in the FTICR experiments. Second, the peak intensities were corrected for isotopic contributions of the free inhibitors. All the (negatively charged) ions in the m/z 380−610 region were considered to be due to the deprotonated free inhibitors. To simplify the isotope calculation, an average relationship between MW and isotope distribution of the inhibitors was obtained by a linear least-squares fitting of theoretical values for several representative members of different masses and with the consideration of the monoisotopic plus two isotopic peaks. The m/z and isotope corrected ion intensities were then divided by theoretical monoisotopic intensity values for an inhibitor mixture of equal contribution with the m/z resolution matching that of the experiments. The data finally obtained were a set of dimensionless values representing the relative intensities of free inhibitor ions. For those inhibitors whose mass difference was less than that resolvable (0.025 Da) under the experimental conditions (including those with identical masses), the final value represents the average for all the possible components in that peak.

Purification and Syntheses of Peptides. All peptides were synthesized by the standard techniques of solid-phase peptide synthesis using Fmoc chemistry. The Fmoc released was determined by UV spectrometry at 267 nm in order to examine if deprotection was completed. A 4-fold molar excess of each Fmoc-amino acid or 4-carboxylbenzenesulfonamide was used in each coupling reaction. The peptides were cleaved from the resin in a solution of 90% TFA, 5% anisole, and 5% water for 3 h. The peptides were lyophilized to give solid materials. Two of the peptides ($AA_1 = AA_2$ = L-Leu or D-Leu) were purified by recrystallization in water. The other peptides were purified by reverse-phase HPLC (C_{18} column, Alltech) using 10% CH_3CN in water as an eluent. All the peptide ligands were characterized by ^1H and ^{13}C NMR in DMSO-d_6 and high-resolution MS.

1 ($AA_1 = AA_2$ = L-Leu): ^1H NMR (400 MHz) δ 8.65 (d, J = 7.9 Hz, 1H), 8.01 (d, J = 8.1 Hz, 2H), 7.90 (m, 4H), 7.47 (s, 2H), 4.47 (m, 1H), 4.23 (m, 1H), 3.20 (m, 2H), 2.35 (t, J = 6.8 Hz, 2H), 1.66 (m, 2H), 1.54 (m, 2H), 1.41 (m, 2H), 0.86 (m, 12H); ^{13}C NMR (100 MHz) δ 172.7, 171.9, 171.7, 165.4, 146.4, 137.0, 128.2, 125.6, 52.1, 51.0, 41.0, 40.1, 34.8, 33.7, 24.4, 24.2, 23.1, 23.0, 21.7, 21.4; HRMS $(M + H)^+$ calcd for $C_{22}H_{34}N_4O_7S$ 499.2228, found 499.2227.

1 ($AA_1 = AA_2$ = D-Leu): ^1H NMR (400 MHz) δ 8.64 (d, J = 7.8 Hz, 1H), 8.01 (d, J = 8.1 Hz, 2H), 7.90 (m, 4H), 7.48 (s, 2H), 4.48 (m, 1H), 4.24 (m, 1H), 3.20 (m, 2H), 2.35 (t, J = 6.6 Hz, 2H), 1.65 (m, 2H), 1.54 (m, 2H), 1.42 (m, 2H), 0.86 (m, 12H); ^{13}C NMR (100 MHz) δ 172.8, 171.9, 171.7, 165.4, 146.4, 137.0, 128.2, 125.6, 52.1, 51.0, 41.0, 40.1, 34.8, 33.7, 24.4, 24.2, 23.1, 23.0, 21.7, 21.4; HRMS $(M + H)^+$ calcd for $C_{22}H_{34}N_4O_7S$ 499.2228, found 499.2227.

1 ($AA_1 = AA_2$ = L-Thr): ^1H NMR (400 MHz) δ 8.36 (d, J = 8.1 Hz, 1H), 8.03 (d, J = 8.2 Hz, 2H), 7.90 (d, J = 8.2 Hz, 2H), 7.83 (m, 2H), 7.48 (s, 2H), 4.54 (m, 1H), 4.12 (m, 2H), 4.01 (m, 1H), 3.21 (m, 2H), 2.30 (t, J = 6.5 Hz, 2H), 1.12 (d, J = 6.1 Hz, 2H), 1.01 (d, J = 6.1 Hz, 2H); ^{13}C NMR (100 MHz) δ 173.2, 169.9, 169.8, 165.7, 146.4, 137.0, 128.1, 125.6, 66.7, 66.2, 59.2, 58.4, 35.1, 34.4, 19.9, 19.7; HRMS $(M + Na)^+$ calcd for $C_{18}H_{26}N_4O_9S$ 497.1318, found 497.1318.

1 ($AA_1 = AA_2$ = D-Thr): ^1H NMR (400 MHz) δ 8.35 (d, J = 8.2 Hz, 1H), 8.03 (d, J = 8.1 Hz, 2H), 7.91 (d, J = 8.2 Hz, 2H), 7.78 (m, 2H), 7.48 (s, 2H), 4.54 (m, 1H), 4.12 (m, 2H), 4.00 (m, 1H), 3.26 (m, 2H), 2.36 (t, J = 7.0 Hz, 2H), 1.12 (d, J = 6.3 Hz, 2H), 1.01 (d, J = 6.2 Hz, 2H); ^{13}C NMR (100 MHz) δ 173.2, 169.9, 169.8, 165.7, 146.4, 137.0, 128.1, 125.6, 66.7, 66.2, 59.2, 58.4, 35.1, 34.4, 19.9, 19.7; HRMS $(M + H)^+$ calcd for $C_{18}H_{26}N_4O_9S$ 475.1510, found 475.1499.

1 ($AA_1 = AA_2$ = L-Ser): ^1H NMR (400 MHz) δ 8.62 (d, J = 7.8 Hz, 1H), 8.15 (d, J = 7.7 Hz, 1H), 8.05 (d, J = 8.2 Hz, 2H), 7.97 (m, 1H), 7.90 (d, J = 8.2 Hz, 2H), 7.48 (s, 2H), 4.59 (m, 1H), 4.17 (m, 1H), 3.73 (m, 2H), 3.60 (m, 2H), 3.20 (m, 2H), 2.20 (t, J = 6.4 Hz, 2H); ^{13}C NMR (100 MHz) δ 172.7, 170.0, 169.8, 165.6, 146.4, 136.9, 128.2, 125.6, 61.6, 61.4, 55.9, 55.3, 34.9, 33.7; HRMS $(M + H)^+$ calcd for $C_{16}H_{22}N_4O_9S$ 447.1187, found 447.1186.

1 ($AA_1 = AA_2$ = D-Ser): ^1H NMR (400 MHz) δ 8.59 (d, J = 7.7 Hz, 1H), 8.04 (m, 3H), 7.90 (d, J = 8.2 Hz, 2H), 7.85 (t, J = 5.6 Hz, 1H), 7.48 (s, 2H), 4.58 (m, 1H), 4.21 (m, 1H), 3.72 (m, 2H), 3.56 (m, 2H), 3.25 (m, 2H), 2.35 (t, J = 7.1 Hz, 2H); ^{13}C NMR (100 MHz) δ 172.7, 170.0, 169.7, 165.5, 146.4, 136.9, 128.1, 125.5, 61.6, 61.4, 55.9, 55.3, 34.9, 33.7; HRMS $(M + H)^+$ calcd for $C_{16}H_{22}N_4O_9S$ 447.1187, found 447.1186.

1 ($AA_1 = AA_2$ = Gly): ^1H NMR (400 MHz) δ 9.00 (t, J = 5.3 Hz, 1H), 8.23 (d, J = 5.6 Hz, 1H), 8.02 (d, J = 8.2 Hz, 2H), 7.91 (m, 3H), 7.49 (s, 2H), 3.91 (d, J = 5.7 Hz, 1H), 3.66 (d, J = 5.8 Hz, 1H), 3.24 (m, 2H), 2.37 (t, J = 6.9 Hz, 2H); ^{13}C NMR (100 MHz) δ 172.8, 169.0, 168.7, 165.7, 146.4, 136.8, 128.1, 125.6, 42.8, 42.0, 34.8, 33.8; HRMS $(M + H)^+$ calcd for $C_{14}H_{18}N_4O_7S$ 387.0976, found 387.0974.

Measurement of Binding Constants. Fluorescence spectroscopy was used to measure the binding affinities of ligands to CAII. Dansylamide was used as a fluorescent ligand, and its K_b (4.0×10^6 M^{-1})[19] for CAII was determined by a direct fluorescence method each time before the measurement of other ligands. Dansylamide at a fixed concentration (3 μM, ca. 10 times above K_d) binds CAII (10 nM); the bound form fluoresces upon excitation. The ligand of interest was allowed to compete with dansylamide for CAII. Analysis of the decreases in fluorescence as a function of the concentrations of the ligand in solution yielded the binding constant for the ligand. The fluorescence binding assay was carried out in 20 mM phosphate buffer (pH 7.5) at 37 °C; excitation wavelength, 280 nm; emission wavelength, 460 nm.

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Supporting Information Available: Tabulated ion intensities of inhibitors in both L- and D-libraries, amino acid analysis for the two libraries, and tabulated solution binding constants for the seven individual inhibitors (4 pages). Ordering information is given on any current masthead page.

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