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Xueheng Cheng, Ruidan Chen, James E. Bruce, Brenda L. Schwartz, Gordon A. Anderson, Steven A. Hofstadler, David C. Gale, and Richard D. Smith*

Chemical Sciences Department and Environmental Molecular Sciences Laboratory, Pacific Northwest Laboratory Richland, Washington 99352

Jinming Gao, George B. Sigal, Mathai Mammen, and George M. Whitesides*

Department of Chemistry, Harvard University Cambridge, Massachusetts 02138

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We report a method based on mass spectrometry for the characterization of noncovalent complexes of proteins with mixtures of ligands; this method is relevant to the study of drug leads and may be useful in screening libraries for tight-binding compounds. It is based on the ability of electrospray ionization (ESI)[1,2] to generate ions of intact noncovalent complexes in the gas phase[3-5] and of Fourier transform ion cyclotron resonance (FTICR) mass spectrometry[6-9] to perform *m/z*-selective ion accumulation,[10] isolation, and multistage ion dissociation to obtain structural information about these complexes (including the identification of the structure of the bound ligand). Here we describe a study of the competitive binding of inhibitors derived from para-substituted benzenesulfonamides to bovine carbonic anhydrase II (BCAII, EC 4.2.1.1) using this technique. Relative binding constants and structural information for a mixture of inhibitors can be obtained in a single experiment using ESI-FTICR-MS.

ESI-MS has been used to detect specific noncovalent biomolecular associations, although questions remain regarding both possible artifacts and the quantitative accuracy of inferences about the stabilities of complexes.[3,4,11] We have used BCAII as a model system, since its binding for ligands has been examined in detail by other techniques.[12,13] We studied two sets of inhibitors based on benzenesulfonamide (structures are shown in Figures 1 and 2), with binding constants ranging from 1.7×10^6 to 1.1×10^9 M^{-1}. Noncovalent complexes of these inhibitors with BCAII gave well-defined ions observable by ESI-MS from solutions in 10 mM NH_4OAc.[14] Typically, only two charge states, for example, 9+ and 10+ in the positive-ion mode, were observed; this observation suggests a tightly folded structure for the complexes of enzyme with the inhibitor.[4] When a mixture of BCAII (1.0 μM) and the 16 inhibitors (equimolar, each 0.05 μM) of the first series was ionized, we observed ions corresponding to all complexes.[15] Relative abundances of the complex ions were consistent with the binding constants of the inhibitors in solution.[16] Adding **1** (2, F) (the second tightest binding inhibitor in the first series) or **1** (3, F) (the tightest binding inhibitor) to concentrations of 0.05 μM in an equimolar mixture of the other 16 compounds (each 0.05 μM) gave mass spectra in which the complex from the added components became the most abundant species. A transition from competitive binding to noncompetitive binding was also observed when the concentration of the inhibitors was decreased relative to that of the enzyme.

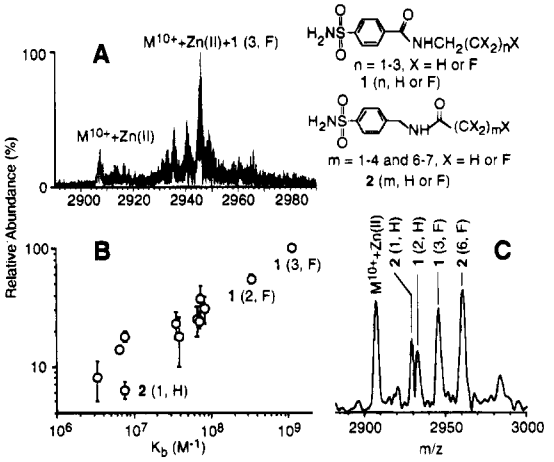

Figure 1. (A) Mass spectra of the 10+ charge state of BCAII (1.0 μM) and 18 inhibitors (0.05 μM each) in 10 mM NH_4OAc (pH 7.0). (B) Correlation of the relative ion abundances with the values of K_b in solution. Six of the K_b values represent the sum of those from two inhibitors with the same mass. The relative errors of solution K_b measurements were 20–30%. (C) Mass spectra of BCAII (1.0 μM) and four inhibitors (**2** (1, H) = 60 μM, **1** (2, H) = 3.0 μM, **2** (6, F) = 1.5 μM, **1** (3, F) = 0.1 μM) in 10 mM NH_4OAc (pH 7.0).

Figure 1A shows the 10+ charge state region of the spectrum from an equimolar mixture of 18 inhibitors (0.05 μM each) with BCAII (1.0 μM). The relative ion abundances correlate well with values of K_b in solution (Figure 1B).[16] The tightest binding inhibitor was readily identified in the 16-, 17-, and 18-component equimolar mixtures based on ion abundances. We also prepared a four-component mixture containing inhibitors with the highest (1.1×10^9) and the lowest affinity (1.7×10^6) from the first series (**2** (1, H), **1** (2, H), **2** (6, F), **1** (3, F)) with their concentrations inversely proportional to their K_b values; the four inhibitors formed 1:1 complexes to BCAII with approximately equal ion abundances (Figure 1C).

The second series of inhibitors[17] (Figure 2), when complexed with BCAII, also gave ions having relative abundances that parallel their binding constants in solution.[18]

* Authors to whom correspondence should be addressed.

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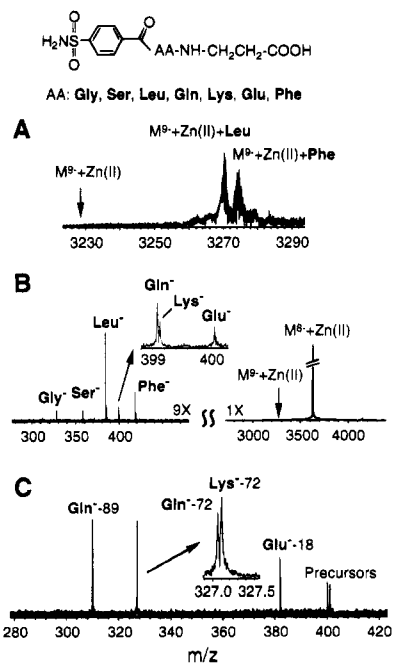

Figure 2. Negative ESI-FTICR mass spectra of BCAII (7.0 μM) with inhibitors (7.0 μM each) in 10 mM NH_4OAc (pH 7.0). The multistep dissociation experiments include the following. (A) Mass spectra of complex ions of BCAII with inhibitors (9− charge state) before ion isolation. (B) Isolation of 9− complex ion species using *m/z*-selective ion accumulation and subsequent dissociation of the complexes with 0.5-s rf irradiation (V_{pp} = 12 V, collision gas nitrogen ~10^{-7} Torr). The intensity of the dissociated inhibitors correlates with their binding affinity. The arrow points to the *m/z* of the precursor ion that has been completely dissociated. (C) SWIFT isolation and dissociation of three of the inhibitor ions (from B), **Glu**$^-$, **Lys**$^-$, and **Gln**$^-$ at *m/z* 399−401 (0.5-s rf irradiation with V_{pp} = 12 V and collision gas nitrogen ~10^{-7} Torr). The precursor ions were attenuated 95%. New peaks result from the fragmentation of the inhibitors.

Two control experiments established that the complexes observed using ESI-MS were the result of active site binding in solution. In one, the pH of the solution was adjusted to below 4 using acetic acid; no noncovalent complexes were observed, and Zn(II) was also lost from higher charge states of the BCAII ions (as indicated by a decrease in mass of 63.4 Da).[19] This result is consistent with acid-induced denaturation of the enzyme.[20] The second experiment used a mixture of BCAII and apoBCAII[21] at pH 7 in 10 mM NH_4OAc. Complexes were observed of inhibitors with BCAII, but not with apoBCAII. The enzyme−inhibitor complexes were studied by both positive and negative ESI from the same solutions at pH 7, and the relative abundance of complexes did not change with polarity of ionization. Collectively, these results provide strong evidence that the complexes observed by ESI-MS involving coordination of sulfonamide anion to the active-site Zn(II) ion have structures related to those in solution.

Some of the inhibitors have very similar masses, and the direct differentiation of their complexes with BCAII is difficult, even with the high-resolution capability of FTICR. These inhibitors (and their relative contribution to the unresolved complexes) can, however, be identified by gas-phase dissociation of the complexes through a tandem mass spectrometry experiment (MS/MS). Dissociation of the *m/z*-isolated 9− charge state complexes produced an 8− ion of BCAII and singly-charged negative ions of all the seven inhibitors (Figure 2B). The three inhibitors having similar masses (**Gln**, **Lys**, and **Glu**) could be distinguished by their exact masses using the high-resolution capability of the instrument (Figure 2B inset). The relative intensities of the inhibitors were similar to those obtained from spectra of the intact complexes and correlated with the relative binding affinities of the ligands to BCAII in solution.[18] However, the direct relative abundance measurement of complex ions can be complicated by the overlapping of isotope envelopes when the components are close in mass and by the presence of adduct peaks that overlap with expected complex ions, whereas the relative ion abundance of inhibitors from gas-phase dissociation of complexes gives a more reliable measure of the relative abundance of complex ions.

To demonstrate the capability of FTICR to carry out structural analysis of the ligands associated with BCAII, the three inhibitor ions (**Gln**, **Lys**, and **Glu**) from the gas-phase dissociation of *m/z*-isolated 9− complex (Figure 2B) were together selected and dissociated in a three-step mass spectrometry experiment. The three inhibitors gave distinctive fragmentation patterns indicative of their structures (Figure 2C). It is a significant observation that structural information identifying these ligands could be obtained using ligands dissociated from their complexes with proteins. Similar types of multistep dissociation experiments should allow the structural characterization of inhibitors of more complex structures and in more complex mixtures. The precise mass measurements and structural information provided by FTICR can also be extended to proteins.[22]

This work demonstrates that ESI-MS has significant potential for measuring relative binding affinities and characterizing the structures of ligands associated noncovalently to proteins. We have detected noncovalent complexes in the gas phase for ligands (**2** (1, H)) having values of K_b as low as 1.7 × 10^6 M^{-1} in solution. The technique also allowed identification of tight-binding ligands from small libraries. The structures of inhibitors having similar masses can be identified by the high-resolution and multistep dissociation mass spectrometry of which FTICR is uniquely capable. This range of capabilities for ESI-FTICR-MS should be widely useful in medicinal chemistry.

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Supporting Information Available: Mass spectra of BCAII with 16 and 17 series 1 inhibitors, of BCAII with series 2 inhibitors at acidic pH, of apoBCAII, and of a mixture of BCAII and apoBCAII with a four-component mixture of series 1 inhibitors, table of MW, solution K_b, and relative complex ion abundances for series 1 inhibitors, table of observed and predicted *m/z* values for ions in Figure 1C, and plot of relative inhibitor ion abundance from dissociation of complexes vs solution K_b values for the series 2 inhibitor mixture (8 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(17) Synthesis followed standard methods of solid-phase peptide synthesis. The molar ratios of these inhibitors were determined by amino acid analysis to be (arbitrary scale) **Gly** (327), **Ser** (255), **Leu** (325), **Lys** (422), **Glx** (661), and **Phe** (329). NMR and MS analyses found no impurities in the sample.

(18) The values of K_b in solution (10^6 M^{-1}), abundances of complex ions (average of five determinations), and abundances of inhibitor ions dissociated from BCAII (average of three determinations) are shown in parentheses, respectively, after each inhibitor (Figure 2): **Leu** (110, 100, 100); **Phe** (77, 81, 61); **Gln** (28, ?, 32); **Lys** (5.3, ?, 15); **Ser** (4.1, 22, 13); **Gly** (3.2, 15, 14); **Glu** (1.9, ?, <9). The complex ions from inhibitors **Gln**, **Lys**, and **Glu** could not be differentiated from each other; the sum of the normalized abundances was 29. The values of K_b were measured by fluorescence in 20 mM phosphate buffer at pH 7.5 (Sigal, G. B.; Whitesides, G. M. Unpublished results).

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