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Protein–inhibitor complexes analyzed by alkaline capillary LC–MS

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Abstract

Liquid chromatography–mass spectrometry (LC–MS) has been used extensively in determination of the molecular weights of proteins, as well as covalent protein–ligand complexes. We have successfully developed LC–MS method for protein molecular weight measurement using small-bore and capillary LC–MS under acidic and basic conditions. A high pH method was critical in studying complexes that were unstable under acidic conditions. Microgram sensitivity was achieved using both methods. A protocol to study the binding mode of protein–ligand complexes under denaturing conditions was developed. These methods were applied to CP88 (a proprietary cysteine protease) inhibitors and revealed different binding modes of inhibitors to proteins that had similar non-reversible behavior in biochemical activity assays. The method also confirmed that one inhibitor studied binds to CP88 in a reversible covalent manner.

Keywords: LC–MS; Intact protein; Alkaline mobile phase; Negative ion; Reversible covalent binding; Sensitivity

1. Introduction

Liquid chromatography–mass spectrometry (LC–MS) has been used extensively in determination of protein molecular weights [1,2], as well as the molecular weights of covalent protein–ligand complexes [3]. Proteins, as large as intact monoclonal antibodies (150 kDa), have been directly analyzed by LC–MS [2,4]. With standard LC–MS methods and analyzers, the accuracy of the molecular weight measurement can exceed 0.01% [2] and is even greater when analyzing small proteins using Fourier transform-ion cyclotron resonance (FT-ICR) MS [5–7]. These measurements are more accurate than classic methods such as SDS-PAGE [8] or analytical ultra-centrifugation [9].

For this paper, we define intact proteins to be undigested, possibly modified proteins, in either their native conformation or denatured. Time-of-flight (TOF) mass spectrometers have traditionally been the top choice of mass analyzer for intact proteins [4,10–13]. This was mainly due to higher mass resolution provided by reflectrons [14], which help resolve peak multiplicity caused by protein inhomogeneity, covalent modifications, and/or salt adducts. The ability of TOF instruments to capture a wide m/z range without sacrificing sensitivity was also beneficial for intact protein analysis. Accurate protein mass measurements have also been reported with ion-trap instruments [15–17], triple quadrupole instruments [18,19], and Fourier transform-ion cyclotron resonance mass spectrometers [5–7]. It should be noted that matrix-assisted laser desorption ionization (MALDI)-TOF-MS usually cannot provide mass accuracies as good as electrospray ionization (ESI)-TOF-MS for intact proteins because the high m/z range of MALDI generated singly charged protein ions were not compatible with reflectrons.

In this paper, intact protein molecular weight measurements were described using atmospheric pressure ionization (API)-TOF and ion-trap instruments. Small-bore (1 mm) and capillary columns (300 μm) were coupled to these mass spectrometers for LC–MS, achieving sensitive detection of proteins in the microgram range. CP88, a proprietary cysteine protease, was studied with inhibitors that were covalently bound to the protein, some of which were suspected to be acid labile. Therefore, it was necessary to perform the measurements in neutral or basic pH, instead of the more

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traditional acidic conditions. Making this drastic change in solvent pH for LC–MS created several new challenges, which were eventually solved.

2. Methods

2.1. Material

Water was from a Millipore Milli-Q Gradient water system (Billerica, MA). Acetonitrile was from Burdick & Jackson (BioSyn grade, Muskegon, MI). Formic acid was from EMD (GR ACS grade, Gibbstown, NJ). Liquid ammonia (NF/FCC grade, contains 27–31% NH_3) was from Fisher Scientific (Fair Lawn, NJ). Carbonic anhydrase, horse heart myoglobin, and yeast enolase were purchased from Sigma–Aldrich (St. Louis, MO), dissolved in water at 1 mg/mL, and stored in –20 °C freezer. CP88 protein, as well as protein–ligand complex, was provided by the Biochemistry Department at Pfizer La Jolla Laboratories.

2.2. Small-bore LC–MS system

Our system for routine LC–MS of proteins was comprised of a LEAP autosampler (CTC LEAP PAL-HTS, Carrboro, NC), an Agilent 1100 LC system (Agilent, Wilmington, DE), a Michrom Micro Protein Trap (1 mm × 10 mm) (Michrom Bioresources, Auburn, CA) heated to 60 °C, and a Micromass LCT API-TOF mass spectrometer (Waters, Milford, MA). Mobile phase A was 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. Wash solutions on the LEAP autosampler were 0.1% formic acid in water for wash 1, and 0.1% formic acid in 90% isopropanol for wash 2. Flow rate was 0.2 mL/min for 1.5 min to load the sample onto the protein trap (higher flow rate caused significant reduction in retention of analytes). The flow rate was raised to 0.5 mL/min for the remainder of the run (8 min total, with 2 min gradient from 5 to 95% B, and a wash at 95% B for 1.5 min, followed by re-equilibration at 5% for 2 min). Mass spectrometer source parameters were: source at 110 °C, desolvation gas at 350 °C and 500 L/h, curtain gas at 20 L/h, cone voltage at 40 V (30 V for yeast enolase), and RF lens at 500 V. The mass spectrometer acquired a spectrum of *m*/*z* 500–3000 every second. Protein mass was obtained by processing the raw data with MaxEnt1 software [20–22].

2.3. Capillary LC–MS system

The capillary LC–MS system was comprised of an Agilent 1100 Micro autosampler, an Agilent 1100 capillary LC system (Agilent Technologies, Wilmington, DE), a Michrom PLRP-S 8um 4000 Å, 0.3 mm × 50 mm LC column (Michrom Bioresources, Auburn, CA) heated to 60 °C, and

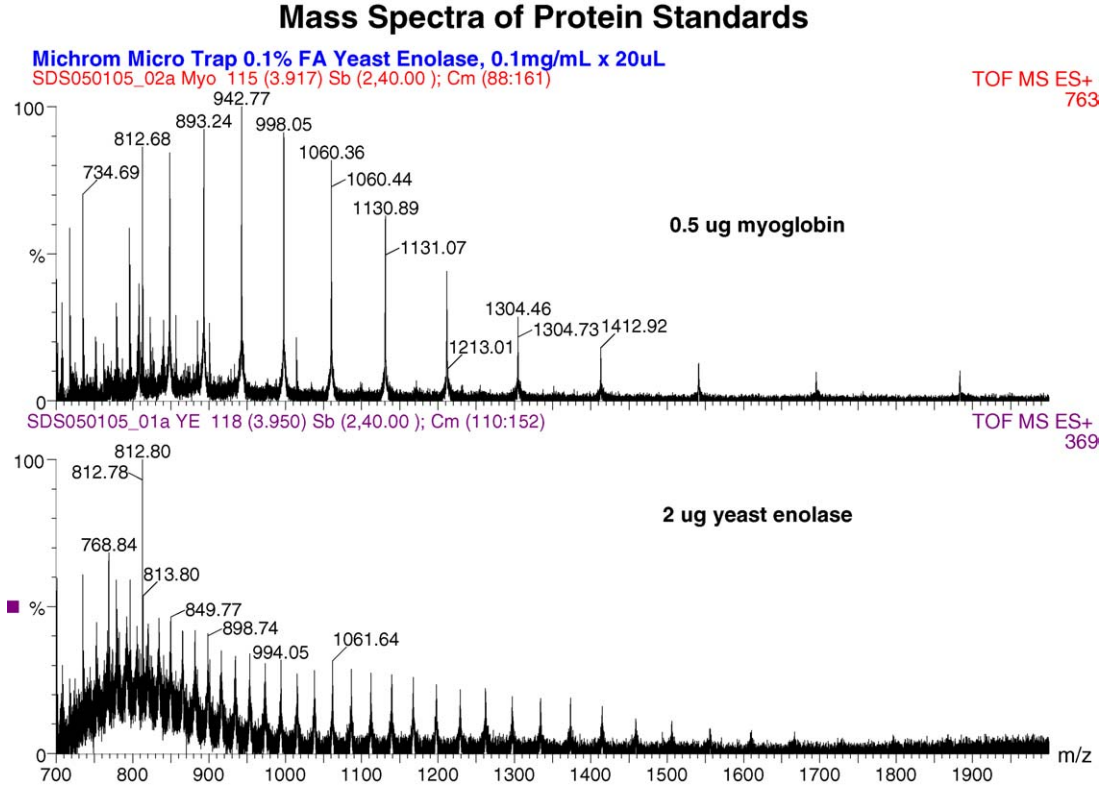

Fig. 1. Representative spectra of protein standards on the TOF instrument. Samples were loaded and desalted on a short trap column (1.0 mm × 10 mm with PLRP-S) and eluted with a gradient. Spectra under the entire protein peak were co-added to prove the spectra.

a Thermo Finnigan LCQ Deca ion-trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with a home-built microelectrospray device with fused silica spray tip (360 μm O.D., 50 μm I.D.). Mobile phase A was 0.1% aqueous ammonia in water (pH 10.5). Mobile phase B was 90% acetonitrile and 10% mobile phase A. The capillary LC pumps generated a gradient of 5–95% B in 15 min at a flow rate of 200 μL/min before splitting to 5 μL/min for the capillary LC–MS system. Total analysis time was 40 min. A union (with 0.1 mm I.D. through hole) replaced the mixer on the Agilent 1100 capillary LC to reduce the gradient delay. A 0.5 μm PEEK frit (Upchurch Scientific, Oak Harbor, WA) was placed in front of the column. The mass spectrometer was operated in negative ion mode with the following source parameters: source temperature 300 °C, spray voltage −3000 V, capillary at 50 V and tube lens at 80 V. The transfer optics were optimized at the following voltages: 5 V on multipole 1, 75 V on lens, 10 V on multipole 2, 400 V on RF and 30 V on entrance lens. The AGC was set to 4×10^7 (twice as recommended) to allow more ions into the trap.

The CP88 protein (30 μM) was incubated with 45 μM inhibitor for 1 h before it was diluted to 3 μM with either water for analysis of the protein–ligand complex, or 6 M urea (incubated at room temperature for 5 min before loading onto the autosampler chilled to 10 °C) for analysis of the denatured complex samples. A volume of 7 μL of either sample was injected into the LC–MS system. The molecular weight of the protein complex was obtained by averaging the mass spectra over the LC peak and deconvoluting with BioWorks software.

3. Results and discussion

3.1. Small-bore LC–MS

The routine protein QC was performed on the small-bore API-TOF LC–MS system. With this system, proteins were usually analyzed at the 1–5 μg level. The protein was loaded onto a short polymer-based reverse-phase trap column (1 mm I.D. × 10 mm length made by Michrom Bioresources), desalted, eluted, and detected. Because the main purpose of the reverse-phase column was to desalt the protein, a very short column [10] was used to speed up analysis. An added benefit of using this particular cartridge format column was the low cost (a fraction of the cost of a regular column).

Splitting the LC effluent before the MS reduces electrospray source contamination by introducing less overall material to the source. Because the electrospray process is concentration dependent, the signal should not be reduced, and increases in sensitivity are often realized at lower flow rates. Although ESI concentration dependence is certainly true in theory and has been observed in practice (this was the

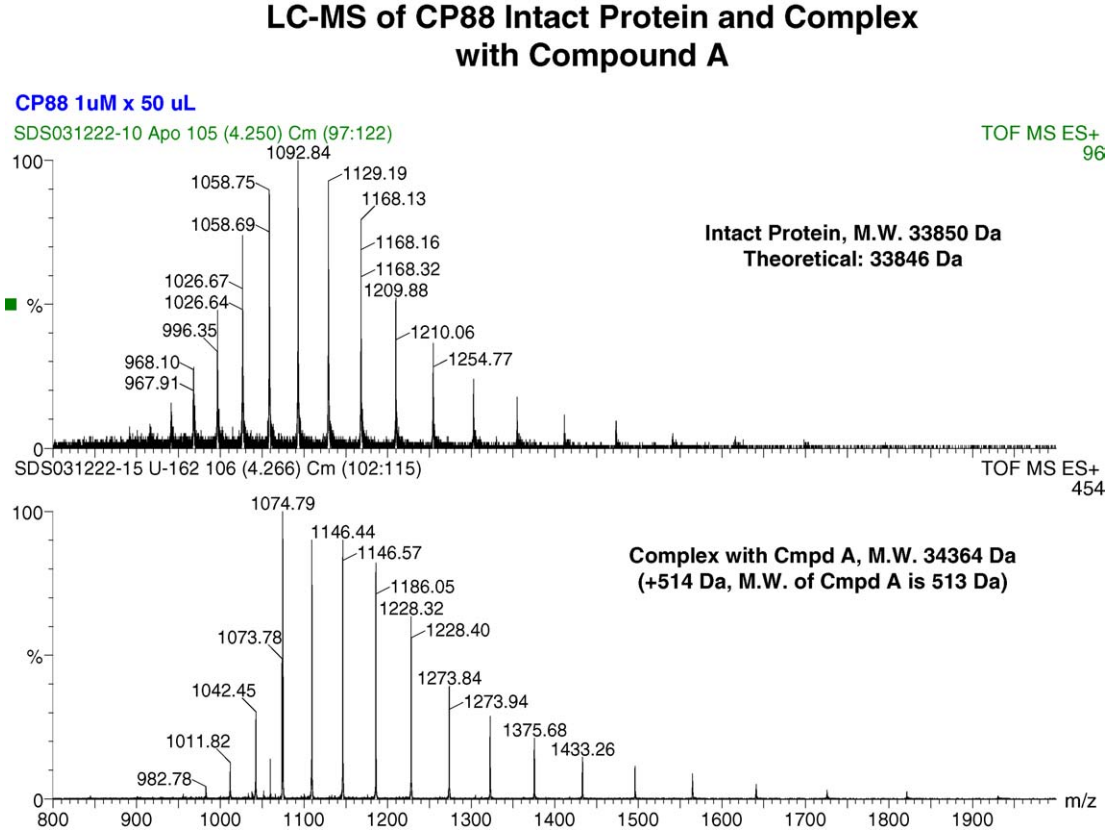

Fig. 2. LC–MS measurement of CP88 and its complex with compound A, measured on the small-bore API-TOF LC–MS system.

basis of important techniques such as capillary chromatography [23,24] and peak-parking [25,26]), we found that flow splitting on the LCT's ESI source lowered the signal abundance significantly and therefore did not split the flow on this system.

The sensitivity of the system was benchmarked with several standard proteins. Horse heart myoglobin was the most widely used protein standard for intact protein analysis. With a 0.5 μg injection, a clean signal with signal-to-noise (S/N) ratio greater than 30:1 (Fig. 1, top panel) was observed. However, several factors made myoglobin a poor choice for use as a daily standard to monitor system performance. Myoglobin is fairly insensitive to changes in electrospray source conditions (which makes it a great standard for vendors to test their instruments), thus the myoglobin signal did not vary with source contamination or desolvation parameter issues. Additionally, myoglobin, at only 17 kDa, was not representative of our usual samples, which were in the 20–60 kDa range. This protein also ionizes much better than most other proteins, therefore can lead to unrealistic expectations on sensitivity.

Yeast enolase (47 kDa) was a much better standard for our application. A 2 μg injection resulted in a spectrum with a S/N of 5:1 (Fig. 1, bottom panel). Yeast enolase was also very sensitive to source conditions and fragmented easily, especially the high charge state ions. On the API-TOF mass spectrometer, spray tip position was optimized so that low cone voltage (30 V) was used to direct ions into the mass spectrometer. Temperature changes have an adverse effect on the TOF mass measurements, which are more pronounced as the protein size increases. Therefore, using yeast enolase at 47 kDa before analyzing unknown protein samples provided a more accurate temperature correction factor than using myoglobin.

The small-bore LC–MS system was used to analyze the CP88 protein (a proprietary cysteine protease), as well as protein–ligand complexes (Fig. 2). The measured molecular weight of 33,850 Da was close to the mass calculated based on the sequence (33,846 Da). The molecular weight of the complex of compound A (proprietary compound) and the protein was measured at 34,364 Da, 514 Da higher than the apo-protein. This mass shift was consistent with the com-

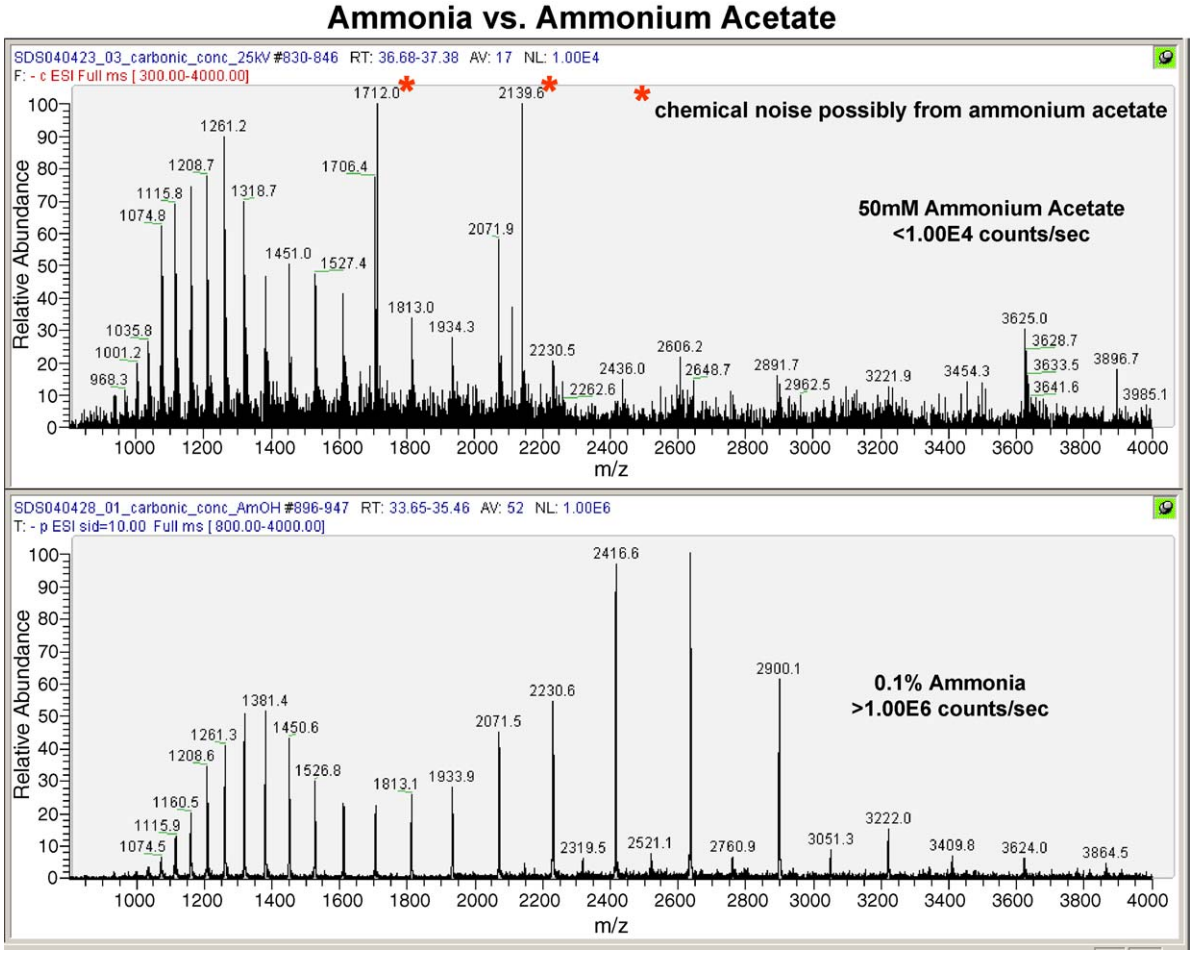

Fig. 3. A buffer of 0.1% aqueous ammonia (pH 10.5) gave 100 times better signal than 50 mM ammonium acetate (pH 6.5). Negative ion mass spectra were obtained on an ion-trap instrument (Thermo Finnigan LCQ Deca). Proteins were loaded and desalted on a capillary column (0.3 mm × 50 mm with PLRP-S) and eluted. Spectra at the chromatographic peak tops were shown.

pound molecular weight (513 Da). These measurements were made with external calibration, and consume 0.17 μg of protein for each analysis.

3.2. High pH capillary LC–MS

Some of the covalent inhibitor adducts to CP88 in this study were suspected to be acid labile. There were concerns that the covalent bond between the protease and the inhibitor could be hydrolysed and the inhibitor dissociated from the complex under acidic LC–MS conditions. Because of this concern, we investigated using a high pH method. Logically, we chose capillary LC to compensate potential sensitivity loss due to basic pH conditions. We also chose the ion-trap instrument because of easier coupling to capillary LC (through our home-made microelectrospray source), as well as better sensitivity due to more efficient electrospray source and ion transfer optics.

Initial efforts with 50 mM ammonium acetate (pH 6.5) as the mobile phase additive were not successful due to poor sensitivity. By switching to 0.1% aqueous ammonia (pH 10.5) [27,28], the signal from 7 μg of tuning standard (in this case, carbonic anhydrase) injected onto the column was ~100 times more abundant (Fig. 3). Note that the 7 μg of protein severely overloaded the capillary column in use (see below, in the protein ghosting section).

Numerous changes were necessary for running LC–MS at high pH. First of all, no silica-based columns could be used, since base catalyzed hydrolysis will not only remove alkyl chains and reduce retention dramatically, but also erode the beads themselves. Fortunately, our column of choice for protein work was a polymer-based column (PLRP-S made by Michrom Bioresources). It has a wide pH range, therefore no changes were needed when using ammonia as the mobile phase additive. Secondly, due to the same concern over silica stability under high pH, it is recommended that all the fused silica capillaries that are used extensively in the capillary LC systems be changed to PEEK tubings. Unfortunately, Agilent does not provide a PEEK version of its non-standard O.D. PEEK lined fused silica tubing, making it impossible to change out the fused silica tubing. The only way to address this concern was to switch to rinse and store the system

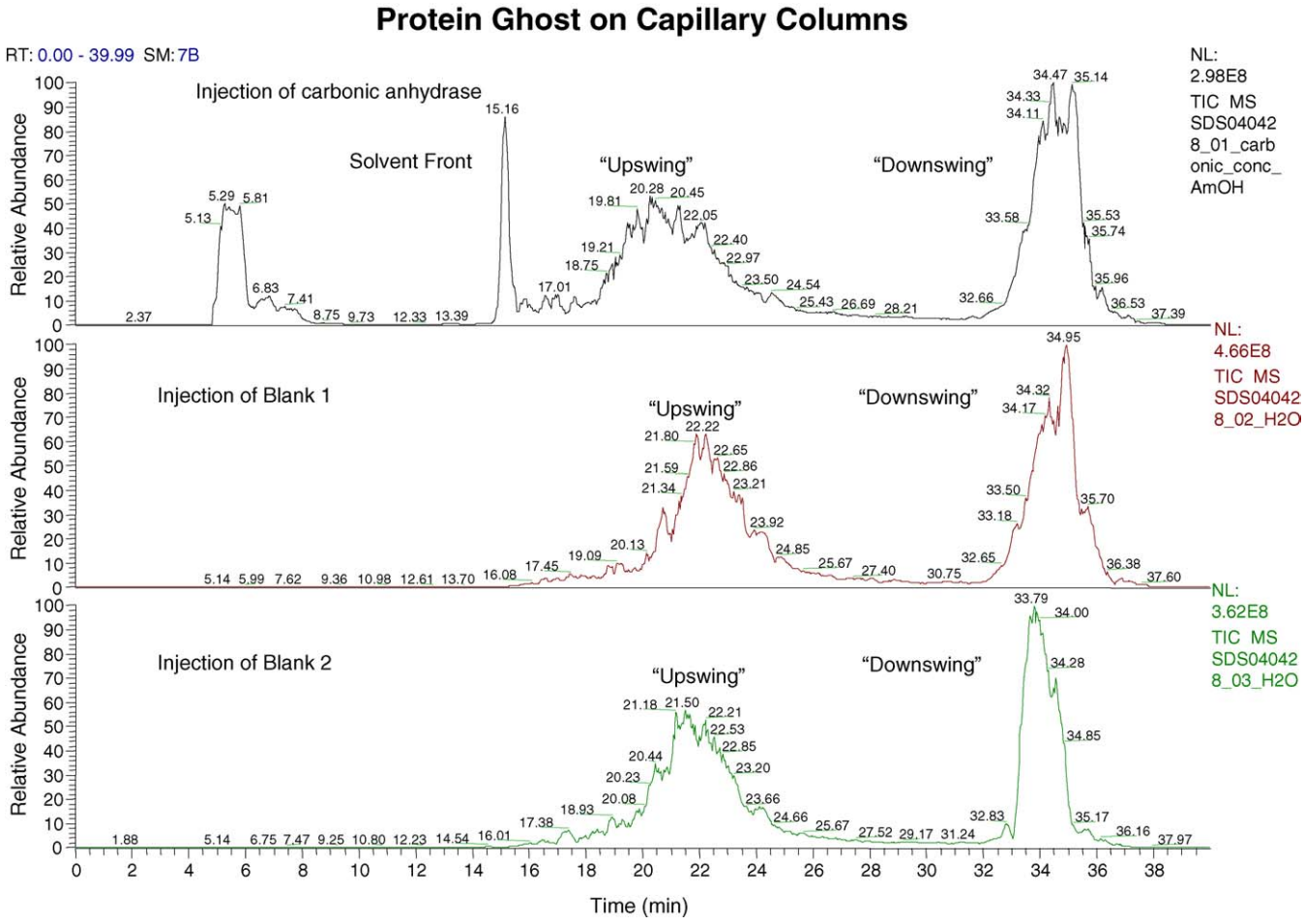

Fig. 4. Protein ghost on capillary columns was demonstrated by grossly overloading carbonic anhydrase. Excess protein (did not bind to column) was eluted in the solvent front (top panel). Bound proteins were eluted twice, once at the intended ("upswing") gradient, the other one at the unintended ("downswing") gradient. These last two protein peaks were observed in the two following blank injection runs.

in pure water/acetonitrile with no additives so that the tubing was not under high pH conditions for extended periods of time. Lastly, after using the system for several hours, the original unmodified fused silica spray tip leaked. The polyimide coating on the capillary spray tip swelled and softened when soaked with 0.1% ammonia. To address this issue, we used heat to remove the coating where the capillary spray tip interfaced to the capillary LC system via a metal union (note the coating on the pulled end was already stripped for better spray). A Teflon sleeve was used to make a seal with the bare silica tubing. Care must be taken to make the connections since the bare silica was brittle.

3.3. Diverting salt away from MS in capillary mode

Since high concentration salt and urea were present in the samples, it was a good practice to divert the LC effluent away from the mass spectrometer to protect the source from contamination and impaired performance. However, conventional divert schemes involving a switching valve could not be used with this low flow capillary format because the extra after column dead volume would cause dramatic peak broadening and loss of sensitivity. Instead of diverting flow, as is traditionally done, we diverted the spray to the mass spectrometer by regulating the spray voltage. In the first 10 min when LC effluent would normally be diverted, the spray voltage was set to 0 V. After 10 min, the excess droplets on the end of the ESI needle were "kicked-off" by setting the spray voltage to 5000 V for 30 s. Normal data acquisition began when the spray voltage was changed to −3000 V, its normal operating condition.

3.4. Ghosting and capillary LC–MS for proteins

It was documented [29] that after injection of certain proteins, the same protein signal was observed when running subsequent blank injections. This phenomenon, called "ghosting", was not caused by autosampler contamination, although the latter can cause the same symptom. Ghosting was caused by protein precipitation inside the HPLC column due to solvent composition change. Proteins are generally not soluble in organic solvents such as acetonitrile. In fact, such low solubility was the basis of protein precipitation protocols used to isolate proteins from small molecules. On

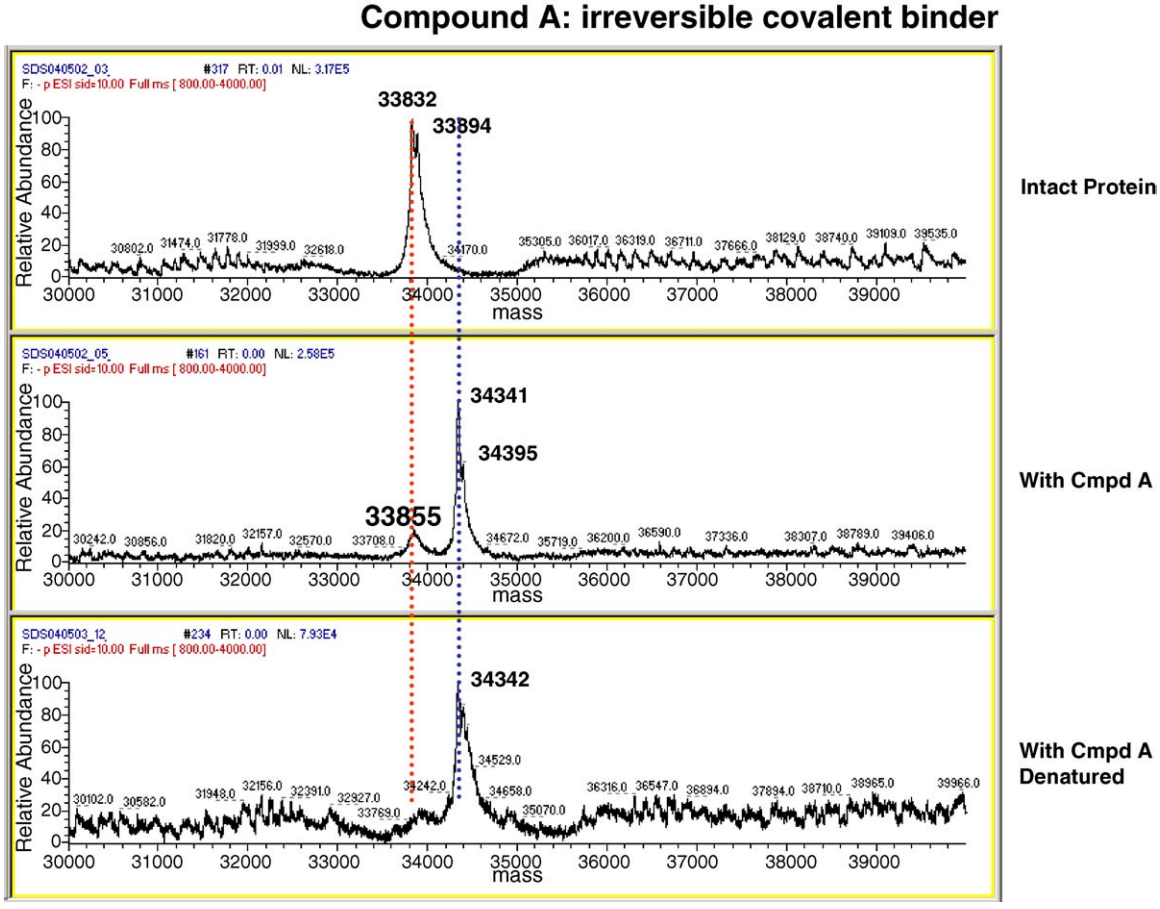

Fig. 5. Binding mode study with LC–MS of intact protein molecular weight. Compounds A and D exhibited similar irreversible covalent binding properties. Compound B was easily dissociated from the protein by the LC–MS conditions and was classified as a non-covalent binder. Compound C was observed partially bound to the protein under LC–MS conditions, but minimal binding was observed under denaturing conditions. Compound C was classified as a reversible covalent binder.

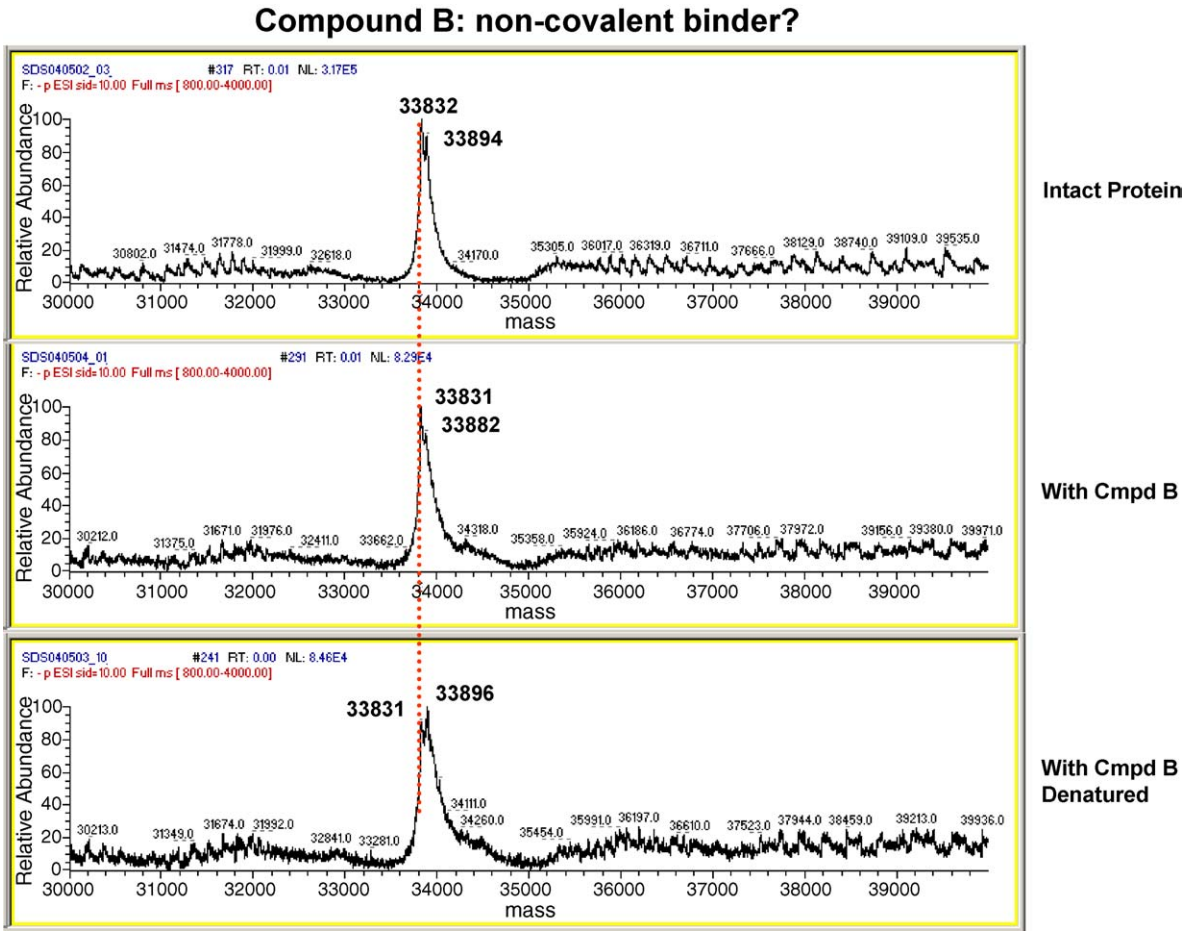

Fig. 5. (*Continued*).

reverse-phase columns, proteins generally do not eluted until organic solvent concentration reaches about 40–60%. If the mobile phase organic concentration rises too quickly, the protein may not have enough time to completely elute from the column, and therefore may precipitate in the column. These precipitates then re-dissolve and elute when the mobile phase conditions becomes favorable again (e.g. during the next run).

Several LC methods have been designed to combat ghosting. One can use a partial gradient only up to 60% acetonitrile, or use isopropanol as the organic phase to avoid protein precipitation on the column. However, these methods have their own problems. The incomplete gradient can cause column performance to degrade over time, since hydrophobic contamination may accumulate on the column. Isopropanol is highly viscous (almost three times as viscous as water) and can cause very high backpressure. As with other alcohol solvents, isopropanol mobile phase needs to be fresh because esters can form in the solvent reservoir in the presence of acid.

Since ghosting is a solubility issue, it becomes more pronounced on smaller bore reverse-phase columns. In other words, the very reason why smaller bore LC offers higher sensitivity [30] could become a liability, because higher concentration of analytes increases chances for precipitation. Fig. 4 demonstrates ghosting on a capillary column. An excess amount of protein (carbonic anhydrase) was injected onto the capillary column. Three protein peaks were identified in the first run. The first protein peak was protein that did not bind to the stationary phase because all binding sites were occupied (column overloading). The second peak was the expected protein elution at the typical retention time. The last protein peak was protein that initially precipitated with the rising gradient and re-dissolved during the “downswing” (reducing organic content down to 5% for re-equilibration).

On conventional HPLC systems, the “downswing” protein peak was never observed, because the downswing was usually too fast for the precipitated protein to re-dissolve and elute. The observation of the downswing protein peak indicates that the capillary LC system used cannot deliver a fast gradient due to large mixing volumes, and therefore the intended sharp “downswing” was actually a gradual change in gradient. Fig. 4 shows the two protein peaks were present in the two blank injections following the standard protein injection.

The ghosting phenomenon put a limit on how much the sensitivity could be improved by increasing the concentra-

tion on the column (i.e. reducing column internal diameter). The focus should be to maximize mass spectrometer signal at protein concentration levels well below the limit of precipitation. Areas for potential improvement include increasing ionization efficiency, reducing ion loss during ion transfer, increasing duty cycle, and increasing detection efficiency.

3.5. Results of CP88 and inhibitor binding analysis

CP88 is a cysteine protease that is marked by a reactive free thiol in the catalytic pocket. Among all cysteine protease inhibitors, reversible covalent inhibitors offer high inhibition of the protease because the covalent "warhead" contributes to tighter binding towards the very shallow binding pocket, and yet show good selectivity. These inhibitors bond covalently only to the catalytic cysteine residue when the inhibitor binds in a suitable location and orientation within the active site [31]. In other words, the covalent complex is dependent on the protein conformation. The goal of this study was to test whether the binding of certain inhibitors to the target protein were of this reversible covalent nature.

We devised an experiment to test binding mode of inhibitors to proteins that was based on the assumption that reversible covalent protein–ligand complexes are conformation dependent, and will dissociate once the protein is denatured. Conversely, irreversible covalently bound complexes are independent of secondary protein structure and should survive this process. Provided the denaturant does not react with the protein–ligand complex and exchange or cause cleavage of the ligand, this method should differentiate the binding modes of ligands to proteins. Also, if the protein–ligand complex was not observed even before intentional denaturation of the protein (see below), one could assume that the binding was non-covalent or very weak.

We tested four proprietary compounds with CP88 (Fig. 5, 0.07 μg of protein consumed in each analysis). The spectra of compounds A and D after incubation with protein showed masses of protein–ligand complexes that were not changed by denaturing the protein, indicating that the binding mode was irreversible covalent. For compound B, no mass of the complex was observed, indicating that the complex was either non-covalent, or unstable in basic conditions. For compound C, two peaks were initially observed, indicating some of the protein was bound to compound C and some was free. However, the protein–compound C complex dissociated after denaturing the protein. These results suggested that compound C binds to the protein in a reversible covalent manner.

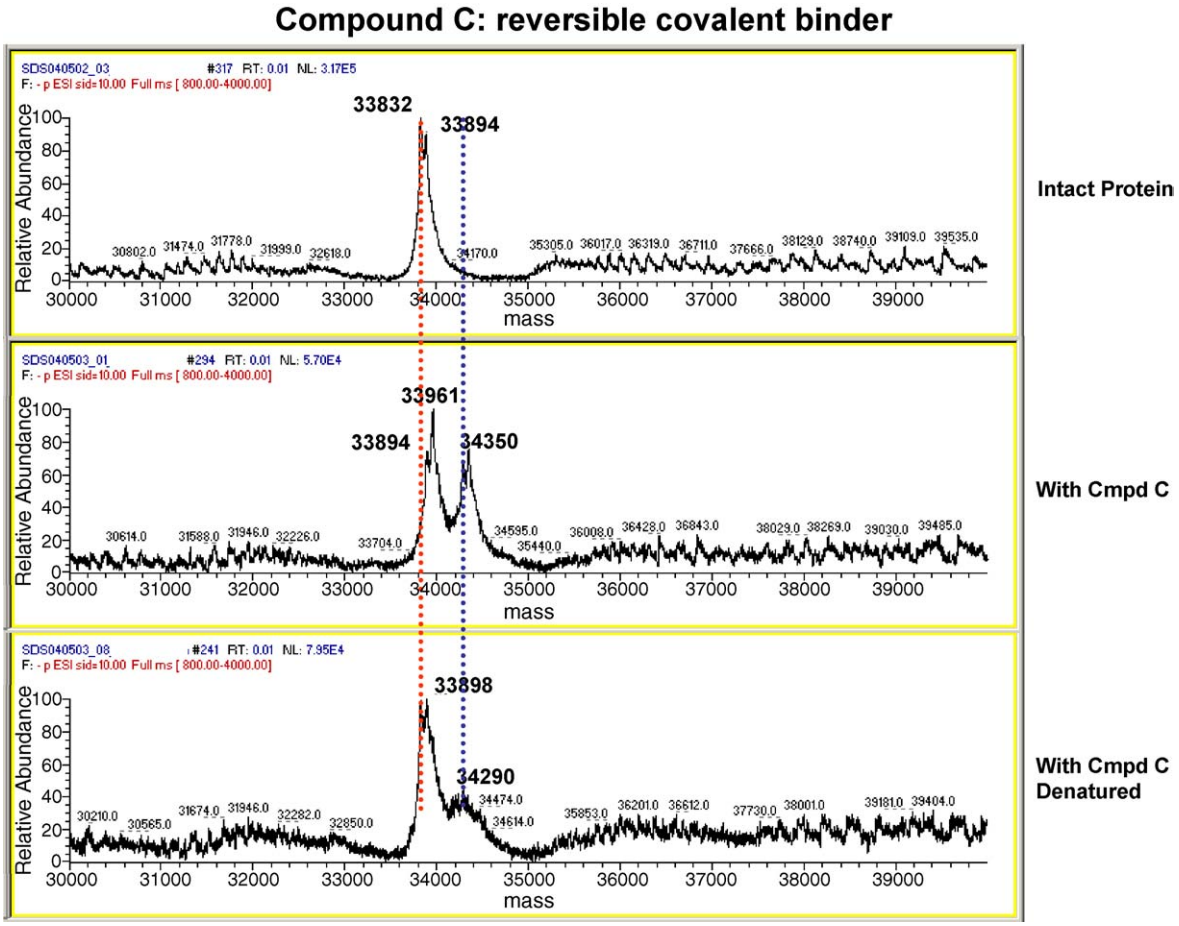

Fig. 5. (*Continued*).

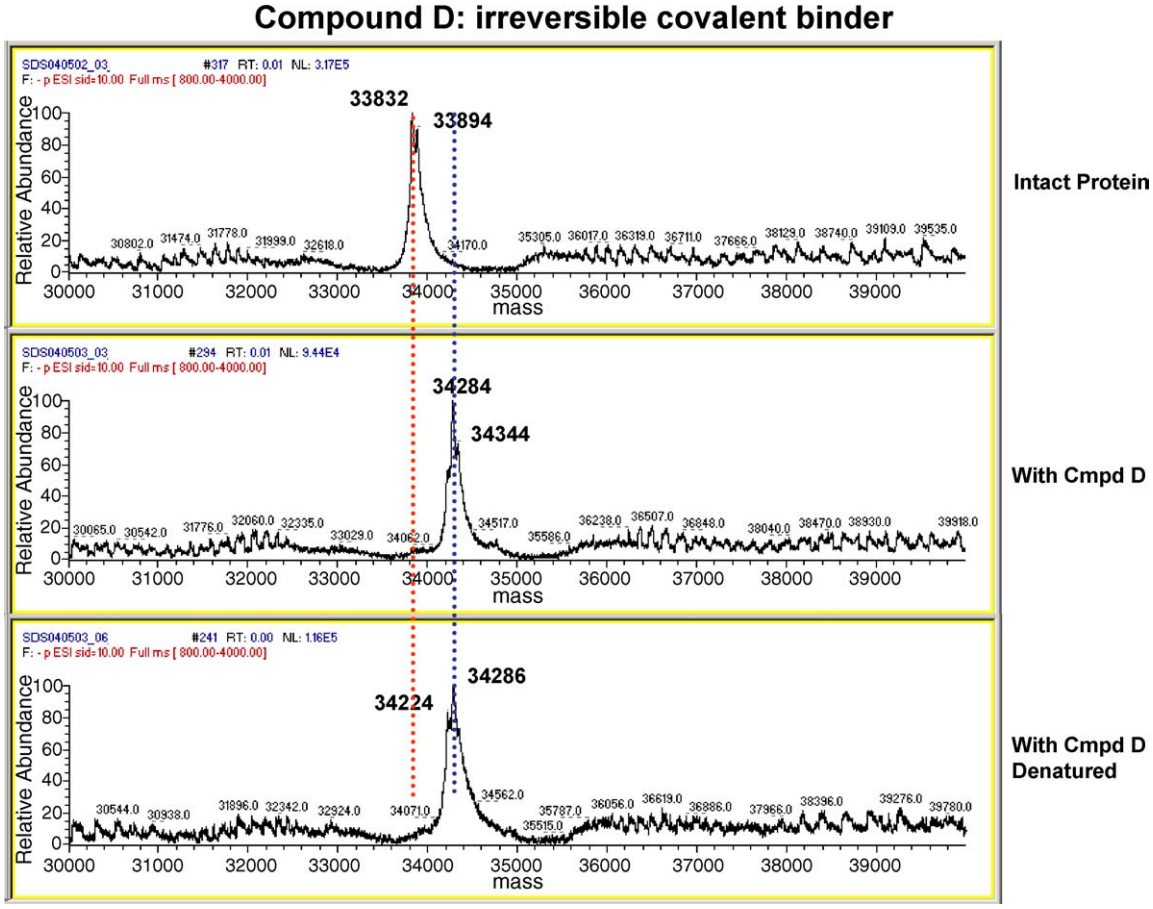

Fig. 5. (*Continued*).

The "wash-out experiment" was another experiment designed to study protein–ligand binding reversibility. It was a test where activity of the recovered protein as described below was measured in a biochemical assay. The protein–ligand complexes were washed with buffer on an ultra-filtration device for 2.5 h to achieve 1.2×10^6 fold dilution, and the recovered protein was tested for activity. If the ligand was irreversibly covalently bound to the protein, the washed protein should remain inactive, or show much reduced activity. No protein activity was observed for washes of the complexes of the protein and compounds A, B and D, and only partial activity was noted for the compound C mixture. These results mostly agreed with the LC–MS results described above. The apparent discrepancy for compound B could be caused by slow off-rate of this compound, and therefore could cause a false positive in the 2.5 h wash-out experiment. Another possibility was that the complex of compound B was not stable in basic conditions and underwent hydrolysis.

One way to resolve the discrepancy between the two techniques on compound B was to perform the wash-out experiment under the same basic pH conditions as the LC–MS method. However, washing the protein with high pH buffer will almost certainly denature the protein. Such change is usually irreversible. Therefore, no activity will be recovered after washout, giving a result of irreversible binding even if the ligand can be washed off. On the other hand, it will be ideal to perform LC–MS at neutral pH (as the conditions used in the wash-out experiments). Unfortunately, at neutral pH, some proteins (including CP88) gave very poor mass spectrometric signal. With pH 6.5, the signal was so weak that no studies could be performed with low micrograms of protein.

3.6. Sensitivity of the binding mode determination experiments

With both LC–MS systems, sub-micrograms of protein were used to produce intact protein mass spectrometric signal with high signal-to-noise ratio. Specifically, for CP88, we injected 0.17 μg of protein onto the narrow-bore LC-TOF system, and 0.07 μg of protein onto the capillary LC–ion-trap system. In both cases, the amount of protein injected was well above the detection limit, especially for the capillary system, where severe peak tailing and ghosting was observed with the amount of protein injected.

The amount of protein used in the wash-out experiment was 30 μM × 100 μL (4.2 μg). This amount was used because it was a convenient quantity to avoid excessive protein loss on the ultra-filtration membrane. The amount used

in the activity assay to check protein activity after the washout experiment was 25 nM × 100 μL (0.0035 μg). This much higher sensitivity was expected since activity assays have a fundamental sensitivity advantage over binding assays in that the signal is amplified (through enzymatic reaction, which is catalytic in nature).

A comparison of the two techniques in terms of overall sensitivity shows that they were comparable. Both techniques could be pushed to reach better sensitivity, although LC–MS method showed more promise. The two methods gave insights to different facets of the same biological problem, and therefore were complementary.

4. Conclusion

We have successfully developed an LC–MS method for protein molecular weight measurement using small bore API-TOF LC–MS and more significantly, another method using high pH on a capillary LC–ion-trap MS system. The latter method was critical in studying protein–ligand complexes that were potentially unstable under acidic conditions. A protocol to study the binding mode of ligands to proteins was developed by studying the complex under denaturing conditions. These methods were applied to CP88, a proprietary cysteine protease, and its inhibitors. Different binding modes of several inhibitors that had similar non-reversible behavior in biochemical activity assays were tested. Also, one compound was proven to bind to CP88 in a reversible covalent manor, consistent with the biochemical activity assay. Submicrogram protein sensitivity was achieved on both systems, making it possible to carry out the binding mode determination experiment with limited amount of protein.

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