Collision-Induced Release, Ion Mobility Separation, and Amino Acid Sequence Analysis of Subunits from Mass-Selected Noncovalent Protein Complexes

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Abstract. In recent years, mass spectrometry has become a valuable tool for detecting and characterizing protein–protein interactions and for measuring the masses and subunit stoichiometries of noncovalent protein complexes. The gas-phase dissociation of noncovalent protein assemblies via tandem mass spectrometry can be useful in confirming subunit masses and stoichiometries; however, dissociation experiments that are able to yield subunit sequence information must usually be conducted separately. Here, we furnish proof of concept for a method that allows subunit sequence information to be directly obtained from a protein aggregate in a single gas-phase analysis. The experiments were carried out using a quadrupole time-of-flight mass spectrometer equipped with a traveling-wave ion mobility separator. This instrument configuration allows for a noncovalent protein assembly to be quadrupole selected, then subjected to two successive rounds of collision-induced dissociation with an intervening stage of ion mobility separation. This approach was applied to four model proteins as their corresponding homodimers: glucagon, ubiquitin, cytochrome c, and β-lactoglobulin. In each case, b- and y-type fragment ions were obtained upon further collisional activation of the collisionally-released subunits, resulting in up to 50% sequence coverage. Owing to the incorporation of an ion mobility separation, these results also suggest the intriguing possibility of measuring complex mass, complex collisional cross section, subunit masses, subunit collisional cross sections, and sequence information for the subunits in a single gas-phase experiment. Overall, these findings represent a significant contribution towards the realization of protein interactomic analyses, which begin with native complexes and directly yield subunit identities.

Keywords: Noncovalent protein complexes, Collision-induced dissociation, Ion mobility, Top-down sequencing

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Introduction

A lthough mass spectrometry (MS) techniques for determination of primary protein structure have reached a state of considerable maturity, MS based approaches for characterization of protein structure at the secondary, tertiary, and quaternary levels continue to evolve. In this regard, one area of particularly keen interest is the development of native MS methods for characterization of noncovalent protein complexes [1–12]. Such experiments involve the application of nanoelectrospray ionization (nESI) to native-like protein solutions, such that the intramolecular and intermolecular noncovalent interactions from solution are preserved in the resulting gaseous ions. This allows for a variety of gas-phase analyses to be performed on intact quaternary protein assemblies. Such capabilities are quite attractive as they can play highly complementary roles in concert with other protein structural determination methods such as X-ray crystallography and nuclear magnetic resonance spectroscopy.

One especially appealing opportunity afforded by native MS is the ability to perform tandem mass spectrometry (MS/MS) experiments on intact protein complexes. Various MS/MS methods have proven quite useful for structural interrogation of quaternary protein assemblies; for instance, collision-induced dissociation (CID) readily supplies information regarding subunit mass and stoichiometry within the complex [13–15], whereas surface-induced dissociation (SID) has been found capable of accessing different dissociation pathways that are informative with respect to the complex substructure and overall architecture [16–18]. Under certain conditions, MS/MS of noncovalent protein complexes can result in the production of covalent bond...
cleavage products. For example, CID on protein oligomers with sufficiently low charge states appears to favor the production of polypeptide backbone fragments rather than subunit losses [19]. Similarly, CID carried out with sufficiently high collision energy has proven to be capable of yielding covalent fragmentation directly from the complex [20]. Experiments involving electron capture dissociation (ECD) and electron transfer dissociation (ETD) of noncovalent protein assemblies have also been shown to provide polypeptide sequence ions, which arise from regions of the complex having relatively high flexibility, or from stretches of sequence residing on the surface of the native structure [21–24]. Such findings are provocative, as they intimate multiple analytical roles for top-down MS/MS strategies applied at the level of intact multi-subunit protein assemblies.

The present work was carried out with the goal of further merging the tools of native MS and top down MS/MS. Here, we provide proof of principle for a multidimensional protein complex analysis method based on the combination of two stages of CID bridged by an intermediate stage of ion mobility (IM) separation. In this experiment, a mass-selected protein aggregate is disrupted to yield monomers by a first round of CID. The released subunits are then sorted by IM and, upon exiting the IM cell, are subjected to the second round of CID such that covalent fragmentation is achieved. Because the released subunits occupy a range of charge states and conformations, they can be resolved to some extent by IM. This results in sequence ions that are aligned in drift time with the released subunit from which they arose [25, 26]. The approach is demonstrated for dimers of glucagon, ubiquitin, cytochrome c, and β-lactoglobulin, resulting in subunit sequence coverage as great as 50%. Overall, this approach provides an additional, novel route to top-down polypeptide sequencing in conjunction with native MS. Moreover, owing to the incorporation of an IM separation step, such an experiment has the potential to yield uniquely information-rich results. In principle, this approach should enable the measurement of complex mass, complex subunit stoichiometry, complex collision cross section (CCS), subunit masses, subunit and subcomplex CCSs, and subunit sequence information in a single gas-phase experiment. The present results represent the first step towards the realization of these capabilities for structural biology and interactomic analysis.

**Experimental**

**Sample Preparation**

All proteins, solvents, and other chemicals were purchased from Sigma (St. Louis, MO, USA) and used without further purification. Various solution conditions were used in order to obtain noncovalent protein aggregates to serve as model analytes for this work. Glucagon (3.5 kDa monomer) was prepared in a nonnative solution of 50% CH3CN and 0.1% HCOOH by volume with H2O constituting the balance. Ubiquitin (8.6 kDa monomer) was prepared in a nonnative solution of 47.5% C2H5OH and 5% CH3COOH by volume with H2O constituting the balance, as described elsewhere [27]. Cytochrome c (12.4 kDa monomer) and β-lactoglobulin (18.3 kDa monomer) solutions were each prepared in H2O, then exchanged into a native-like buffer of 200 mM NH4OAc at pH 7.4 using Micro Bio-Spin 6 spin columns (Bio-Rad Laboratories, Hercules, CA, USA). To promote dimerization, protein concentrations were on the order of 100 μM (monomer basis).

**Mass Spectrometry**

Mass spectrometric analysis was performed using a Synapt G2 HDMS quadrupole time of flight (Q-TOF) hybrid mass spectrometer (Waters Corporation, Manchester, UK). Protein solutions were subjected to nESI in static mode using a custom-built nESI stage and home-pulled borosilicate glass emitters. The emitters were fashioned from melting point capillaries (Corning Pyrex 0.8–1.1×100 mm; Corning, NY, USA) with the aid of a vertical micropipette puller (David Kopf Instruments model 720; Tujunga, CA, USA). Ionization via nESI was performed by introducing approximately 10 μL of protein solution into a pulled glass emitter and applying the capillary potential by means of a platinum wire in direct contact with the analyte solution. The nESI capillary potential was set to 1.0–1.8 kV, as optimized for each individual emitter and sample. Other instrument parameters were tuned to ensure the preservation of noncovalent dimers, and to maximize the transmission of ions with relatively high mass to charge ratios. Ion source temperature was kept at approximately 25°C (i.e., ambient temperature), and the source region backing pressure was increased to approximately 5 mbar by adjusting a valve (Edwards SpeediValve; Crawley, UK) between the roughing pump and the source region vacuum chamber. Ion source potentials were set to their lowest practical values for each analyte, with sampling cone voltages ranging from 30 to 110 V and extraction cone voltages ranging from 0.3 to 4.0 V. The rf amplitudes applied to all stacked ring ion guides were maximized: in the source region, the amplitude was set to 350 V; in the trap and transfer regions, the amplitude was set to 380 V; and in the IM cell region, the amplitude was set to 250 V. Gas flow rates to the IM cell were 180 mL/min He (injection cell) and 90 mL/min N2 (mobility separator). The pressure in the mobility separator was approximately 3.2 mbar. For experiments including IM separation, the IM cell traveling DC wave heights and wave velocities were set to 40 V and 550–850 m/s, respectively, depending on the analyte. The injection potential applied to introduce ions into the IM cell (the DC offset between the trap region and the IM cell) was set for 40–75 V, depending on the analyte. CID was carried out in the trap and transfer regions of the instrument using argon as the collision gas. The pressures in the trap and transfer region stacked ring ion guides were held on the order of 10⁻² mbar by introducing argon at a flow rate of 4.0 mL/min. The collision energies for trap region CID were set by adjusting the DC offset between the ion source region stacked ring ion guide and the trap region of the instrument (ΔUTrap). Likewise, the collision energies for transfer region CID were set by adjusting the DC offset between the IM separator and the transfer region of the instrument (ΔUTr). These ΔU values were set over a range of 20–120 V, depending on the analyte.
On occasion, ions having a given m/z were observed at low intensities over the entire range of drift times. When this unexpected behavior was noted, the spreading of mass spectral signals throughout the mobility dimension was corrected by decreasing the DC offset between the helium cell body and the body of the IM cell (i.e., “helium cell DC,” in this work, set over a range of 20–30 V). It was also noted that setting transfer cell traveling DC wave velocities to excessively high values could lead to a similar broadening of signals in the mobility domain (in this work, the transfer cell traveling DC wave velocity was set over a range of 250–300 m/s).

Data Processing

Acquisition and rudimentary processing of mass spectra was performed using MassLynx v4.1 (Waters). DriftScope v2.1 (Waters) was used to generate heat maps of IM-MS data and to extract mass spectra from selected drift time regions. Mass spectra were deconvoluted to give centroided monoisotopic masses and charge states using Decon2LS v1.0 (http://ncrr.pnl.gov/software/). These experimentally observed masses were compared with predicted sequence ions using Protein Prospector (http://prospector.ucsf.edu/). The monoisotopic masses and charge states of all matching sequence ions were manually verified. Further processing and visualization of data was carried out in IGOR Pro v6.1 (WaveMetrics, Lake Oswego, OR, USA).

Results and Discussion

With the objective of gathering subunit sequence information directly from noncovalent complexes, characteristics of both top-down MS/MS and native MS approaches were combined in a single experiment. This was enabled by the configuration of the Waters Synapt G2 HDMS instrument, which provides the facility for vibrational activation both before and after the IM separator. As diagrammatically summarized in Figure 1, this design was exploited such that two successive stages of CID could be performed with an intervening stage of IM separation. Protein dimer ions generated by nESI were first quadrupole selected, then subjected to CID in the trap cell according to the DC offset between the quadrupole and the trap cell. This served to disrupt the dimer, rendering individual subunits that were folded to different extents and had correspondingly different charge states. Although some exceptions have been noted [19, 27–32], it has been well-established that CID of noncovalent protein complexes typically results in the release of an unfolded subunit, which carries a disproportionately high number of charges with respect to the mass of the subunit [14, 28, 33–38]. This charge partitioning is often described as “asymmetric” with respect to the mass of the released subunit; however, the charge states generally appear to be proportional to the surface areas of the dissociation products. The conformationally distinct subunits released by this first stage of CID were then introduced to the IM cell, where they were sorted in drift time according to their conformations and charge states. In light of the considerations discussed above, the size-to-charge ratios of the CID products vary widely, facilitating their sorting by IM into high charge state (extended) subunits and low charge state (compact) subunits. As ions reached the transfer cell according to their IM drift times, they underwent a second stage of CID driven by the potential difference between ion mobility and transfer regions of the instrument (ΔUtrap). This enabled the further fragmentation of the collisionally released and mobility selected subunits, thus yielding polypeptide sequence information. The products of this second round of CID were finally subjected to TOF-MS analysis.

The applicability of this Q-CID-IM-CID-TOF approach was assessed using several well-characterized model proteins. The first of these was glucagon, a 3.5 kDa protein that is known to dimerize under appropriate solution conditions [27]. The nESI mass spectrum of this protein contained glucagon monomers and dimers with charge states ranging from z=2+ to 6+. Figure S1 of the Supplementary Material). The z=5+ charge state of the glucagon dimer (m/z=1393) was quadrupole selected and subjected to CID in the trap region at ΔUtrap=24 V. This resulted in monomeric product ions having charge states of z=2+ and 3+, which were separated by IM into distinct drift time regions.

Figure 1. Schematic overview of a CID-IM-CID-TOF experiment. Quadrupole selection of a dimer is followed by CID in the trap region stacked ring ion guide, yielding monomers of differing charge states and conformations. These monomers are then separated on the basis of size to charge ratio in the ion mobility cell. As the monomers exit the mobility cell, they are subjected to a second stage of CID in the transfer region stacked ring ion guide, yielding sequence ions. The second stage of CID has the effect of producing subunit fragment ions, which are aligned with the IM drift time of the corresponding precursor ion.
Further dissociation of monomers was brought about by applying CID in the transfer cell of the instrument at $\Delta U_{\text{Xfer}} = 60$ V (Figure 2b). Because this second stage of CID took place as ions exited the IM cell, the resulting fragment ions were “time-aligned” with their respective mobility-selected precursors. This resulted in a range of fragment ions of various $m/z$ occurring within a given range of drift times. Such a region is delineated by the dotted rectangle in Figure 2b, which encompasses the drift time region of approximately 9.5–11.5 ms and, thus, captures all of the product ions originating from the $z=3^+$ monomer. As shown in Figure 2c, extracting the corresponding $m/z$ and intensity data from this specific range of drift times yielded the mobility-selected CID spectrum, which exhibited signals arising from the triply charged subunit. As expected, the spectrum was

![Figure 2](image_url)
dominated by singly and doubly charged \( b \)- and \( y \)-ions. The cleavage map (Figure 2c, inset) highlights the resulting sequence coverage obtained for the monomer; 91 of the observed peaks matched predicted \( b \)- and \( y \)-ions. These fragment ions represent 16 of 28 possible polypeptide amide bond cleavages, corresponding to 57% coverage. A total of 56% of the peak intensity of the spectrum was matched to these \( b \)- and \( y \)-ions. By contrast, the doubly charged subunit did not dissociate as efficiently because of its lower charge state. This observation is consistent with expectations based on the lower overall kinetic energy imparted to the doubly charged subunit when accelerated through \( \Delta U_{\text{Xfer}} \), as well as proton mobility (we note that glucagon contains two arginine residues and one lysine residue) [39, 40].

Ubiquitin (8.6 kDa, monomer) was next analyzed in the same manner. MS of ubiquitin from denaturing solution exhibited dimers and monomers carrying charges

![Graphs and images illustrating mass spectrometry data and spectra](image)

**Figure 3.** Pre-IM CID of the 11+ ubiquitin dimer, with IM separation of the resulting monomers (a), with addition of post-IM CID (b), and sequence ions generated by CID of the collisionally released and mobility separated ubiquitin monomer (c). The mass spectrum in (c) was extracted from the boxed drift time region shown in (b). The inset legends indicate the sequence of events which yielded the data shown (for further explanation of the legend, please see the caption to Figure 2).
ranging from $z=10^+$ to $12^+$ and $z=5^+$ to $13^+$, respectively (Figure S2 of the Supplementary Material). The $z=11^+$ charge state of the dimer ($m/z=1558$), being the only odd charge state with appreciable intensity, was chosen for dissociation. The dimer was then subjected to trap region CID, yielding monomers with a rather symmetric distribution of charge with respect to mass (presumably because of the dimer being sampled from non-native solution), which were mobility separated (Figure 3a). In this case, product ions from monomer $z=4^+$ onwards fell into a drift time range that was difficult to isolate for any particular charge state because of the wide variety of conformations occupied by the released subunits in the $z=4^+$, $5^+$, and $6^+$ charge states. The released subunits were then subjected to post-IM transfer region CID, resulting in a variety of covalent

![Image](image_url)

**Figure 4.** Pre-IM CID of the 11+ cytochrome c dimer, with IM separation of the resulting monomers (a), with addition of post-IM CID (b), and sequence ions generated by CID of the collisionally released and mobility separated cytochrome c monomer (c). The mass spectrum in (c) was extracted from the boxed drift time region shown in (b). The inset legends indicate the sequence of events which yielded the data shown (for further explanation of the legend, please see the caption to Figure 2)
fragments arising from the higher charge state subunit ions (Figure 3b). Mass spectral data were extracted from the drift time region encompassed by the dotted rectangle in Figure 3b, resulting in Figure 3c. This spectrum displays b- and y-ions from ubiquitin that cover 25% of the sequence (42 peaks matched out of the 75 observed).

Moving to subunits of even higher mass, cytochrome c (12.4 kDa, monomer) dimeric aggregates were next probed. Performing nESI of this solution from native like conditions yielded both monomers and dimers with relatively narrow charge envelopes as typical of native MS (Figure S3 of the Supplementary Material). The

Figure 5. Pre-IM CID of the 13+ β-lactoglobulin dimer, with IM separation of the resulting monomers (a), with addition of post-IM CID (b), and sequence ions generated by CID of the collisionally released and mobility separated β-lactoglobulin monomer (c). The mass spectrum in (c) was extracted from the boxed drift time region shown in (b). The inset legends indicate the sequence of events that yielded the data shown (for further explanation of the legend, please see the caption to Figure 2). Owing to space limitations, not all assigned fragments are labeled in the spectrum; for the same reason, some of the protein sequence has been omitted as indicated by the solid circles.
dimer with $z=11$ ($m/z=2248$) was mass selected for a round of CID in the trap cell and afforded a population of released monomeric subunits ranging in charge state $z=3+ \text{ to } 8+$ (Figure 4a). Finally, the subunits were passed through the transfer region, where they experienced another potential drop in the transfer region cell, eventually fragmenting to $b$- and $y$-ions (Figure 4b). A range of drift times was then chosen to capture the CID products arising from released subunits of multiple charge states. The corresponding mass spectrum contained $b$- and $y$-ions that covered 22% of the sequence (Figure 4c).

Finally, the native dimeric form of β-lactoglobulin (18.3 kDa, monomer) was studied in the same manner. Native nESI-MS analysis of this protein revealed a mixture of monomeric and dimeric β-lactoglobulin (Figure S4 of the Supplementary Material). The $z=13+$ dimer ($m/z=2812$) was targeted for CID-IM-CID-MS dissociation. Dissociation of this dimeric precursor ion furnished released monomers with charge states ranging from $z=5+$ to $8+$ (Figure 5a). The released monomers most prevalently occupied the $z=6+$ and $z=7+$ complementary pair, representing a highly symmetric dissociation. Although this is not considered typical CID behavior for native dimers in general, β-lactoglobulin is well-known to contain two disulfide bridges per monomer. The presence of these covalent linkages would be expected to stabilize each monomer such that extensive subunit unfolding is prevented, thereby blocking the events that lead to asymmetric division of charge. Indeed, symmetric CID of dimers with disulfide bridged monomers has been previously observed [28]. After sorting by IM and subsequent transfer region CID, the released monomers having charge states of $z=7+$ and $z=8+$ dissociated to provide $b$ and $y$ type sequence ions (Figure 5b). Mass spectral data exported from the relevant range of drift times provided 24% sequence coverage. This represents coverage comparable to that observed for cytochrome $c$, despite the substantially greater subunit mass and the presence of stabilizing disulfide bonds.

Conclusions

A novel combination of nESI, IM, and multiple stages of CID was used to analyze simple noncovalent protein complexes in a top-down manner. Release of subunits from mass-selected noncovalent protein assemblies with subsequent mobility sorting and fragmentation of the subunits afforded significant coverage of the subunit sequences. We note that this approach is similar in concept to that very recently reported by Kelleher and coworkers [41], although our approach differs in that it has the potential to simultaneously interrogate multiple mobility-resolved released subunits in a single experiment. The incorporation of IM also provides performance gains for top-down sequence analysis, as noted by Russell and coworkers [42]. The addition of IM also presents the possibility of measuring CCS values for both the initial complex as well as the first stage CID products. In concert with measurements of complex mass, complex subunit constitution, subunit mass, and subunit sequence information, this method has the potential to yield a uniquely informative data set from a single gas-phase experiment.

We acknowledge some challenges toward extending this approach to larger and more heterogeneous systems. For example, TOF resolution may be a limitation when interpreting top-down data sets for larger proteins. Another instrumental drawback is that range of CID collision energies is somewhat limited in commercial instruments designed for low-energy beam-type CID (for example, in the Waters Synapt G2 instruments, the limit is 200 V each for $\Delta U_{\text{Trap}}$ and $\Delta U_{\text{Xfer}}$). This introduces some constraints as to what complexes and subunits can be efficiently fragmented. Nevertheless, ongoing improvements in TOF resolution, together with instrument modifications designed to increase the ability to deposit vibrational energy into precursor ions [20, 43], would suggest that such limitations are temporary. Since dissociation occurs more efficiently for ions of higher charge states, we envision that supercharging agents could also significantly extend the capabilities of this method [44–47]. Our current efforts are aimed at incorporating such modifications into our approach in order to achieve top-down analysis of subunits released from larger native complexes, with the ultimate goal of obtaining top-down subunit identification directly from the complex. Overall, these data indicate significant potential for a uniquely information-rich protein interactomic analysis method, which begins with native complexes and directly yields subunit identities.

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References


