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# A Quadrupole Ion Trap Mass Spectrometer with Three Independent Ion Sources for the Study of Gas-Phase Ion/Ion Reactions

Ethan R. Badman, Paul A. Chrisman, and Scott A. McLuckey*

*Department of Chemistry, Purdue University, West Lafayette, Indiana 47907-1393*

**An instrument for the study of gas-phase ion/ion reactions in which three independent sources of ions, namely, two electrospray ionization sources and one atmospheric sampling glow discharge ionization source, are interfaced to a quadrupole ion trap mass analyzer is described. This instrument expands the scope of gas-phase ion/ion reaction studies by allowing for manipulation of the charge states of multiply charged reactant and product ions. Examples are provided involving the formation of protein−protein complexes in the gas phase. Complexes with charge states that cannot be formed from reactant ion charge states present in the normal electrospray charge state distributions can be formed in the new apparatus. Strategies that rely on both reactant ion charge state manipulation and product ion charge state manipulation are demonstrated. In addition, simplification of product ion spectra generated from dissociation of complexes formed via ion/ion reactions can be effected by using the discharge source to reduce the charge state of the product ions to primarily 1+.**

The study of gas-phase ion/ion reactions of biomolecules constitutes a relatively new area of research. Ion/ion reactions have several advantages over gas-phase ion/molecule reactions,[1] including larger rate constants and the ability to easily control both the identity of reactants and reaction times, because unlike neutral reagents, the reactants can be manipulated using conventional ion optical approaches. The first published results of ion/ion reactions in the gas-phase involving biomolecules were from Loo et al., who used a simple y-tube flow reactor to generate products from ion/ion reactions before mass analysis in a quadrupole mass spectrometer.[2,3] In the y-tube design, ion beams generated either by two electrospray ionization (ESI) sources or an ESI and a discharge source were merged at the base of the y-tube in the source interface region and reacted before entering the mass analyzer. A number of reactions were observed, including charge inversion of small singly charged negative ions when reacted with multiply charged positive ions,[2] partial neutralization of multiply charged ions upon reaction with oppositely charged ions from air discharge and electrospray,[3] and solvent transfer products.[3]

Smith and co-workers have also studied ion/ion reactions prior to sampling ions into a mass spectrometer. The emphasis of the work was on charge-state reduction of multiply charged biological molecules with either a radioactive polonium source[4,5] or corona discharge[6,7] used to generate charge-reducing ions. They have demonstrated controlled charge reduction of multiply charged both positive and negative ions using each method coupled to a time-of-flight mass spectrometer.

The majority of ion/ion reaction studies has been performed using quadrupole ion traps, as both the reaction vessel and the mass analyzer.[8] Most of the work has focused on methods for charge state reduction of multiply charged either positive ($n+$) or negative ($n-$) ions. Charge state manipulation has served three major purposes in these studies: the generation of charge states that cannot be produced directly from the electrospray source[9−16] for subsequent study by tandem mass spectrometry, the reduction of the charge states of the parent and product ions after collision-induced dissociation (CID) to primarily singly and doubly charged ions to simplify interpretation of tandem mass spectra,[10−19] and

* Corresponding author. E-mail: mcluckey@purdue.edu.

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for gas-phase concentration and purification in mixture analysis.[20−24] In most cases, atmospheric sampling glow discharge ionization (ASGDI) was used to generate the charge-reducing ions, which were injected through a hole in the ring electrode of the ion trap.[25] For charge reduction of $n+$ ions, the reagent ion has been generated primarily from perfluorocarbon molecules, such as perfluoro-1,3-dimethylcyclohexane (PDCH).[9,25] For $n-$ ions, charge reduction has been achieved through electron transfer with oxygen[24] and xenon[26] cations and through proton transfer with pyridine[27] and isobutane.[24] In addition to proton-transfer reactions, other reaction types that have been observed include electron transfer,[25] anion attachment,[28,29] and anion transfer[29] to multiply charged cations and cation attachment to multiply charged anions.[30]

Recently, we described a new instrument for ion/ion reactions designed to accommodate two ESI sources.[31] This instrument uses the two ESI sources to generate a wider variety of positive and negative ions for reaction in the ion trap than is possible with the use of ESI with ASGDI. In this design, all ions are injected through an endcap electrode, a process that is less energetic and much more efficient than injection through the ring electrode, which allows a wider variety of ion sources to be used for generation of reactant ions. The main type of new reaction possible with this instrument is the reaction of multiply charged positive ions with multiply charged negative ions. To date, both proton transfer and complex formation has been observed. The most studied example of the latter process has involved protein−protein complex formation,[31,32] but a number of other species can also be generated, such as protein−metal and protein−ligand complexes.

In the study of ion/ion complexes with the dual ESI source instrument, it has become apparent that the addition of the ability to charge reduce reactant and product ions is desirable for a number of reasons. First, for studying the fundamental properties of complexes formed by condensation of oppositely charged ions in the gas phase, the systematic study of a wide range of charge states of the complex is desirable. The ability to manipulate the charge states of both the reactant and product ions would allow easier generation of charge states needed for these studies. Second, upon generation of the ion/ion complex, it is often desirable to perform tandem mass spectrometric experiments ($MS^n$) to address issues of ion structure and stability. As the first generation products of ion/ion reactions become larger and more complex, the dissociation spectra (second generation products) become more complex and difficult to interpret; therefore, the ability to reduce the complexity of the second generation product ion spectrum using charge reduction methods becomes desirable.

Here, the properties of a new instrument to study ion/ion reactions in the gas phase are demonstrated. This instrument is based upon a quadrupole ion trap mass spectrometer and has three separately controllable ion sources: two ESI sources with ion injection through an endcap electrode and an ASGDI source used to inject singly charged anions through the ring electrode of the ion trap. Several novel ion/ion reaction experiments made possible with this instrument are described.

## EXPERIMENTAL SECTION

Positive and negative ion nanoelectrospray were performed using drawn borosilicate glass capillaries with tip diameters of ~10 $\mu$m. Electrical contact with the solution was made through a stainless steel wire inserted through the back of the glass capillary; typical operating voltages were ±1200−1500 V.

Nanoelectrospray solutions for the proteins were 10−20 $\mu$M in either 1% aqueous acetic acid or 50/50% methanol/water with 1% acetic acid for positive electrospray or in aqueous 4% ammonium hydroxide for negative ions. Bovine heart cytochrome *c* and bovine ubiquitin (Sigma, St. Louis, MO) were used without further purification. Potassium hexacyanoplatinate (IV) (Aldrich, Milwaukee, WI), $K_2Pt(CN)_6$, was nanosprayed in pure water solutions at a concentration of 0.1 mg/mL. Perfluoro-1,3-dimethylcyclohexane (PDCH) was obtained from Aldrich.

## RESULTS AND DISCUSSION

**Instrument Design.** A schematic diagram of the three-ion source instrument (drawn to scale) is shown in Figure 1. The instrument chamber consists of a 6-in. ConFlat cube housing the quadruple ion trap and detector system, connected by a flange adaptor to an 8-in. ConFlat cube housing the turning quadrupole. The primary pumping for the chamber was performed by two turbomolecular pumps (Turbovac 361, 400 L/s, Leybold Vacuum, Export, PA; and TurboV 550ICE, 350 L/s, Varian, Lexington, MA) with one mounted on each cube, and each was backed by a rotary vane pump (Varian SD301; and Alcatel 2008AC, Hingham, MA). A typical chamber pressure with all sources closed is $5 \times 10^{-7}$ Torr; with all sources operating, $2 \times 10^{-5}$ Torr, to which helium is added to a pressure of ~$1.5 \times 10^{-4}$ Torr (all pressure measurements are uncorrected).

All of the electrospray (ESI) ion sources were identical and were designed similarly to those described previously.[25] The interface region of each source was constructed in an 8-in. ConFlat flange. The front plate aperture hole between atmosphere and the interface region was 150 $\mu$m in diameter, and the aperture between the interface region and the main vacuum chamber had a diameter of 250 $\mu$m. The interface region was a 2.00-in.-diameter hole with a depth of 0.625 in. cut into the flange, with two small lenses in the interface to aid in focusing ions into the vacuum chamber. The pressure in the interface was ~550 mTorr, maintained in the negative electrospray source by a rotary vane pump (Leybold


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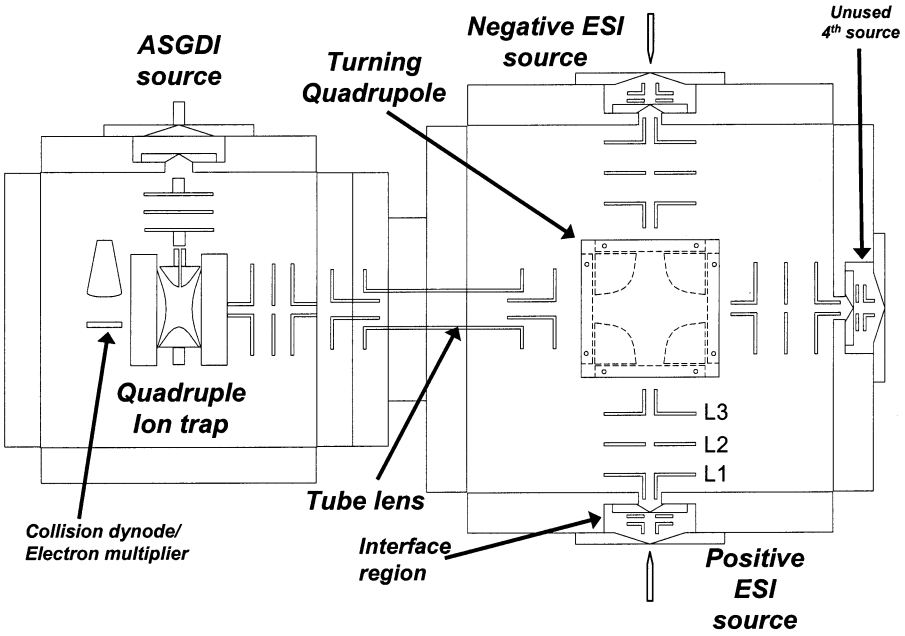


Figure 1. Schematic diagram of the three-source quadrupole ion trap instrument showing two electrospray ionization (ESI) sources and one atmospheric sampling glow discharge ionization (ASGDI) source interfaced to a quadrupole ion trap mass spectrometer. The turning quadrupole allows the ion beams generated by ESI to be deflected 90° to the axis of the ion trap.

D25B) and in the positive electrospray source by a molecular drag pump (Alcatel Drytel 100) (It should be noted that a third electrospray/discharge ion source is shown in Figure 1, but its advantages and use will be described in a future publication.)

After exiting the ion source interface region, ions were focused through a three-element Einzel lens stack (L1, L2, and L3), with the center lens split into half-plates to provide some directional focusing, into a DC turning quadrupole (Quadrupole Deflector Energy Filter, ABB Extrel, Pittsburgh, PA). The turning quadrupole allowed the ion beam to be deflected at a 90° angle from the source to the axis of the mass analyzer (as well as to be focused on-axis). To achieve 90° deflection of the ion beam, identical voltages are applied to diagonally opposing rods of the quadrupole. Typically, this is achieved by electrically connecting the rods inside the vacuum chamber, but in the case here, it was done outside the vacuum chamber to allow the front and back rods pairs to be connected. In the future, this will allow ions to be injected on-axis through the turning quadrupole to allow for three ESI sources to be operated. After the turning quadrupole, ions passed through a tube lens assembly and, finally, through another three-element lens stack before being focused onto the entrance endcap aperture.

For both of the ESI sources, the voltages for the interface and focusing lenses up through L3 were constant throughout the entire experiment (except as noted below). The front plate voltage was supplied from a separate power supply (model 556, EG & G Ortec, Oak Ridge, TN), whereas the rest of the lens voltages were supplied from four nine-output power supplies (Spectrum Solutions Inc., Russellton, PA) with outputs of ±500 V. Because ions of both polarities must be transmitted through them during the course of a single experiment, the lenses of the turning quadrupole, tube lens, and final lens stack were switched between two states necessary for transmission of ions from the positive or negative ESI sources. Voltage switching was achieved via two custom built fast voltage switches based on a Coto-Wabash 3540 reed relay (Coto Technology, Providence, RI). With this switch, the 10 outputs available on the device could be switched in 1 ms from ±500 V upon input of a software controlled TTL level control voltage.

The ASGDI source was used as described previously.[25] The source was orthogonal to the main ion optical axis; ions were focused through a three-element lens stack with the center lens split into half-plates, and entered the quadrupole ion trap through a hole in the ring electrode. The glow discharge was operated in a pulsed mode, using a fast high voltage pulser (GRX-1.5K-E, Directed Energy Inc., Fort Collins, CO) controlled via a software TTL trigger. The high voltage for the pulser was supplied via an Ortec 556 power supply. PDCH vapors in air were sampled through a leak valve, and the source was operated at a pressure of ~850 mTorr. The PDCH anions were used to reduce the charge states of multiply charged positive biomolecules through ion/ion proton-transfer reactions.[9,25]

The mass analyzer used in the instrument was a modified Finnigan ITMS quadrupole ion trap mass spectrometer (ThermoFinnigan Corporation, San Jose, CA) that has been described previously.[25] The voltages applied to the ion trap electrodes for trapping, mass isolation, collision-induced dissociation, and resonance ejection were controlled by ICMS software.[33] Mass selection was performed via application of a single frequency resonance ejection voltage to the endcap electrodes during an rf ramp.[34] Mass range extension was achieved by application of an appropriate ac frequency to the end caps during the mass analysis rf scan.[35] Upon exiting the ion trap, ions were accelerated by a high-voltage lens held at ± 1.5 kV for negative and positive ions, respectively, which

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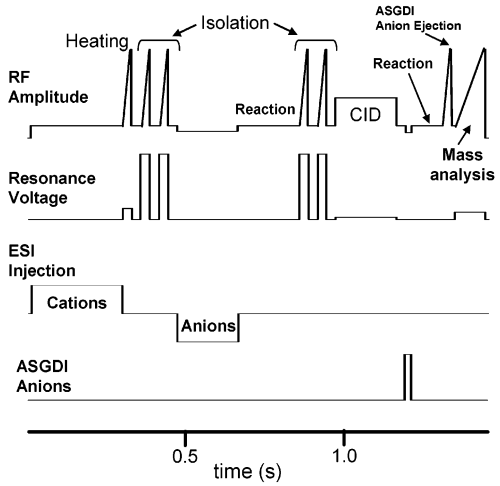



Figure 2. Example timing diagram for the operation of the three-source instrument. The top plot shows the amplitude of the rf trapping voltage applied to the ring electrode of the ion trap. The second plot shows the relative amplitudes and the time during which ac voltages are applied, in dipolar fashion, to the endcap electrodes for resonance ejection and excitation. The lower two plots show the times when ions from the electrospray and discharge sources are injected into the ion trap. Details in text.

aided in the detection of high-$m/z$ ions. Ions were detected with a collision dynode/electron multiplier detector.

Control of the sequential injection of ions from the three ion sources was controlled via TTL triggers from the ITMS data system. With the TTL trigger low ("on" in the software), the switching box was set to pass ions from the positive ESI source. With the trigger high ("off") the switching box was set to pass ions from the negative ESI source. To prevent ions from entering the trap at all times from the negative ESI source (when the trigger is in the default "off" state), one of the L2 half-plates in the negative source was connected to the gate control of the ITMS system. The gate control was originally used by the ITMS to control injection of electrons from a filament by switching a tube lens from −180 to +180 V to allow electrons through to the ion trap. Now this is used to control injection of negative ions from the ESI source in the same way through a software-controlled parameter. Another TTL trigger was used to control the timing and duration during which the ASGDI source was pulsed and PDCH anions were injected through the ring electrode.

An example timing diagram for one typical three-source experiment is shown in Figure 2. Times shown are typical times, but can vary depending on the conditions used and the types of experiments performed. The top trace shows the rf voltage level during the experiment, the second trace shows when the resonance excitation/ejection voltages were applied to the endcaps electrode (with relative amplitudes), and the bottom two traces show when ions were injected from the positive and negative ESI and ASGDI sources, respectively. In this example, $n+$ ions were injected for 300 ms, followed by an rf heating ramp (30 ms) to aid in parent ion desolvation and adduct removal and two rf ramps (30 ms) to achieve $m/z$ selection of the positive reactant ion with 30-ms ion-cooling times between each of the ramps. During both ramps, an ac voltage was applied across the endcaps, ~3 $V_{p-p}$ for the heating and 16 $V_{p-p}$ for the isolation steps. Then, $n-$ ions were injected for 200 ms and allowed to react with the $n+$ ions for 200 ms. After the reaction time, two more rf ramps were used to isolate the product ion charge state of interest. Following this step, an activation step (typically 200 ms) could be used to dissociate the complex during which a low-level ac voltage was used to resonantly excite the ion to be dissociated. Finally, ASGDI-generated anions were injected through the ring electrode for 20 ms and reacted with the complex for 100 ms to effect proton-transfer reactions and reduce the charge states to 1+ and 2+. Mass analysis was performed by an rf ramp of 100 ms with the appropriate ac voltage (amplitude and frequency) applied across the endcaps, depending upon the mass range extension used.

**Manipulation of Reactant Protein Ion Charge State Prior to Ion/Ion Complex Formation.** The first novel type of experiment made possible with this instrument is that in which the reactant $n+$ ion charge state is manipulated via a proton transfer ion/ion reaction prior to complex formation by reaction with an $n-$ ion. This experiment is useful for a number of reasons, including the ability to study ion/ion complexes formed from reagent ion charge states that are lower than those that can be formed directly from solution. In addition, for the study of protein−protein complexes with a net negative charge, this step is essential, because typically, for a protein, the negative charge states produced from ESI are lower than those of the corresponding positive ions; thus upon reaction, a positive complex will be formed. To form negative complexes of this type, it would, therefore, be necessary to charge-reduce the positive ion population to a charge state lower than that formed from the ESI process.

Figure 3 shows an example reaction of $n+$ and $n-$ cytochrome $c$ ions in which the charge state of the cations was reduced prior to the reaction. The pre-ion/ion charge state distribution for positive cytochrome $c$ (3a) shows charge states from 19+ to 12+ with no appreciable signal corresponding to the 10+ ion. To reduce the charge state of the ion population, PDCH anions generated from the ASGDI source were injected into the ion trap for 3 ms, followed by a reaction time of 175 ms. During the ion/ion reaction time, a resonance voltage at 31.5 kHz, and 1.9 V was applied to perform ion parking[22] and concentrate the ion signal primarily in the 10+ charge state (3b). After the PDCH ions were ejected, the 10+ ion was mass selected, followed by an 8 ms injection of the cytochrome $c$ anions (3c) and a 130-ms reaction time (no isolation was performed on the protein anions prior to the reaction). The resulting spectrum (3d) shows the 1+ to 4+ cytochrome $c$ dimers formed from the ion/ion reactions of the 10+ cations with the 11− to 6− anions. Because the reaction of the 10+ ion with the 10− and 11− ions results in neutral or negative complexes, respectively, the product complexes observed here in the positive ion mode reflect only the reaction with the 9− to 6− anions.

**Charge Reduction of First-Generation Products of Ion/Ion Reactions.** A second type of experiment using two ESI sources and the ASGDI source involves generating an ion/ion complex, a first generation product ion, and subjecting it to a charge-reducing ion/ion reaction (Figure 4). In this example, the 15+ cytochrome $c$ charge state was isolated from the parent ion distribution (Figure 3a) and was allowed to react with the population of negative cytochrome $c$ ions (as shown in Figure 3c, again without mass selection) to give cytochrome $c$ dimers with charge states of 4+ to 10+ (4a). In addition to dimer formation,

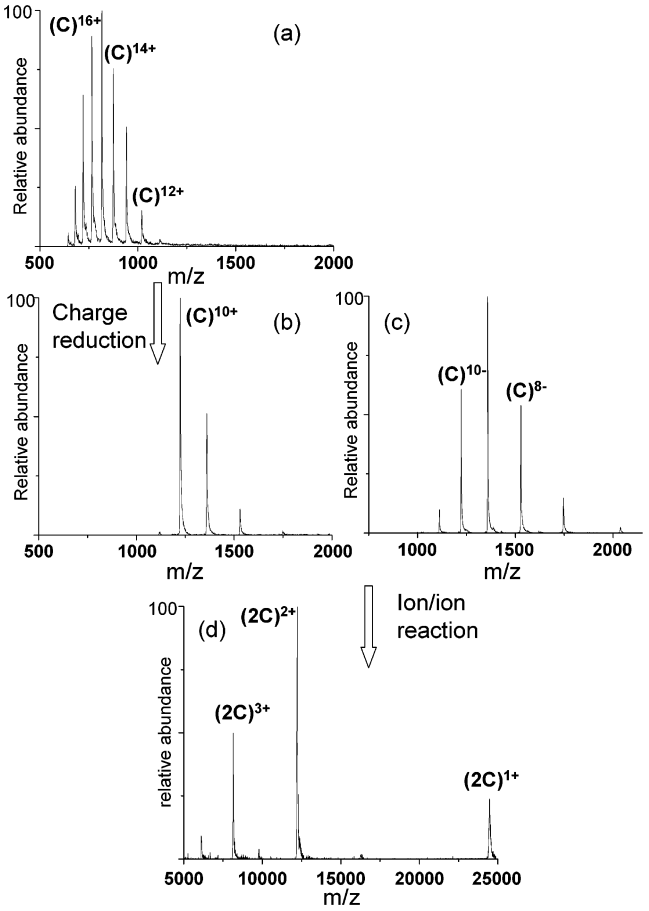



Figure 3. Charge-state reduction of positive cytochrome *c* ions via proton transfer to PDCH anions prior to ion/ion reaction with negative cytochrome *c* to form cytochrome *c* dimers. (C) = cytochrome *c*. (a) Charge state distribution of positive cytochrome *c* from nanospray of a 50/50 water/methanol solution with 1% acetic acid. (b) Positive ion mass spectrum of cytochrome *c* after proton-transfer ion/ion reaction with PDCH anions and ion parking of 10+ charge state. Cation injection time = 200 ms; PDCH anion injection time = 3 ms; ion/ion reaction time = 175 ms; and ion parking resonance voltage at 31.5 kHz, 1.9 V. (c) Charge state distribution of negative cytochrome *c* from nanospray of aqueous 4% ammonium hydroxide solution. (d) Cytochrome *c* dimer reaction products after ion/ion reaction of isolated 10+ ions with negative cytochrome *c* ions from part c. Anion injection time = 8 ms, ion/ion reaction time = 130 ms.

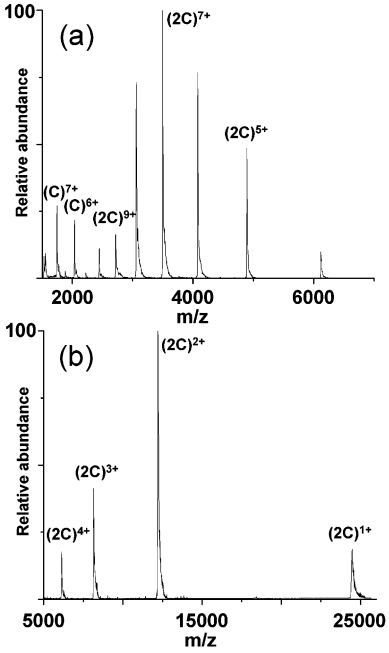



Figure 4. Charge-state reduction of first-generation product ions from ion/ion reaction of positive and negative cytochrome *c* ions. (a) Product ion distribution from ion/ion reaction of 15+ cytochrome *c* with negative cytochrome *c* ions. Cation injection = 400 ms, anion injection time = 10 ms, ion/ion reaction time = 150 ms. (b) Charge reduction of 7+ cytochrome *c* dimer using ion/ion reactions with PDCH anions. PDCH anion injection time = 10 ms, ion/ion reaction time = 130 ms.

minor products from proton transfer to form 8+ to 6+ monomers were observed. The 7+ cytochrome *c* dimer was then mass-selected and allowed to react with the PDCH anions. The resulting spectrum (4b) shows the 1+ to 4+ charge state of the complex. This type of experiment is also useful for generating charge states of complexes that cannot be formed from the ion populations generated directly by ESI. Either the charge states of the parent ions can be manipulated, as illustrated in Figure 3, or the charge states of the product ions can be manipulated, as in Figure 4, or both. It remains to be determined, however, if the different routes to producing a complex of an arbitrary charge state are equivalent, in terms of ion structure.

**Charge Reduction of Second-Generation Product Ions from Ion/Ion Complexes.** In addition to the manipulation of the charge states of reactants and first-generation products of ion/ion reactions, the new instrumentation can be used to aid in interpretation of the MS/MS spectra of complexes by charge reduction of the second-generation product ion spectra formed from CID. After the parent ion/ion complex has been created, dissociation can lead to product ion spectra that are complicated by charge state ambiguities. Charge reduction of the product ions to primarily 1+ and 2+ charge states with the PDCH anions greatly simplifies the spectrum and allows for easier interpretation and assignment of the fragment ions.

An example is shown in Figure 5 for the dissociation of the protein−metal complex species $[\text{ubiquitin} + \text{Pt(CN)}_6 + 7\text{H}]^{5+}$. Positive ubiquitin ions were injected for 300 ms, and the 7+ charge state was isolated. Then anions generated from ESI of $K_2Pt(CN)_6$ (primarily $Pt(CN)_6^{2-}$) were injected for 200 ms and allowed to react with the cations for 200 ms. The product ion corresponding to $[\text{ubiquitin} + \text{Pt(CN)}_6 + 7\text{H}]^{5+}$ was isolated and activated at a resonance excitation frequency of 89 kHz with an amplitude of 570 mV for 300 ms. After activation, PDCH anions were injected for 4 ms and reacted with the product ions for 120 ms. The resulting charge-reduced product ion spectrum is shown in Figure 5a. For clarity, only the 1+ product ions are labeled; the notation X in the labels refers to the entire $Pt(CN)_6$ complex.

Several noteworthy observations can be made regarding the dissociation of this protein−metal complex. First, there is no evidence that the metal complex dissociates from the ion to leave only the ubiquitin ion or that the metal complex itself dissociates during activation. Second, ignoring the presence of the platinum complex, the identities and relative ratios of the fragment ions are comparable to those observed previously[10,13] for dissociation of the $[M + 5H]^{5+}$ ion of ubiquitin; it appears that adding the platinum complex does not affect the relative contributions of the various fragmentation pathways observed under these activation

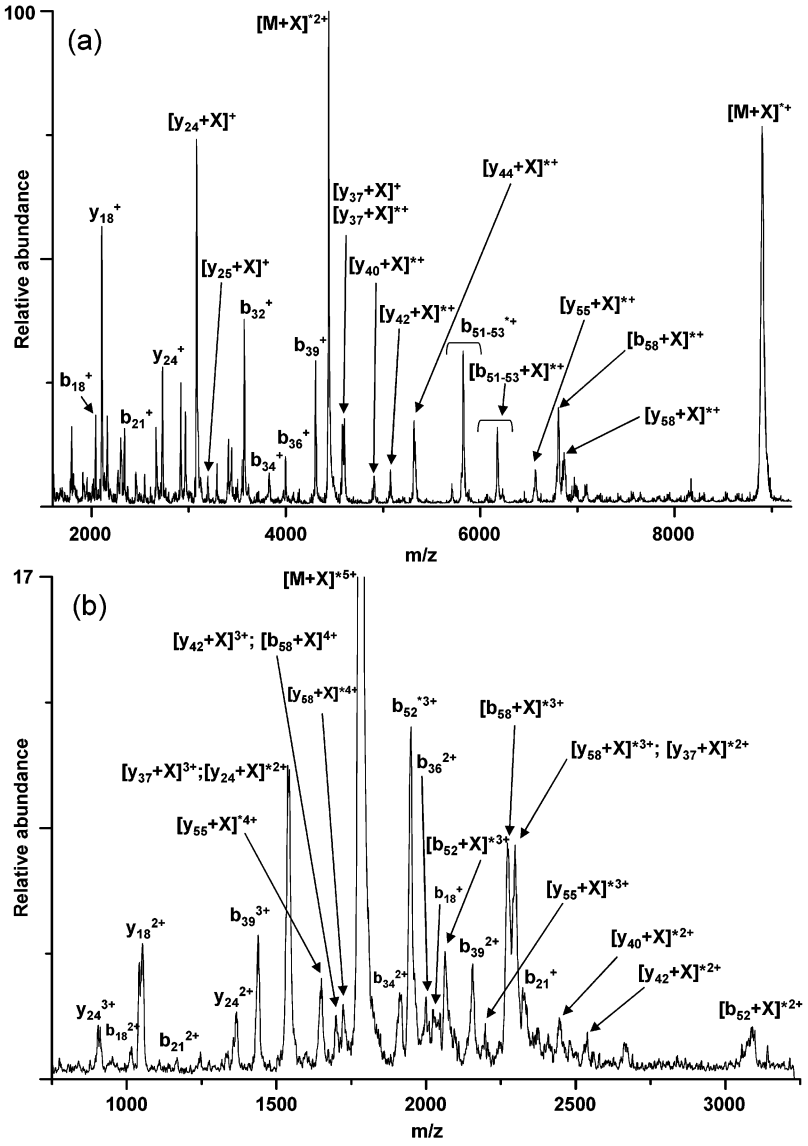
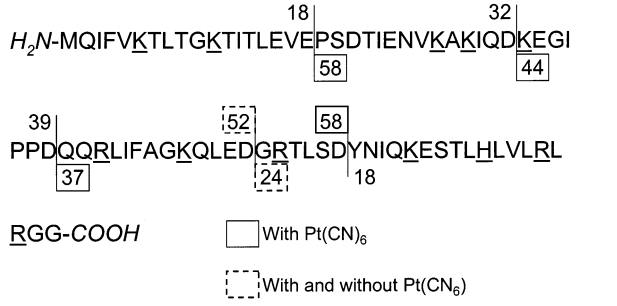



Figure 5. (a) Charge-reduced, tandem mass spectrum of [ubiquitin + $Pt(CN)_6$ + 7H]$^{5+}$. Ubiquitin cation injection time = 300 ms; $Pt(CN)_6^{2-}$ injection time = 200 ms; ubiquitin/$Pt(CN)_6$ reaction time = 200 ms; activation for 300 ms at 89.0 kHz, 570 mV; PDCH anion injection time = 4 ms; PDCH/complex reaction time = 120 ms. Note: X = $Pt(CN)_6$, and the asterisk (*) denotes a small neutral molecule loss ($NH_3$ or $H_2O$) from the ion. (b) Pre-ion/ion MS/MS spectrum of the complex with the fragment ions labeled to shown the charge state of the fragments prior to charge reduction.

conditions. The loss of a small neutral molecule ($NH_3$ or $H_2O$) from the parent ion is also observed to be a major process.

Scheme 1 shows a summary of the locations of the prominent product ions and the presence (solid box around the fragment ion label) or absence of the platinum complex. One complementary pair of the ions are present both with and without the platinum complex (dashed box around the fragment ion label). The most basic amino acid residues (R, K, and H), where the protons are most likely located, are underlined. These results clearly show that the complex is located in one specific stretch of sequence between the 40th (Q) and 58th (D) amino acid residues and is not distributed throughout the molecule. The fact that two major fragments are present both with and without the platinum complex suggests that the platinum complex binds in two locations and is able to dissociate from one of the binding sites during the CID process but still remain attached at the second binding site.

Scheme 1. Summary of the Locations of the Prominent Product Ions from the Dissociation of [Ubiquitin + $Pt(CN)_6$ + 7H]$^{5+}$, and the Presence (Solid Box around the Fragment Ion Label) or Absence of the Platinum Complex on the Fragment[a]




[a] A dashed box around the label indicates that the fragment ion is present both with and without the platinum complex.

To gain further understanding of the behavior of this complex, the pre-ion/ion MS/MS spectrum is shown in Figure 5b. The fragment ions have been labeled using the post-ion/ion spectrum to determine which fragment ions are present. It should also be noted that all of the major fragment ions observed from the post-ion/ion spectrum have been identified except the $[y_{44} + Pt(CN)_6]/b_{32}$ complements, which would fall under the parent ion peak if they have a charge state of 3+ and 2+, respectively; thus, they have been assigned to those charge states. There are complementary fragment ion pairs observed at $y_{18}^{2+}/[b_{58} + Pt(CN)_6]^{*3+}$, $b_{32}^{2+}/[y_{44} + Pt(CN)_6]^{*3+}$, and $b_{39}^{2+}/[y_{37} + Pt(CN)_6]^{3+}$ and $b_{39}^{3+}/[y_{37} + Pt(CN)_6]^{2+}$ ions. This suggests that there are two or three protons located N-terminal to the 39th residue and two that are C-terminal to the 58th residue; therefore, two or three protons can be located in the region where the platinum complex attaches, where there are also three possible basic sites (two arginines and one lysine). Thus, there are multiple locations where the two negative charge sites on the metal complex can attach to the region between the 39th and 58th residue, which may also play a part in the observation that the $b_{51-53}$ fragment ions are present both with and without the complex.

The three types of experiments described above constitute a subset of novel ion/ion reaction sequences and reactant combinations that are made possible by the three-source instrument. A similar set of experiments, for example, can be executed whereby a negatively charged complex composed of ESI-generated ions can be subjected to cations formed by ASGDI. Therefore, a wide range of first- and second-generation ion/ion reaction products can be subjected to reactions with singly charged ions generated by ASGDI. For example, we are currently exploring metal ion−protein complexes in more detail as well as enzyme−inhibitor complexes formed in the gas phase. In addition, we are currently exploring new experiments made possible by using all three ESI sources available in this instrument as well as the discharge source.

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