"Dueling" ESI: Instrumentation to Study Ion/Ion Reactions of Electrospray-generated Cations and Anions

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Novel instrumentation has been developed which allows for the sequential injection and subsequent reaction of oppositely-charged ions generated via electrospray ionization (ESI) in a quadrupole ion trap mass spectrometer. The instrument uses a DC turning quadrupole to sequentially direct the two ion polarities into the ion trap from ESI sources which are situated 90° from the axial (z) dimension of the trap, and 180° from one another. This arrangement significantly expands the range of ionic reactants amenable to study over previously-used instrumentation. For example, ion/ion reactions of multiply-charged positive ions with multiply-charged negative ions can be studied. Also, reactions of multiply-charged ions with singly-charged ions of opposite polarity that could not be generated by previously used ionization methods, or that could not be efficiently injected through the ion trap ring electrode, can be studied with the new instrument. This capability allows, for example, the charge state manipulation of negatively-charged precursor and product ions derived from proteins and oligonucleotides via proton transfer reactions with singly-charged cations generated by ESI. (J Am Soc Mass Spectrom 2002, 13, 614–622) © 2002 American Society for Mass Spectrometry

Gas-phase ion chemistry has come to play important roles in both the chemical and, increasingly, the biological sciences. Tandem mass spectrometry utilizing unimolecular dissociation of gas-phase ions is a primary method for identifying unknown species in complex mixtures [1]; a recent example which is rapidly increasing in significance is the use of gas-phase dissociation of proteolytic peptides to identify individual proteins in protein mixtures [2, 3]. Ion/molecule chemistry provides a means to study novel chemical species that may be impossible to generate in solution, and is the primary method by which intrinsic (i.e., free of solvation effects) thermochemical information is obtained [4–7]. Ion/molecule reactions that have been applied to large, biologically-derived ions such as proteins include hydrogen/deuterium exchange [8–11] and hydroiodic acid attachment [12–14], which can both be used as probes of gas-phase conformation. Ion/molecule reactions between multiply-protonated ions and strong neutral bases have been studied as a means for reducing ion charge state [15–20]. Modification of gas-phase oligonucleotide ions via nucleophilic substitution has also been demonstrated [21]. Less attention has been given to reactions of multiply-charged gas-phase ions with ions of opposite polarity, largely because the instrumentation needed to carry out such ion/ion reactions is not readily available. However, it has recently been shown that ion/ion reactions of multiply-charged ions with singly-charged ions of opposite polarity may have important analytical utility. For example, ion/ion proton transfer reactions can be used to simplify ESI mass spectra of complex mixtures by reducing the charge states of the mixture components, thereby minimizing *m/z* overlap and relaxing the *m/z* resolution requirements of the mass analyzer [22, 23]. Also, collision-induced dissociation spectra of multiply-protonated protein ions may be simplified via product ion charge-state reduction, minimizing product ion *m/z* overlap and allowing otherwise uninterpretable MS/MS spectra to yield information on protein sequence [24–26] and post-translational modifications [27]. The analytical usefulness of these charge reduction reactions suggests that further study of ion/ion reactions is warranted.

To date, ion/ion reactions studies have been carried out using one of three mass spectrometry-based apparati. The first, used by R. D. Smith and coworkers, employed a Y-shaped flow tube arrangement in front of a linear quadrupole mass spectrometer [28, 29]. Ions from the two sources were conducted down the arms of the Y, and were allowed to react in the base of the Y before sampling into a linear quadrupole mass analyzer. This arrangement was used to study reactions of

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multiply-charged protein ions generated by ESI with ions of opposite polarity generated by discharge methods and by ESI. A limitation of this system is that there is no prior *m/z* selection of either reactant ion polarity, increasing the complexity of the product ion spectra. Also, the ion/ion reaction time is not well controlled or variable, and the limited *m/z* range of the mass filter can preclude observation of high *m/z* products. L.M. Smith and coworkers recently described an interface that uses either a polonium decay ionization source [23, 30] or a corona discharge ionization source [31] to generate singly-charged ions for reaction with multiply-charged, ESI-generated ions for charge-state reduction, followed by mass analysis via time-of-flight (TOF) mass spectrometry. This arrangement has been demonstrated to reduce the charge states of electrosprayed protein and oligonucleotide mixtures largely to + or −1, simplifying spectral interpretation; however, for the study of ion/ion reactions, the device has some of the same limitations as the Y-tube arrangement described above, in that no prior *m/z* selection of the reactants is allowed and the ion/ion reaction time is not well controlled. An advantage of both the ion/ion reaction devices just described is that they can in principle be used with any form of mass analyzer, although they place *m/z* range at a premium.

The third, and most extensively used, ion/ion reaction apparatus takes advantage of the ability of electrodynamic quadrupole ion traps (Paul traps) to simultaneously store oppositely-charged ions in overlapping regions of space. This ion storage capability, together with ion isolation techniques developed for tandem mass spectrometry in quadrupole ion traps, allows the device to overcome the limitations described above, viz., precursor ion selection and control of ion/ion reaction time. A given polarity may be admitted to the trap and manipulated as desired, for example, via isolation and collision-induced dissociation of a particular ion of interest. Ions of opposite polarity may then be admitted to the trap, allowed to react for a given period of time, and then removed either via an appropriate change in the trapping RF voltage amplitude or some other isolation mechanism. A variety of ion/ion reaction phenomenologies have been observed for multiply-charged ions reacting with singly-charged ions of opposite polarity, and this subject has recently been reviewed [32]. Multiply-charged anions of oligonucleotides and peptides have been shown to react with singly-charged cations (from electron and chemical ionization) via proton transfer (e.g., from protonated pyridine), electron transfer (e.g., to xenon cations), and cation attachment. Multiply-charged cations reacting with singly-charged anions yield products from proton transfer, electron transfer, anion attachment, and anion transfer. Extensive study of the reactions of multiply-protonated proteins with singly-charged anions of fluorocarbons (principally perfluorodimethylcyclohexane, or PDCH) generated by atmospheric-sampling glow discharge ionization (ASGDI) [33] has shown that these reactions proceed primarily by proton transfer, resulting in the charge-state reduction of the proteins, with no evidence for cation dissociation upon reaction [34]. Even non-covalent complexes, such as holo-myoglobin, can be reduced in charge to the +1 state with no evidence for dissociation of the non-covalently bound heme group [35]. These observations have led to the use of glow discharge generated anions for the simplification of whole protein MS and MS/MS spectra [24–26], with the goal of protein identification and characterization via a top-down approach. Protein mixture analysis has also been facilitated via charge state reduction [22], which relaxes the mass resolution requirements of the mass analyzer by making the charge, *z*, in the *m/z* measurement equal to one.

All quadrupole ion trap ion/ion reaction experiments performed to date have utilized an arrangement wherein ESI generated ions were admitted to the trap through an ion trap end cap, while either ionizing electrons (in the case of electron and chemical ionization) or ASGDI generated ions were admitted through a hole in the ion trap ring electrode. While the analytical utility of the positive ion ESI/negative ion ASGDI combination has been established [24–26], the use of ASGDI with injection through the ring electrode is somewhat limiting, in terms of the possible ion/ion reaction combinations and reactants that can be studied, for a number of reasons. The ASGDI source only yields singly-charged ions, and only relatively volatile compounds yield sufficient vapor pressure to give adequate ion current from the ion source. Also, injection through the ring electrode has been shown to be very inefficient (less than 1%) [34], and to be substantially more energetic than injection through the end cap, leading to extensive dissociation during ion injection [34]. These factors preclude, for example, the study of reactions of multiply-charged ions of opposite polarity.

In this paper, an arrangement of ion sources and ion optics which allows for the sequential injection of ions of opposite polarity through one end cap of the ion trap is described. Two ion sources are mounted 180° from one another, and 90° from the axial dimension of the ion trap, and a DC-only turning quadrupole is used to sequentially direct ions from the sources into the trap. This dueling ESI arrangement overcomes the ion source and ring electrode injection limitations described above and allows for a number of heretofore inaccessible ion/ion reactions to be studied. Examples of a number of such reactions are given to illustrate the capabilities of the instrument.

Experimental

Bovine heart cytochrome *c* and bovine ubiquitin were purchased from Sigma (St. Louis, MO) and used without further purification. Protein solutions for nano-electrospray were prepared by diluting aqueous 1.0 mg/mL stock solutions to final concentrations of 0.1 mg/mL in aqueous 1% acetic acid for positive ions, or 5

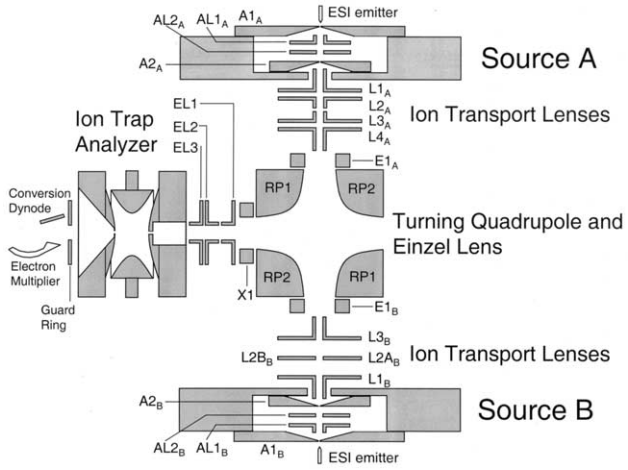

Figure 1. Schematic diagram (not to scale) of the dueling ESI ion trap mass spectrometer.

mM NH_4HCO_3 or 2% NH_4OH for negative ions. Benzene sulfonic acid and 1,3-benzene disulfonic acid were purchased from Aldrich (Milwaukee, WI), and were nano-electrosprayed at concentrations of 0.1 mg/mL from pure water. Benzo(f)quinoline was purchased from Aldrich and was nano-electrosprayed at a concentration of 1 mg/mL from 1% acetic acid. Nano-electrospray was effected by loading 10 μL of solution into a drawn borosilicate glass capillary with a tip diameter of approximately 10 μM. The electrical connection to the solution was made by inserting a stainless steel wire through the back of the capillary, with typical nano-electrospray voltages of approximately $+/-1.2$ kV. The interface used to transport ions to the high vacuum region and inject them into the ion trap is described below. Isolation of ions of interest was effected via the application of resonance ejection voltages to the end caps of the ion trap at the appropriate frequencies while the RF trapping voltage amplitude was scanned [36]. Mass analysis was also performed via resonance ejection during an RF amplitude scan, at a frequency selected to give the desired mass range extension [37].

Results and Discussion

Description of the Dueling Source Arrangement

Figure 1 is a diagram (not to scale) of the new instrument arrangement. The modifications described were made to a Finnigan Ion Trap Mass Spectrometer (ITMS, ThermoFinnigan Corp., San Jose, CA). The two electrospray interfaces are similar to those described previously [34], each consisting of a six-inch ConFlat-type flange with a 2.5 inch diameter by 0.563 inch deep chamber machined into one side to act as an intermediate pressure region. A 150 μm aperture (A1) separates this interface region from atmosphere, and a 250 μm aperture (A2) separates it from the main vacuum chamber of the mass spectrometer. Each interface region is pumped by a Leybold D25B rotary vane pump (Leybold Vacuum Products, Export, PA) to a pressure of approximately 600 mTorr. Two DC lenses (AL1 and AL2) facilitate transport of ions through the interface region.

The main vacuum chamber of the mass spectrometer is pumped by a Leybold TMP361 turbomolecular pump to a pressure of approximately 1×10^{-4} torr when both ESI sources are open. During operation, helium is admitted to this region to a gauge pressure of 2×10^{-4} torr (approximately one mTorr corrected pressure) to provide collisional cooling of ions in the ion trap. A series of DC lenses transports ions from the inner aperture of the interface to the turning quadrupole. Note that the current system is asymmetric with respect to these transport lenses. Source A utilizes four lenses in sequence ($L1_A$–$L4_A$), as shown in Figure 1, while Source B has three lenses ($L1_B$–$L3_B$), with the center lens divided into two half-plates ($L2A_B$ and $L2B_B$) to allow this lens to act as an ion gate (see below). Note also that other types of ion sources, e.g., an ASGDI source, can be installed in place of one or both of the ESI sources shown.

The DC turning quadrupole is an ABB Extrel Quadrupole Deflector Energy Filter (Part number 811989, ABB Extrel, Pittsburgh, PA). Turning quadrupoles allow for 90° turns in ion beams when the beam is injected on the xy plane between two rods (as opposed to z-axis injection parallel to the rods as is done with RF mass analyzing or ion transport quadrupoles). The turning quadrupole is operated with opposing rod pairs electrically connected (RP1 and RP2), so that only two voltages are applied to turn ions through 90°. The turning quadrupole has cylindrical entrance and exit lenses ($E1_A$, $E1_B$, and X1) which are connected to the rod pairs through a resistive voltage divider to hold each at a potential equal to one-half the difference between the RP1 and RP2 potentials. After the turning quadrupole, an Einzel lens (Part number 812174, ABB Extrel) is used to focus ions into the entrance aperture of the ion trap.

Figure 2 shows simulated ion trajectories calculated using SIMION 7.0 [38] for *m/z* 1000 ions having 50 eV kinetic energy and a $+/-$ 5° angular dispersion traveling on the Source B ion optical path described above. Note that the simulation is only of the DC components, and does not attempt to address the efficiency of ion injection into the quadrupole ion trap. The simulation shows that ions can be focused into the turning quadrupole and turned through 90° with good efficiency. Experimentally, we have found no significant loss in overall ion injection efficiency with the new ion optics; i.e., for a given analyte concentration, the same signal-to-noise ratio can be acquired with the same ion injection time on the dueling ESI instrument as can be acquired with the standard ESI/ion trap interface that we have described previously [34]. Figure 2 suggests that most ion losses in the main vacuum region occur in the sampling of the ion beam emitted from A2 by the first ion transport lens, $L1_B$. Furthermore, the turning quadrupole does not appear to introduce any apprecia-

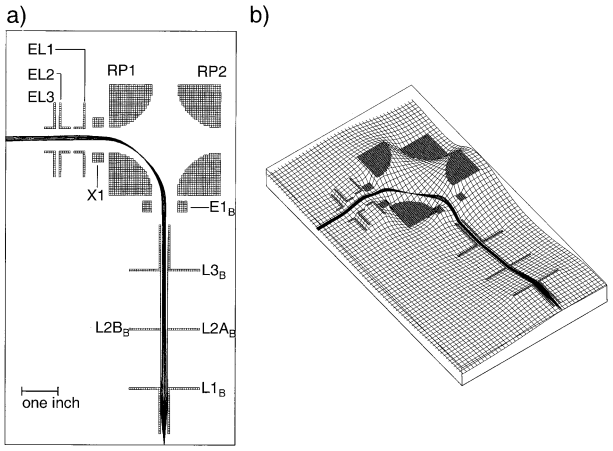

Figure 2. Simulation of the trajectories of *m/z* 1000 cations having an initial kinetic energy of 50 eV and an initial spread of +/−5° through the DC portion of the ion optical path for Source B. (**a**) Two-dimensional view of ion trajectories, (**b**) potential energy surface experienced by the ions. The calculations were done using SIMION 7.0, with the following potentials on the lens elements: $L1_B = -10$ V, $L2A_B = L2B_B = -160$ V, $L3_B = -220$ V, $E1_B = -105$ V, RP1 = −10 V, RP2 = −220 V, X1 = −105 V, EL1 = −50 V, EL2 = −80 V, EL3 = −120 V.

ble *m/z* discrimination effects; i.e., charge-state distributions for a given protein electrosprayed under given solution conditions show similar relative abundances for the various charge states as observed with the standard ESI/ion trap interface.

The interface apertures and lenses, and the ion transport lenses for each source, are connected to their own independent power supplies and the potentials applied thereto are manually tuned for optimum ion injection efficiency. Typical voltages applied to various lens elements are summarized in Table 1. However, because the turning quadrupole and Einzel lens must sequentially transport both polarities of ions, the polarity of the voltages applied to each must be switched in a time frame compatible with an ion trap experiment (i.e., on the ms time scale). In addition to the necessary change in polarity, it is possible that ions of different *m/z* value may require different turning quadrupole and/or Einzel lens voltage amplitudes for maximum injection efficiency. Therefore, it was decided that each element of the turning quadrupole and Einzel lens should have two independent power supplies associated with it, with a mechanism for switching between the two under software control, to allow independent optimization of the applied voltages for each ion polarity. A custom switching box was designed and constructed to accomplish this task. The switching function is based on Coto-Wabash 3540 reed relays (Coto Technology, Providence, RI). Two relays are used for each of 10 output channels, and the box is capable of switching the voltages on all 10 channels between +500 and −500 V in approximately 1 ms under the control of a TTL level trigger signal generated by the ITMS Scan Acquisition Processor Adaptor PCB and controlled by the

Table 1. Typical lens voltages for the dueling ESI interface

Lens element	Voltage applied (V)
Source A (anions)	
$A1_A$	−65
$AL1_A$	−70
$AL2_A$	−75
$A2_A$	−5
$L1_A$	20
$L2_A$	205
$L3_A$	210
$L4_A$	220
RP1	200[a]
RP2	20[a]
EL1	50[a]
EL2	100[a]
EL3	30[a]
Source B (cations)	
$A1_B$	75
$AL1_B$	80
$AL2_B$85	
$A2_B$10	
$L1_B$	−2.5
$L2_B$	−160
$L2B_B$	−160[b]
$L3_B$−	−250
RP1	−10[a]
RP2	−200[a]
EL1	−50[a]
EL2	−100[a]
EL3	−30[a]

[a]The applied voltage changes depending on the ion polarity injected as described in the text.
[b]The applied voltage is −160 V when cations are to be injected, and +160 V otherwise, as described in the text.

ICMS software [39]. A manual mode, where either set of lens voltages is selected with a physical switch rather than with the TTL trigger signal, is also built into the switching box for tuning and optimization of each ion polarity prior to ion/ion reactions. The switching box was used in the work reported here to control the voltages applied to the elements RP1, RP2, EL1, EL2, and EL3. Oscilloscope waveforms for the output of one channel of the switching box, with input voltages of +150 V and −150 V, and a trigger frequency of approximately 20 Hz, are shown in Figure 3 to illustrate the capabilities of the box.

A timing diagram for the sequential injection of positive and then negative ions into the ion trap is shown in Figure 4. The cations from Source B are gated into the ion trap via manipulation of the voltages applied to the two half-plates of the central ion transport lens ($L2A_B$ and $L2B_B$), while the switching box holds the turning quadrupole and Einzel lens at the voltages necessary to inject cations and reject anions. $L2A_B$ is held at approximately −160 V at all times with an external power supply. The voltage applied to $L2B_B$ is under software control, and switches between +160 V when cations are to be rejected, to −160 V when cations are to be passed on to the turning quadrupole and so admitted into the ion trap during the cation

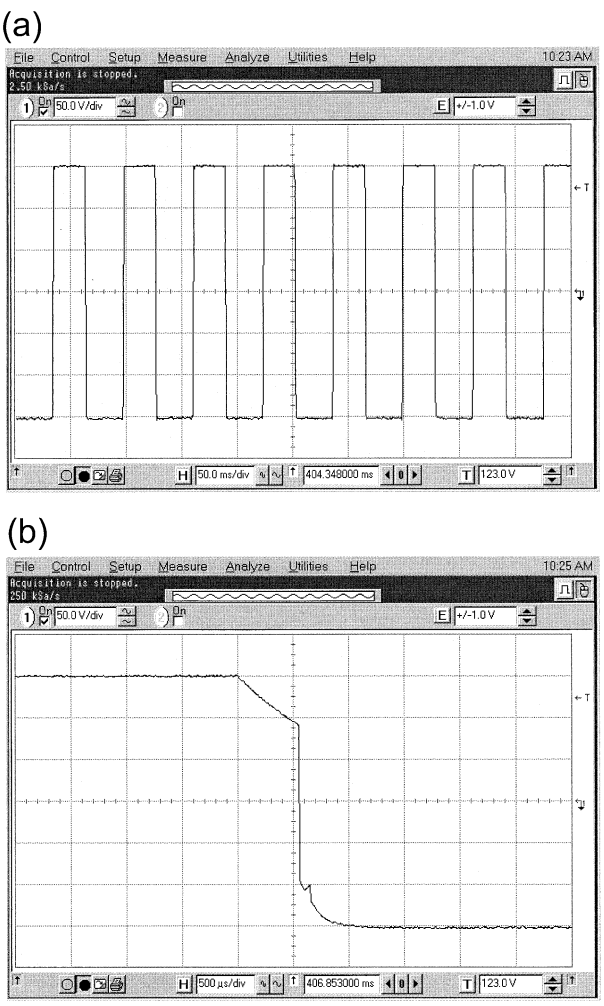

Figure 3. (**a**) Oscilloscope traces of the output of one channel of the custom switching box. The inputs to the box were +150 V and −150 V, and the box was triggered to switch at a rate of approximately 20 Hz by the ITMS electronics. The zoom in (**b**) shows the positive-to-negative transition occurring in approximately 1 ms.

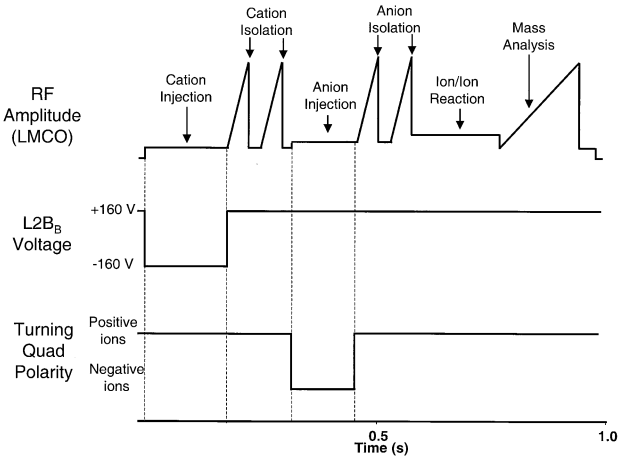

Figure 4. Timing diagram for the injection and isolation of cations from Source B, followed by the injection and isolation of anions from Source A and an ion/ion reaction period before *m/z* analysis. Turning Quad Polarity refers to the voltages on RP1, RP2, EL1, EL2, and EL3, which allow either positive or negative ions to be turned toward the ion trap and injected.

injection time. This software-controlled voltage is the electron gate voltage generated by the ITMS electronics and originally used to gate ionizing electrons into the ion trap for internal EI. In the current instrument this voltage is inverted with a custom circuit that has been described previously [34] to allow gating of positive ions. After injection of cations, manipulation of the ion population, e.g., isolation of a particular ion of interest, can occur prior to anion injection. When injection of anions is desired, a TTL trigger is toggled on at the appropriate point in the scan function. This trigger causes the switching box to switch the voltages on the turning quadrupole and Einzel lens to allow anions to be turned and focused into the ion trap. Note that if cations are already present in the trap, then ion/ion reactions may occur during the anion injection time, so that although isolation of a particular anion of interest can be effected prior to ion/ion reaction, as shown in Figure 4, some undesired side reactions of the previously injected and isolated cations with other anions may result. A possible solution to this problem, which has not been implemented on our system to date, is to use a broadband isolation waveform during the anion injection, so that only anions of interest are allowed into the ion trap. Note also that it is of course possible to reverse the order of injection and allow anions to enter the trap first.

Ion/Ion Reactions Facilitated by the Dueling Source Arrangement

Multiply-charged cations/singly-charged anions (n+/1−). Reactions of multiply-charged cations with singly-charged anions have been the subject of extensive study using the ASGDI source to generate the anions, with ion injection through the ring electrode [34]. Such an arrangement has proven to be very useful for protein analysis applications and is still in extensive use in our laboratory. This $n+/1-$combination is still possible with the source arrangement described here, if one of the ESI sources shown in Figure 1 is converted to an ASGDI source. This is readily accomplished by removing the interface lenses ($AL1_A$ and $AL2_A$), and connecting the outer aperture plate ($A1_A$) to the appropriate high voltage power supply to generate a discharge. Alternatively, ESI of hexafluoroisopropanol yields $(M - H)^-$ ions that appear to react with multiply-charged cations only by proton transfer (data not shown). Another alternative is to effect corona discharge ionization at atmospheric pressure with a needle placed a few millimeters from the $A1_A$ aperture. An adequate ion signal for PDCH molecular anions can be generated in this way to reduce protein ion charge states via proton transfer (data not shown).

The dueling ESI instrument allows singly-charged

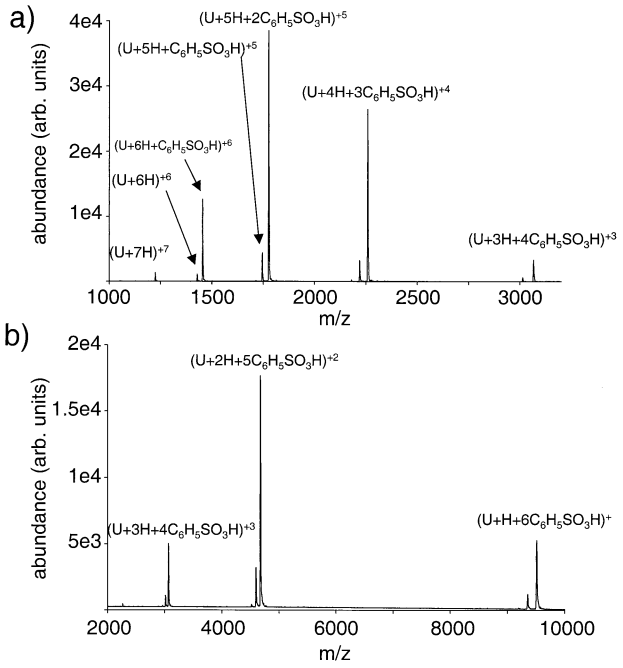

Figure 5. Mass spectra resulting from ion/ion reactions between isolated $(U + 7H)^{+7}$ ubiquitin and deprotonated benzene sulfonic acid $[(C_6H_5SO_3]^-)$. (**a**) Ubiquitin injection time = 100 ms, $(C_6H_5SO_3)^-$ injection time = 100 ms, ion/ion reaction time = 200 ms. (**b**) Ubiquitin injection time = 100 ms, $(C_6H_5SO_3)^-$ injection time = 200 ms, ion/ion reaction time = 500 ms.

anions that are difficult to form via ASGDI and/or are not amenable to injection through the ring electrode to be generated via ESI and injected into the ion trap through the end cap. This capability opens up the possibility for a variety of new chemistries to be explored. In the example given here, the interactions of multiply-protonated proteins with sulfonic acid species are studied. Benzene sulfonic acid ($C_6H_5SO_3H$) is a solid at room temperature, hence it is not expected that ASGDI will generate a large ion current for this species. Given that injection of ions through the ring electrode is approximately two orders of magnitude less efficient than injection through the end cap, [34] it is unlikely that a sufficient number of the ions generated could be trapped to give an observable ion/ion reaction on a time scale of a few hundred ms or less. Furthermore, we observed during ESI of benzene sulfonic acid that the deprotonated ion $[(C_6H_5SO_3)^-]$ readily dissociated in the ESI interface unless the interface lens voltages were carefully tuned to minimize ion acceleration in the interface. Based on this observation, and given that injection through the ring electrode is much more energetic than injection through the end cap [34], it is expected that any molecular ions generated by ASGDI and injected through the ring electrode would dissociate extensively during the injection process.

Nano-ESI of benzene sulfonic acid from pure water yielded a high abundance of $(C_6H_5SO_3)^-$ ions, provided low interface voltages were used to prevent dissociation. Figure 5 shows the results of reacting multiply protonated ubiquitin $[(U + 7H)^{+7}]$ with $(C_6H_5SO_3)^-$ ions. Benzene sulfonic acid anions react with the protonated protein primarily via attachment, although a small amount of proton transfer also appears to occur. Attachment of anions to multiply-protonated proteins has been observed previously [14] The transfer of a proton from $(U + 7H)^{+7}$ ubiquitin to $(C_6H_5SO_3)^-$ is approximately 82 kcal/mol exothermic, based on the apparent gas-phase acidity of $(U + 7H)^{+7}$ ubiquitin measured by Cassady [40] to be 218 kcal/mol and the gas-phase acidity of benzene sulfonic acid of 300 kcal/mol [41]. The energy released by proton transfer is present in the collision complex formed between the protein and the acid, and can drive dissociation of the complex after proton transfer. Collisional cooling which removes this excess energy competes with dissociation of the complex; cooling rates of multiply-protonated proteins in the one mTorr of helium bath gas used in these experiments are expected to be on the order of 10^2 to 10^3 s^{-1} [42]. The results in Figure 5 indicate that collisional cooling competes favorably with dissociation of the complex, yielding primarily protein/acid complexes. There is no evidence in Figure 5 for fragmentation of the protein or the acid via cleavage of covalent bonds.

Multiply-charged anions/singly-charged cations (n−/1+). The dueling source arrangement also allows the *n*−/1+ experiment to be carried out with generation of the singly-charged cations via ESI. Previous work on this reaction type, which used internal EI and/or CI to generate the cations, was limited to gaseous or volatile reagents, e.g., rare gases such as xenon [43] or small organics such as pyridine [44]. This arrangement has a number of disadvantages. The presence of neutral reagents in the ion trap can lead to undesired ion/molecule reactions. More importantly, the use of EI/CI to generate cationic reactants limits the *m/z* range of the anions that can be studied, as the trapping RF amplitude level (or ion trap low mass cut-off, LMCO) must be kept low enough to store the low *m/z* cations, and so the potential well depths for higher *m/z* anions are not sufficiently deep to allow efficient trapping. Recent work in our laboratory has shown that these limitations may be overcome by allowing ion/ion reactions to occur during the period when the cations are injected from an external ASGDI source, and relying on the space charge potential of the cation cloud to store the high *m/z* anions until the RF level is raised [45]. This approach requires the ability to generate a relatively bright beam of singly-charged ions (i.e., on the order of a nano-amp). The dueling arrangement provides an alternative by allowing for the generation of higher *m/z* singly-charged cations with ESI. For example, Figure 6 shows the ion/ion reaction of multiply-deprotonated cytochrome *c* with protonated benzo(f)quinoline. Protonated benzo(f)quinoline (*m/z* 180 Da) can be efficiently injected into the trap at a LMCO of 30 to 40 Da, and ion/ion reactions can be carried out at LMCO up to

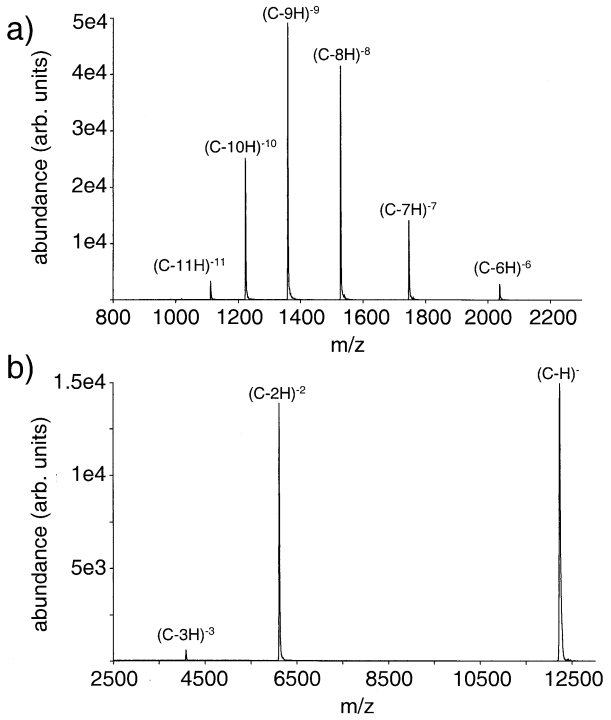

Figure 6. (**a**) Pre-ion/ion reaction mass spectrum of cytochrome *c* anions formed via nano-ESI from 2 % NH_4OH and injected into the ion trap for 30 ms. (**b**) Mass spectrum resulting from reaction of the ion population in (**a**) for 500 ms with protonated benzo-(f)quinoline injected for 270 ms.

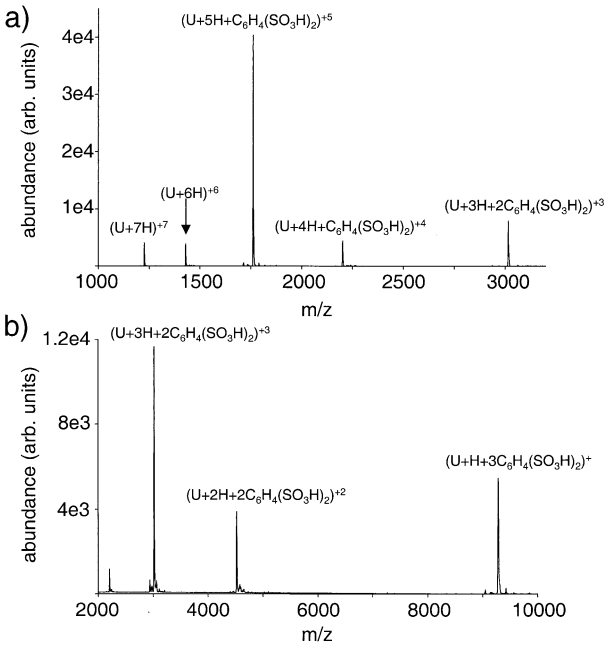

Figure 7. Mass spectra resulting from ion/ion reactions between isolated $(U + 7H)^{+7}$ ubiquitin and $[C_6H_4(SO_3)_2]^{-2}$ ions from benzene disulfonic acid. (**a**) Ubiquitin injection time = 30 ms, $[C_6H_4(SO_3)_2]^{-2}$ injection time = 150 ms, ion/ion reaction time = 200 ms. (**b**) Ubiquitin injection time = 100 ms, $[C_6H_4(SO_3)_2]^{-2}$ injection time = 250 ms, ion/ion reaction time = 600 ms.

approximately 170 Da, which is sufficiently high to store the singly-charged cytochrome *c* anions above *m/z* 12,000. Protonated benzo(f)quinoline appears to react with multiply-deprotonated proteins exclusively by proton transfer. We are currently investigating the dissociation behavior of multiply-deprotonated cytochrome *c* and other protein anions, using benzo-(f)quinoline as a charge reduction reagent to simplify interpretation of the product ion spectra. While the $n-/1+$ reaction combination is illustrated here with this relatively utilitarian reaction, the comments made in the $n+/1-$ section with regards to the possibility for novel new chemistries that can be studied with the dueling ESI instrument of course applies to the $n-/1+$ ion combination as well.

Multiply-charged cations/multiply-charged anions (n+/m−). Reactions of multiply-charged ions with multiply-charged ions of opposite polarity are of interest for a variety of reasons. For example, multiple simultaneous or near simultaneous charge recombinations within a given ion/ion collision complex may deposit sufficient energy into the complex to cause fragmentation of covalent bonds, perhaps through novel pathways not accessed by more conventional excitation methods. Also, the ability to reduce charge states in steps greater than one may allow for novel ion isolation and ion charge-state parking [46] experiments to be carried out on complex mixtures.

Continuing the example of interactions of multiply-protonated proteins with sulfonic acid containing species, Figure 7 shows the product ion spectrum resulting from reaction of $(U + 7H)^{+7}$ ubiquitin with doubly-deprotonated 1,3-benzene disulfonic acid $[(C_6H_4\{SO_3\}_2)^{-2}]$. As observed for benzene sulfonic acid, benzene disulfonic acid anions attach to the protein cations, and, as expected, the charge state of the protein/acid complex is reduced in steps of two. Interestingly, there is evidence that single proton transfer reactions also occur. This is probably due to dissociation of the collision complex after the first proton transfer because of the excess energy liberated by the charge recombination [47]. As discussed above, there is a competition between complex dissociation and removal of energy via collisional cooling. Note, however, that we cannot rule out the possibility that the single proton transfer product is the result of a side reaction with a singly-charged species introduced during the anion injection period. There are a number of singly-charged ions formed at relatively low abundance during ESI of benzene disulfonic acid, which are presumably fragments of the $[C_6H_4(SO_3)_2]^{-2}$ ion formed in the ESI interface. As noted above, a broadband isolation waveform applied during the anion accumulation period, which would only allow the $[C_6H_4(SO_3)_2]^{-2}$ ions to be accumulated in the ion trap, would remove this ambiguity.

Another $n+/m-$ reaction is illustrated in Figure 8, which shows the results of reacting +13 and −5 ions of

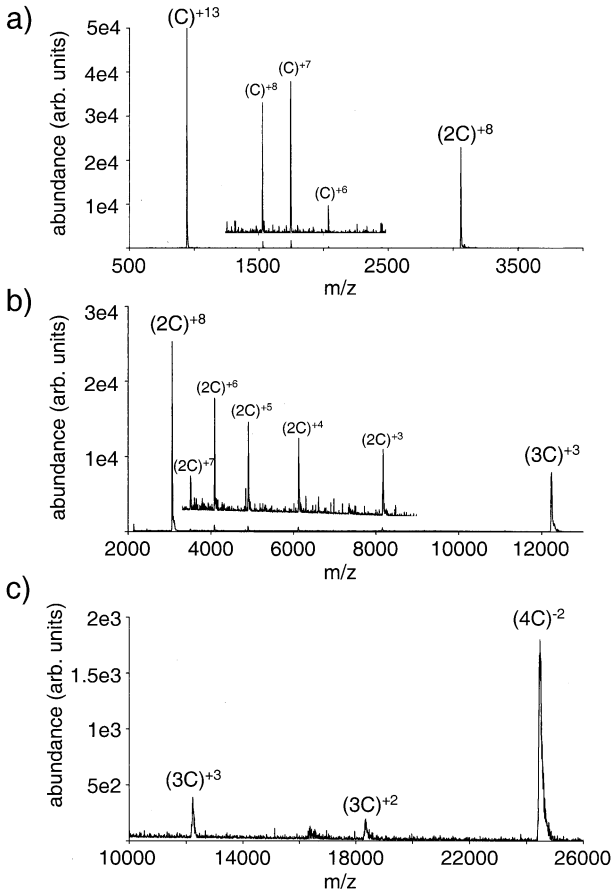

Figure 8. Mass spectra resulting from ion/ion reactions between isolated +13 cytochrome *c* and −5 cytochrome *c*. The insets in (**a**) and (**b**) are ×20 zooms over the portion of the spectra where proton transfer products were observed. (**a**) Cation injection time 100 ms, anion injection time = 100 ms, ion/ion reaction time = 100 ms. (**b**) Cation injection time = 100 ms, anion injection time = 100 ms, ion/ion reaction time = 300 ms. (**c**) Cation injection time = 100 ms, anion injection time = 250 ms, ion/ion reaction time = 600 ms. Note that at the conditions used to acquire the data shown in (**c**), the main product is the $3C^{+3}$ trimer, hence the observation of a small peak for this product, and for the proton-transfer product $3C^{+2}$, even though the detector is set to detect negative ions.

cytochrome *c*. During this experiment, the negative ions of cytochrome *c* were generated via nano-electrospray from 5 mM NH_4HCO_3, which results in a product ion distribution composed primarily of −5 ions (i.e., the distribution of negative cytochrome *c* ions is substantially different from that shown in Figure 6a). The major product of the reaction after 100 ms is a cytochrome *c* dimer with eight positive charges (Figure 8a). There is also some evidence for proton transfer reactions, which result from complex dissociation occurring after a given number of proton transfers but before collisional cooling can remove the reaction energy. This result is consistent with previous studies we have conducted on protein/protein reactions between cytochrome *c* and ubiquitin using the dueling ESI instrument [47]. Interestingly, even though each proton transfer is expected to be exothermic by approximately 100 kcal/mol, so that the complex can have on the order of 500 kcal/mol after the five proton transfers necessary to neutralize the cytochrome *c* anion, there is no evidence in Figure 8 for fragmentation of the proteins via cleavage of covalent bonds. Collisional cooling of the complex as well as dissociation of the complex into individual protein ions clearly occur at higher rates than covalent bond cleavage. If the reaction time is extended, formation of a cytochrome *c* trimer with three positive charges (Figure 8b) and even a tetramer with two negative charges (Figure 8c) can be observed. Further study of the gas-phase formation of protein/protein complexes is underway in our laboratory using the dueling ESI instrument.

Conclusions

The dueling ESI instrument described here allows for a wider variety of ion/ion chemistries to be accessed, with greater control of the reactant species and the reaction conditions than has previously been available. Interactions of multiply-charged protein and oligonucleotide ions with a wide variety of reactant ions is facilitated by the interface, which may lead to new analytical capabilities for biological ion identification and characterization. For example, studies of the dissociation behavior of negatively charged protein ions, with simplification of the resulting product ion spectra via charge state reduction, are now possible. Novel complexes can also be formed in the gas phase, between, for example, proteins and small molecules or between two macro-ions.

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