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Charge-state dependent dissociation of a trypsin/inhibitor complex via ion trap collisional activation

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Abstract

Ion trap collision-induced dissociation (CID) of a non-covalent complex formed between porcine trypsin and bovine pancreatic trypsin inhibitor (BPTI) has been studied for charge states +10 to +5. Fragmentation of the +10 and +9 complexes formed directly from solution shows separation of the two subunits as the predominant dissociation channel. Lower charge states of the complex were formed by ion/ion (or, in one case, ion/molecule) proton transfer reactions. The +8 complex shows a mixture of fragmentation behavior, including subunit separation and losses of small neutral and charged species. The neutral loss is also a dominant pathway for the +7 to +5 charge states and the loss of a small cation is also common to the +7 and +6 charge states. The identity of the small cation lost was investigated and is likely to be the b_2^+ ion from the BPTI subunit. This identification was supported by examination of fragmentation of various charge states of BPTI cations and a "fast" collisional activation experiment performed on the +7 complex. These results suggest that precursor ion charge state can play a dramatic role in the gas phase dissociation of protein–protein complexes such that covalent bond dissociation can come to dominate over subunit separation when Coulombic repulsion is decreased.
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Keywords: Trypsin/trypsin inhibitor complex; Tandem mass spectrometry; Gas-phase protein–protein complex; Gas-phase ion/ion proton transfer

1. Introduction

The development of electrospray ionization (ESI) [1] and matrix-assisted laser desorption ionization (MALDI) [2,3] has allowed the study of a variety of biomolecules in the gas phase. In particular, these two ionization techniques have been widely used in the field of protein mass spectrometry (MS) to identify and characterize proteins [4–10]. Significant effort continues to be expended in improving the tools of tandem mass spectrometry, in particular, for the structural characterization of proteins including those with post-translational modifications. The determination of protein function, another major objective in proteomics, is aided by the study of protein interactions, such as protein–protein interactions. The gentle nature of ESI has allowed the ionization of intact complexes, even those that are weakly bound [11–13]. Early studies showed that ESI-MS was capable of preserving both specific [14,15] and non-specific [16] non-covalent interactions. Since then, much effort has been expended using mass spectrometry to investigate the structures and binding strengths of protein complexes and to elucidate information about protein interactions with other proteins, DNA, RNA, ligands, and various cofactors. Some studies rely on mass spectrometry solely for the mass-to-charge measurements of species related to complexes of interest presumed to be formed in solution while others subject complexes to structural interrogation in the gas-phase.

The information sought about a protein–protein complex can range from the stoichiometry of the complex and the identities of its subunits to binding energies and three-dimensional structure. Gas-phase studies are clearly useful for the determination of complex stoichiometry and subunit identification. However, the usually unknown relationship between gas-phase binding and three-dimensional structure, relative to the corresponding condensed-phase characteristics, requires caution in interpretation of the gas-phase data when it is the condensed phase structure that is ultimately of interest. In any case, it is desirable to maximize the information obtainable from a gas-phase protein–protein complex via tandem mass spectrometry.

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In this regard, it is of interest both to explore the behavior of gaseous protein complexes under a wide range of conditions and to develop technologies that facilitate the collection of data from conditions that prove to be particularly informative.

To date, most studies of non-covalent complexes have been performed using time-of-flight (TOF) mass spectrometers [17]. The essentially unlimited mass-to-charge (*m*/*z*) range provided by TOF instruments is conducive to the analysis of the relatively high *m*/*z* ions formed from non-covalent complexes. Other attractive attributes of TOF instruments include high speed and sensitivity, and the potential for relatively high resolving power. Quadrupole TOF (Q-TOF) instruments, which have tandem mass spectrometry (MS/MS) capabilities, have also been used. Standard commercial Q-TOF instruments are not optimized for work with non-covalent complexes, as the mass filter (*Q*) generally has an upper *m*/*z* limit of 3000–4000 for isolation, while many non-covalent complexes are formed with *m*/*z* values in excess of this limit. In addition, the pressures in the source region used for collisionally cooling and focusing the ion beam often require significant elevation to adequately focus the larger non-covalent complex ions [18]. Instrument modifications to overcome these problems have been reported [18,19]. Studies of non-covalent complexes with other forms of mass analyzers, such as sector instruments [20], and Fourier transform ion cyclotron resonance (FT-ICR) [21] instruments have also been reported. FT-ICR mass spectrometers have the advantage of trapping ions in the ICR cell, thereby allowing for the execution of sophisticated MS^n experiments involving a number of means for probing the ions, such as collisional activation, blackbody radiation, etc. However, the significant advantage in mass resolution that FT-ICR mass spectrometry typically enjoys over other forms of mass analysis is adversely affected by the fact that mass resolution is inversely related to *m*/*z* and that many non-covalent complex ions formed via ESI are observed at relatively high *m*/*z*.

To date, quadrupole ion trap (QIT) mass spectrometers have not been routinely used for the analysis of non-covalent complexes. Although the *m*/*z* range of a QIT instrument can be extended [22] (one commercial ion trap system has been shown to be capable of mass analysis up to roughly *m*/*z* 150,000 [23]), most commercial instruments support an upper limit to *m*/*z* of 2000–4000. In addition, the modest mass resolving power of QIT mass spectrometers can limit the information that can be obtained from the analysis of high *m*/*z* ions. Wang et al. have reported the use of a QIT instrument, with the *m*/*z* range extended, to detect multimeric non-covalent assemblies of bovine serum albumin (BSA) and alcohol dehydrogenase (ADH). The largest assembly that they reported was an ADH octamer with a molecular weight of 290 kDa [24]. Heck and van den Heuvel have reported the study of vanillyl-alcohol oxidase (monomer ∼65 kDa) using an LCQ-Deca [13]. By extending the *m*/*z* range, they were able to observe both the multiply charged dimeric (*m*/*z* 6000) and octameric (*m*/*z* 11,000) assemblies.

Despite their mass analysis limitations, electrodynamic ion traps can be useful in the study of non-covalent complexes due to their capabilities for sophisticated MS^n experiments. Ion storage capabilities allow for a variety of reactions to be studied between stages of ion isolation and mass analysis. Ion trap collisional activation, a slow heating method [25], is the most common means for inducing unimolecular dissociation. Charge state manipulation within the context of an MS^n experiment is uniquely effective in electrodynamic ion traps via ion/ion reactions [26]. The first example of ion/ion proton transfer reactions involving a non-covalently bound complex was a study of multiply protonated holomyoglobin (a prosthetic group/protein non-covalent complex) [27]. It was noted that such ions subjected to multiple sequential ion/ion proton transfer reactions showed no evidence for heme loss, the most facile fragmentation channel, despite the highly exothermic nature of ion/ion proton transfer. Chrisman et al. have subsequently described the charge state dependent fragmentation behavior of holomyoglobin ions under ion trap collisional activation conditions [28]. In this case, ion/ion proton transfer reactions were used to form charge states of the complex that were lower than those formed directly via ESI. From the dissociation of the various charge states of holomyoglobin, loss of heme dominated in all cases but the extent to which it was lost as an ion or as a neutral species depended strongly on the precursor ion charge state. Studies of protein ions (covalent bonds) have shown that complementary information can be gained from dissociating various charge states of a protein ion, as the charge state plays a major role in determining which of the possible competing cleavages dominate. The results with holomyoglobin suggested that, at least under ion trap collisional activation conditions, charge state dependent fragmentation of protein complexes might also be commonplace [29–32].

In this study, the ion trap collision induced dissociation behavior of a model protein–protein (enzyme–inhibitor) complex formed between porcine trypsin and bovine pancreatic trypsin inhibitor (BPTI) is examined. Early reports from Mar et al. employed ESI-MS to detect the intact complex between trypsin and inhibitor [33]. Kraunsoe et al. have reported the use of ESI-MS in conjunction with nozzle-skimmer dissociation to investigate the binding strength of trypsin with various trypsin inhibitors [34]. Nesatyy has reported the use of ESI-MS/MS with beam-type collision-induced dissociation (CID) to determine the binding properties of complexes between trypsin and various inhibitors [35,36]. Here, the results from a systematic study of the charge state dependent fragmentation behavior of the complex are presented. Emphasis is placed on the information obtained via ion trap collisional activation while ion/ion proton transfer reactions are used to manipulate parent ion charge states, and, where necessary, product ion charge states.

2. Experimental

Bovine pancreatic trypsin inhibitor (BPTI) was purchased from Roche (Indianapolis, IN). Porcine trypsin, perfluoro-1,3-dimethylcyclohexane (PDCH), and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, MO). All samples were used without further purification. To make the BPTI/trypsin complex, a solution composed of the following was prepared and allowed to sit for 1 h at room temperature: BPTI (60 μM), porcine trypsin (20 μM), and ammonium acetate (2 mM). Samples were ionized directly from this solution using

wire-in-capillary nano-electrospray [37,38]. Nanospray emitters were pulled from borosilicate glass capillaries (1.5 mm o.d., 0.86 mm i.d.) using a P-87 Flaming/Brown type micropipet puller (Sutter Instruments, Novato, CA). Approximately 1.2 kV was applied to a stainless steel wire inserted into the nanospray emitter to induce ionization.

All experiments were performed on a Hitachi M-8000 quadrupole ion trap mass spectrometer adapted to allow for ion/ion reaction capabilities [23]. Ion/ion proton transfer reactions with anions generated by atmospheric sampling glow discharge ionization (ASGDI) of PDCH anions were used to reduce the charge states of precursor and/or product ions as described by Stephenson and McLuckey [39]. Typical ion accumulation time for the complex was 1000 ms during which $[C+10H]^{10+}$ and $[C+9H]^{9+}$, where C is the complex, were formed. $[C+10H]^{10+}$ and $[C+9H]^{9+}$ ions were isolated directly from solution using resonance ejection ramps to eject ions of *m/z* higher and lower than the ions of interest [40]. $[C+8H]^{8+}$, $[C+7H]^{7+}$, $[C+6H]^{6+}$, and $[C+5H]^{5+}$ ions were formed by subjecting the $[C+10H]^{10+}$ and $[C+9H]^{9+}$ ions to proton transfer reactions with PDCH anions injected through the ring electrode. "Ion parking" [41] was used to concentrate ions into a specific charge state and, once again, RF ramps were used to isolate the ions [40]. A single frequency resonance excitation voltage produced by an external waveform generator (Model 33120A, Agilent, Palo Alto, CA) was applied to the end-caps for 300 ms to achieve collision-induced dissociation (with ∼1 mTorr helium as the target gas). In selected experiments, a technique known alternately as pulsed Q dissociation (PQD) [42] or high amplitude short time excitation (HASTE) [43], was used to induce dissociation of the complex. The acronym PQD is used herein for the sake of brevity. PQD is similar to a previously reported technique, "fast excitation" CID [44], in that it applies a high amplitude resonance excitation voltage for a short period of time, but it also incorporates changes in the rf level during the activation. In PQD, the rf level of the ion trap is raised to a high level (a high *q*) for a short time period (∼2 ms in this case) and a high amplitude resonant activation is applied to deposit energy into the target ions. The activation is then discontinued and the rf is reduced to a lower level (or a low *q*) for some defined period of time. Product ions formed from activated ions that fragment after reduction of the rf level are collected during this time period. This technique allows for the detection of product ions of lower *m/z* ratio than are usually accessible with the conventional ion trap collisional activation experiment. Product ion spectra were acquired via resonance ejection [22] of the ions. In some cases, further isolation and/or proton transfer ion/ion reactions were performed to determine the charge state of an ion. The mass spectra shown are an average of approximately 300 scans.

3. Results and discussion

3.1. Charge state dependent fragmentation of the trypsin/inhibitor complex

ESI of non-covalently bound protein complexes typically gives rise to species with fewer charge states than is often observed with proteins, particularly when the latter are formed from denaturing solution conditions. Nevertheless, several abundant charge states are often noted and it is important to determine the role of parent ion charge in the fragmentation behavior of the protein complex. Jurchen and Williams have reported results for the effect of charge state on the dissociation of several non-specific protein homodimers [45,46]. They have shown that charge state plays a role in how the homodimers dissociate, with low charge states favoring a more asymmetric dissociation and high charge states favoring a more symmetric dissociation. Asymmetric dissociation is a phenomenon that has been previously reported for dissociation of non-covalent protein complexes, where one subunit of a multimeric complex dissociates taking a disproportionately large amount of the total charge on the complex [36,47–58]. For all charge states reported, however, separation of the subunits was the exclusive process. That is, no dissociation of covalent bonds was noted. Under the experimental conditions used here, $[C+10]^{10+}$ is the highest charge state of the trypsin/inhibitor complex formed directly from nano-ESI and fragmentation of this charge state via ion trap CID is shown in Fig. 1A. The fragmentation of the complex shows dissociation into the individual BPTI (B) and trypsin (T) components: B^{4+}/T^{6+} and B^{3+}/T^{7+}. Dissociation of a complex into monomeric subunits is typical behavior for multiply charged non-covalent complexes as the strength of the non-covalent interaction would be predicted to be weaker than the covalent bonds. Product ions with what is believed to be a phosphoric or sulfuric acid adduct were also observed, particularly on the BPTI fragments. When a BPTI-only solution is subjected to MS, phosphoric acid adducts are commonly observed. The resolution with which precursor ions can be isolated with the instrument used in this study does not allow for the efficient ejection of $[C+10H+H_3PO_4]^{10+}$ ions that may be adjacent to the $[C+10H]^{10+}$ peak, so it is likely these acid adduct products arise from some heterogeneity in the initial ion population. The most abundant products in the dissociation of $[C+10H]^{10+}$ are the B^{3+}/T^{7+} pair with a much smaller contribution from the B^{4+}/T^{6+} complementary pair. The trypsin

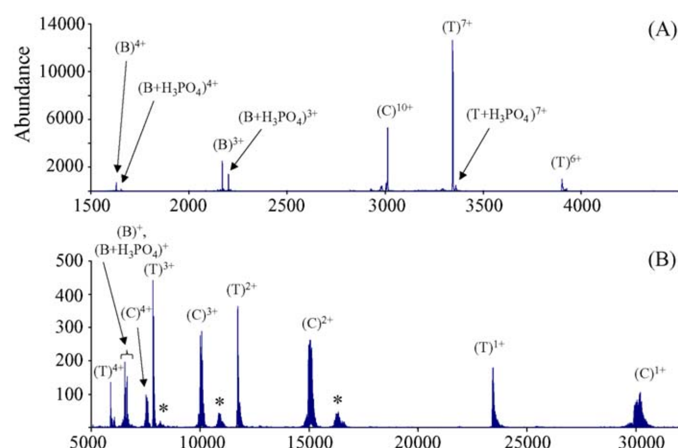

Fig. 1. (A) MS/MS product ion spectrum of complex $[C+10H]^{10+}$ and (B) Post ion/ion MS/MS product ion spectrum of complex $[C+10H]^{10+}$. C = complex, B = BPTI, T = trypsin. (*) indicates peaks from ions with *m/z* ratios initially similar to $[M+10H]^{10+}$, but different mass and charge following proton transfer ion/ion reactions.

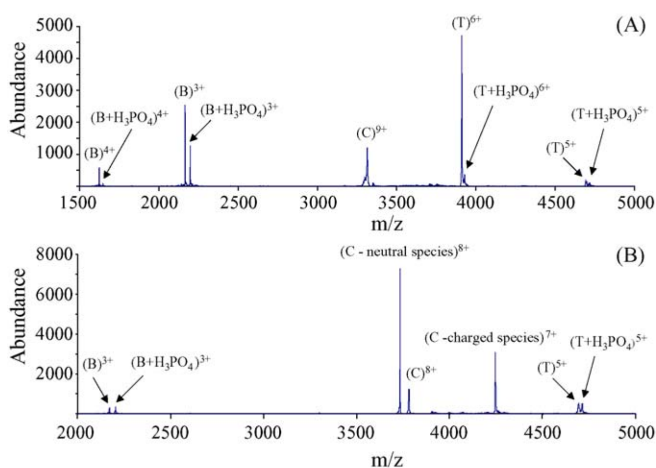

Fig. 2. (A) MS/MS product ion spectrum of complex $[C + 9H]^{9+}$ and (B) MS/MS product ion spectrum of complex $[C + 8H]^{8+}$. C = complex, B = BPTI, T = trypsin.

product carries more charge than the BPTI product, which is consistent with the expectation that the larger subunit (23.5 kDa for porcine trypsin versus 6.5 kDa for BPTI) will carry more charge. However, on a per mass basis, the BPTI fragment is more highly charged. The complex has 10 arginine residues with 6 residing in BPTI and only 4 in porcine trypsin. Hence, on the basis of the numbers of highly basic arginine residues, it is not surprising that BPTI carries proportionately more charge when the total charge of the complex is relatively low. Fig. 1B shows the product ion spectrum obtained after the ions of Fig. 1A were subjected to ion/ion proton transfer reactions with anions derived from PDCH and the mass range was extended to about *m/z* 32,000. From the widths of the peaks from the parent ion population, which reflect the width of the precursor ion isolation window, it is clear that adduct species can be present. The peaks indicated with asterisks arise from species of different mass and charge than the $[C + 10]^{10+}$ ions, but similar *m/z* ratios, such that they are also present in the initial precursor ion population. Some of the products of very low abundance in the product ion spectra may arise from collisional activation of these species.

The $[C + 9H]^{9+}$ complex ions fragment similarly to the $[C + 10H]^{10+}$ complex (Fig. 2A), where the complex breaks into monomeric subunits, with the two major charge partitioning channels represented by the B^{4+}/T^{5+} and B^{3+}/T^{6+} complementary pairs. Notably, in the two fragmentation channels for the $[C + 9H]^{9+}$ complex, the charges retained by the BPTI fragments are the same as in the dissociation of the $[C + 10H]^{10+}$ complex. The reduction in parent ion charge is therefore largely reflected in the reduction of the trypsin product, which is consistent either with a lower net charge on trypsin in the complex or with dissociation dynamics that favor higher relative charge in the BPTI product. As with the dissociation of the +10 complex, the fragmentation channel in which BPTI has a +3 charge is the major fragmentation channel. The fragmentation of the $[C + 8H]^{8+}$ complex (see Fig. 2B), which was formed via ion/ion proton transfer from the $[C + 10H]^{10+}$ and $[C + 9H]^{9+}$ ions, shows a dramatic change in fragmentation behavior. A small extent of cleavage into the BPTI and trypsin components is observed, as reflected by the appearance of the B^{3+}, $(B + H_3PO_4)^{3+}$, T^{5+} and $(T + H_3PO_4)^{5+}$ ions. This result again reflects the tendency for BPTI to more effectively retain charge as the precursor ion charge state decreases. Two fragmentation channels not present in the more highly charged precursor ions dominate the fragmentation of the $[C + 8H]^{8+}$ ion. These correspond to the losses of a small neutral species and a small charged species, respectively. Ion/ion proton transfer reactions were performed (data not shown) on the isolated fragment ions from these reactions to confirm their charge states. The measured *m/z* of the neutral loss fragment ion varied by several Th in measurements conducted over several months, which, when converted to Da for the +8 charge state, gave a range for the neutral species of 76–134 Da. This rather large range probably results from variations in the relative contributions of the adducts within the precursor ion population from one experiment to the next (e.g., a different solution was associated with each replicate). Differences in the relative contributions of the adduct species results in differences in the locations of the centroids of the peaks thereby resulting in the uncertainty in the neutral mass lost in the process. It is not possible to positively identify the neutral loss species due to this uncertainty. However, the loss of phosphoric or sulfuric acid is a strong possibility.

The activation of $[C + 8H]^{8+}$ also gives rise to the loss of a relatively small charged species from the parent, (C—charged species)$^{7+}$. Based on multiple measurements made over months, the mass of the ion lost from the precursor ion falls within the range of 220–348 Da, indicating that it is not a charged version of the species that is lost as a neutral. It is not likely that a non-covalently bound adduct species could account for this loss, as the fragment is relatively large. As discussed further below, this channel arises from cleavage of a covalent bond. Covalent bond cleavage from the fragmentation of a non-covalent complex has been reported for a variety of complexes in the literature [59–63], but it has not been a common observation for non-covalently bound protein–protein complexes.

No evidence for separation of the subunits was present in the ion trap CID data of the +7, +6, and +5 charge states of the trypsin/inhibitor complex. Fragmentation of the +7 and +6 charge states of trypsin/inhibitor complex show exclusively the losses of the small neutral and charged species. For the +7 complex, based on mass measurements of the product formed by loss of an ion, the charged species appeared to range in mass from 234 to 327 Da, and for the +6 complex it appeared to range from 250 to 284 Da. Activation of the +5 charge state shows only the neutral loss. A summary of the fragmentation of all the charge states of the complex studied is shown in Table 1. Subunit separation, which is the most commonly noted phenomenon resulting from collisional activation of protein–protein complexes, is clearly a charge-state dependent process, which is presumably facilitated by electrostatic effects (i.e., Coulombic repulsion associated with subunits of like charge). As the total charge decreases, other channels can become competitive. Both the emergence and dominance of other channels was noted for charge states that were not formed directly via ESI. Rather, they were formed from ESI-derived ions via ion/ion proton transfer

Table 1
Summary of charge state dependent fragmentation of trypsin/inhibitor complex

Charge state (n)	B4/T($n-4$) (%)	B3/T($n-3$) (%)	Neutral loss (%)	Charged loss (%)
+10	10	90	0	0
+9	10	90	0	0
+8	0	14	61	26
+7	0	0	86	14
+6	0	0	95	5
+5	0	0	100	0

reactions. In every case where ion trap CID of a charge state of a protein formed directly from ESI has been compared with that of the same charge state formed via ion/ion proton transfer, the results were essentially indistinguishable. While a similar comparison cannot be made in this case, the previous comparisons just mentioned provide no basis on which to suspect that the use of ion/ion proton transfer is the reason for the change in observed dissociation mechanism for the BPTI/trypsin complexes. Furthermore, an experiment employing pyridine vapors in the spray plume [64] was successful in forming the $[C + 8H]^{8+}$ ion and the subsequent CID of this species also showed the losses of the small neutral and charged species to dominate the product ion spectrum. Hence, the method by which the charge state is reduced appears not to be key to the observation of this behavior. It appears that, at least for the +10 through +8 charge states of the complex, the BPTI subunit shows a strong tendency to retain three charges upon separation from the trypsin subunit, while the charge carried by the trypsin is reduced as the charge state of the complex is reduced. Little can be said from these data alone about the charges of the subunits in the complexes that show no subunit separation from these data alone. However, at least some information might be apparent from studies designed to provide more specific information regarding the identity of the charged species lost from the +8 and +6 complexes (see below).

3.2. Small ionic fragment loss via covalent bond dissociation in a non-covalent complex

The loss of a relatively small charged species from a 30 kDa protein complex at relatively low charge states is an unusual observation. As a rule, charge separation processes tend to decrease relative to neutral loss processes from large multiply protonated species as the charge state decreases. Indeed, as the charge state decreases from +8 to +5, the relative contribution of the charge loss process decreases. However, in this case, the major neutral loss process and the major charge loss process are not simply the same cleavage in which there is a competition for a proton. That is, the species lost are not simply protonated and unprotonated forms of the same fragment. The two processes occur via cleavages of different bonds. The process giving rise to loss of the small ionic fragment is of particular interest because it cannot be readily accounted for by loss of an adduct. Attention was therefore focused on identifying the likely origin of these charge separation products.

Cleavage of the complex involving charge separation creates at least two charged fragments, both of which are presumably amenable to mass analysis. While the masses of both fragments can, in principle, be determined from the measurement of the high *m/z* ion of the complementary pair, the uncertainty in the mass measurement in the high *m/z* region leads to a relatively high degree of uncertainty in determination of the *m/z* of the low mass species, as reflected by the ranges of mass values for the small fragment mentioned above (i.e., for +8, 220–348 Da, for +7, 234–327 Da, and for +6, 250–284 Da). A direct measurement of the low *m/z* fragment would yield a much more precise value for its mass. However, under conventional ion trap collisional activation conditions, it was not possible to effect dissociation of the precursor ion at a sufficiently low value of the rf amplitude applied to the ring electrode to trap the low *m/z* fragment. At rf amplitudes sufficiently low to store the low *m/z* fragment ion, the trapping well depths for the precursor ion were too low to avoid ejection of the precursor ion by the resonance excitation voltage. This well-known practical limitation of conventional ion trap CID [65] gives rise to a lower *m/z* limit for the collection of product ions and, in this case, prevented observation of the singly charged fragment ion with conventional CID.

An alternative implementation of ion trap CID, referred to as pulsed Q dissociation (PQD) or high amplitude short time excitation (HASTE) [42,43], was used in an effort to obtain a more precise measurement of the low mass charged species lost from the +8 to +6 charge states of the complex. In PQD, the precursor ion is activated for a relatively short period of time with a high resonance excitation voltage using a fairly high rf amplitude applied to the ring electrode. In this case, activation was performed for 2 ms using an rf voltage that yielded a low *m/z* cut-off of 1995 and a resonance excitation voltage of frequency = 116.5 kHz and amplitude = 8.79 V. Then the resonance excitation voltage was turned off, the rf level was reduced to the point where ions of *m/z* ratio as low as 200 could be stored, and the ions were allowed some time to fragment. Mass analysis followed. Product ions below *m/z* 1995 observed in the subsequent mass analysis scan arise from the fraction of the parent ion population that fragments after the rf amplitude was reduced. The PQD process for these ions is distinct from conventional ion trap CID, in which ion excitation and fragmentation occur in parallel. In this sense, the PQD experiment has analogies with a beam-type CID experiment described for a hybrid triple quadrupole/linear ion trap instrument, whereby ions are activated relatively quickly by beam-type collisional activation but products formed only after a time delay are collected and subjected to mass analysis [66]. The results of the conventional ion trap CID and PQD experiments for the $[M + 7H]^{7+}$ ion are summarized in Fig. 3. Fig. 3A shows the conventional ion trap CID spectrum of the complex obtained under conditions in which the parent ion was essentially completely dissociated. The major products arose from the losses of a small neutral fragment and a small ionic fragment, the charge states of which were confirmed with ion/ion proton transfer reactions, with essentially no evidence for separation of the subunits.

The PQD results from the +7 complex are shown in Fig. 3B and C. Fig. 3B shows the product ion spectrum obtained over the

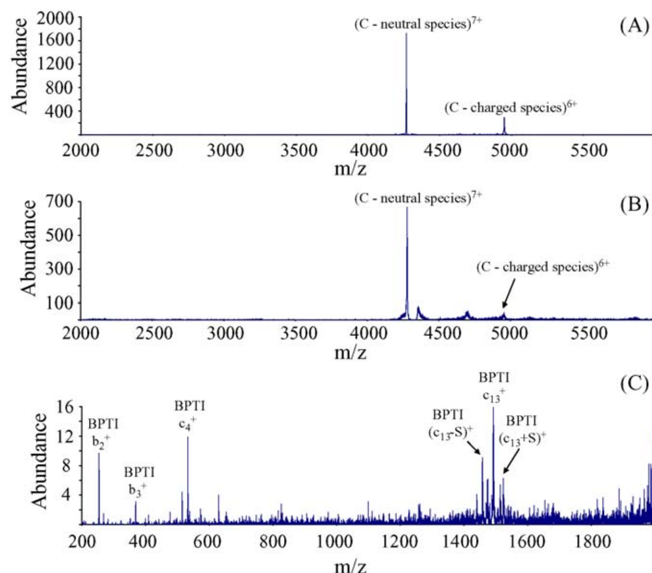

Fig. 3. (A) MS/MS product ion spectrum of complex $[C+7H]^{7+}$ using conventional ion trap CID; (B) high *m/z* range of PQD MS/MS product ion spectrum of complex $[C+7H]^{7+}$; (C) low *m/z* range of PQD MS/MS product ion spectrum of complex $[C+7H]^{7+}$.

same *m/z* range as that used for the traditional CID experiment. It is clear that the different activation conditions give rise to differences in the spectra. The PQD data shows more extensive fragmentation, as reflected by the appearance of the signal along much of the baseline. However, the (complex-charged species)$^{6+}$ peak is still observed, indicating the possibility for capture and analysis of its low *m/z* complementary ion. Fig. 3C shows the lower *m/z* region of the PQD experiment. This spectrum shows a fragment ion of *m/z* 254, which is the only low *m/z* fragment that falls within ranges established from the high mass regions of the +8 and +6 complex spectra obtained with conventional ion trap CID. The most abundant products that appear in Fig. 3C are assigned as products from the BPTI subunit, which were made based on experiments described below.

The PQD experiment provides a more precise measure of the *m/z* ratio of the cation that is likely lost from the +8 to +6 complexes than can be obtained from the high *m/z* complementary product. The ion trap CID behavior of the individual subunits was examined for possible insights into the identity of the fragment. Native porcine trypsin showed only losses of small neutral molecules (e.g., H_2O and NH_3) at low charge states. Previous studies of the charge state dependent fragmentation of various proteins under conventional ion trap collisional activation conditions have shown that, as charge state is decreased, the losses of small neutral molecules dominate the spectra [31,32]. Given that small neutral losses dominate the spectra of native porcine trypsin at charge states as high as $[T+10H]^{10+}$ (data not shown), it is unlikely that either the major neutral loss (which is significantly higher in mass that the losses of one or two molecules of ammonia or water) or small ion loss observed with the +8 and +6 complexes arise from the trypsin subunit. Furthermore, as discussed below, no loss of a neutral species within the range of values determined from the high *m/z* region of the product ion spectra of the complex was noted in the charge state dependent dissociation behavior of BPTI (see below). On the other hand, in all cases in which subunit separation was noted, at least some of the products showed an adduct consistent with H_3PO_4 (or H_2SO_4). Hence, the loss of this adduct as a neutral, the mass of which falls within the window determined from the high *m/z* region of the spectrum, is the most likely origin of the abundant neutral loss peak in the spectra of the +8 and +5 ions.

Table 2
Summary of charge state dependent fragmentation of bovine pancreatic trypsin inhibitor

Charge state	y_{56} (%)	y_{55} (%)	z_{54} (%)	z_{45} (%)	c_{37} (%)	Neutral loss (%)
+7	37	17	0	0	0	47
+6	35	12	0	2	2	49
+5	16	5	3	6	10	60
+4	13	4	9	5	9	60
+3	19	2	7	7	4	61
+2	2	0	0	0	0	98

The relative contributions for each fragmentation channel were calculated from post ion/ion MS/MS spectra.

BPTI product ion spectra derived from conventional ion trap CID were obtained for the precursor ion charge state range of +7 through +2. The charge state dependent fragmentation of BPTI is summarized in Table 2 for the major observed dissociation channels while Fig. 4 shows the data for the $[B+3H]^{3+}$ ion, for illustration. There are several noteworthy aspects about the dissociation behavior of BPTI ions. For the +5, +4, and +3 charge states, c- and z-type ions are observed N-terminal to cysteine residues. Cleavage of disulfide bonds to give c- and z-type ions N-terminal to the cysteine residues involved in the bond is the primary dissociation mechanism observed for disulfide linked protein anions [67]. It has also been previously reported as a result of the fragmentation of insulin and elastase cations [29,68]. For positive protein ions, cleavage of disulfide bonds was previously observed only with the lowest charge states studied. The appearance of such ions at low charge state was interpreted on the basis of limited proton mobility,

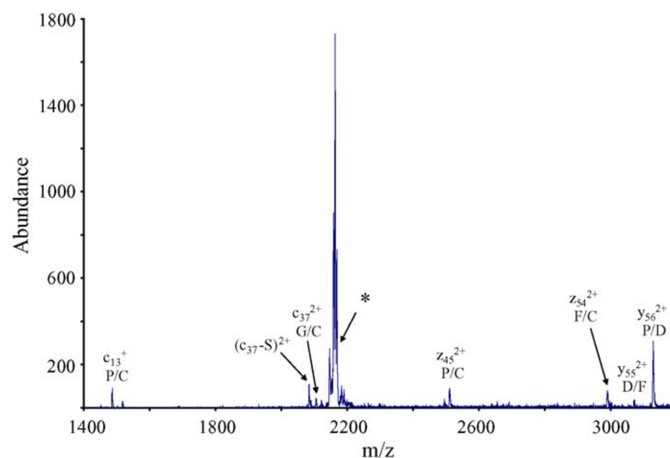

Fig. 4. MS/MS product ion spectrum of BPTI $[B+3H]^{3+}$. The asterisk indicates the position of the precursor ion prior to activation.

which allowed for non-charge catalyzed cleavages to compete. The BPTI results are also consistent with this interpretation considering the relatively high number of arginine residues in the protein. Two prominent amide bond cleavages are also observed in BPTI, both located outside the disulfide loop. In all cases, the more prominent process of the two is cleavage of the Pro^2–Asp^3 bond to yield the b_2/y_{56} complementary pair. Evidence for this cleavage is apparent for each charge state examined, although its relative abundance in the +2 charge state is much smaller than for the higher charge states. The b_2^+ ion (*m/z* 256) was not observed directly, due to the low *m/z* storage limitation associated with conventional ion trap CID, but its formation can be inferred from the observation of the complementary y_{56} ion with a net charge one unit lower than that of the parent ion. No evidence for loss of a neutral b_2 fragment was noted. The second amide bond cleavage is the Asp^3–Phe^4 channel, resulting in b_3 and y_{55} product ions. This cleavage is present in all charge studies studied, with the exception of +2, and its contribution is always less than that of the Pro^2–Asp^3 channel. Like the b_2/y_{56} cleavage, the charges on the product ions are always apportioned so that the b_3 carries one charge and the y_{55} product carries the remainder. The observation of these two channels is not entirely surprising, as cleavage C-terminal to aspartic acid residues is known to be a favored fragmentation channel [31], particularly at low charge states, and cleavage to yield a b_2 ion is often a prominent process in peptide fragmentation. The loss of a b_2^+ fragment has also been noted to be a dominant process for some charge states of apomyoglobin under ion trap CID conditions and its appearance was taken as evidence for protonation at the N-terminus [32]. Arginine is the N-terminal residue of BPTI, which favors protonation at that site. Hence, the simplest explanation for the origin of the loss of the relatively small cation from those complexes that show this channel is that it arises from cleavage of the BPTI subunit, which happens to be N-terminally protonated, to the b_2^+ and its complement composed of trypsin bound to the y_{56} fragment of BPTI.

The ion trap CID spectrum of the $[B + 3H]^{3+}$ ion was selected for illustration because the charge state dependent fragmentation behavior of the trypsin/inhibitor complex suggests that the BPTI component of the complex that loses a small cation probably has fewer than four charges (see Fig. 2B). Furthermore, the greater relative abundance observed for the b2/y56 channel from $[B + 3H]^{3+}$ than from $[B + 2H]^{2+}$ might also suggest that the BPTI subunit in the complexes that yield a b_2^+ fragment carries three charges. The decrease in the relative contribution from b_2^+ loss as the charge state decreases from +8 to +6, and the absence of the charged loss in the +5 complex data, is consistent with a reduction in the total charge of the BPTI subunit or, at least, a reduced extent of N-terminal BPTI protonation. Finally, it is also interesting to note that the prominent ions in the PQD spectrum of the +7 complex are readily assigned on the basis of the fragmentation of the $[B + 3H]^{3+}$ ion.

Given that covalent bond cleavage has not been commonly reported upon CID of protein–protein complexes, the generality of the observations made here is unknown. However, it might be noteworthy that examination of the CID behavior of the subunits was able to provide as much support in the assignment of the charged loss channel as did that of the BPTI data and trypsin data (especially the former). It could easily be imagined that the dissociation behavior of the subunits within a complex would be impacted by the interactions with the other subunit(s). When the crystal structure of the trypsin/BPTI complex is examined (see Fig. 5 for a ribbon diagram of the crystal structure), it can be seen that the N-terminus of the BPTI portion is well away from the area in which the BPTI and trypsin interact. While little can be said about the structures of the gas-phase ions interrogated here, other than they likely were initially present in solution in an arrangement more or less like that in Fig. 5, the fragmentation of the complex is not consistent with protection of the N-terminal end of BPTI via interaction with trypsin. In other words, the fact that the N-terminus of PBTI is uninvolved with trypsin in the native complex is consistent with the dissociation behavior noted here.

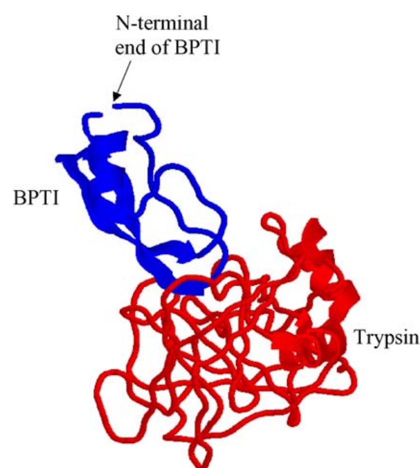

Fig. 5. BPTI/trypsin complex from the Protein Data Bank file 1TPA.

4. Conclusions

Ion trap CID of the charge states of the porcine trypsin/BPTI complex formed directly via ESI (i.e., +10 and +9) show essentially exclusive separation of the subunits. Upon reduction of charge by ion/ion proton transfer reactions (as well as by ion/molecule proton transfer in one case) subunit separation decreases dramatically in the +8 charge state and disappears for charge states +7 and below. The loss of either phosphoric or sulfuric acid from complexes within the isolated parent ion population becomes a prominent process for ions of all charge states below +9. A much more interesting observation, however, is the appearance of a dissociation channel in the +8 through +6 charge states that involves the loss of a small cation. The evidence suggests that this fragment is the b_2^+ ion from the BPTI subunit and that, in those complexes that fragment in this manner, BPTI is N-terminally protonated. This channel is no longer apparent upon dissociation of the +5 charge state of the complex. Most of the fragments noted from low *m/z* data collected via pulsed Q dissociation were also observed as major products from dissociation of isolated BPTI in the +3 charge state.

These results show that processes other than subunit separation can become dominant in the CID of protein-protein complexes, although from this work it is not possible to determine if such behavior is common for all protein–protein complexes, common only for already relatively strongly bound protein–protein complexes, as might be the case for many specific complexes formed originally in solution, or relatively uncommon. It is known that subunit separation in non-covalently bound complexes becomes less facile as charge state decreases due to decreasing electrostatic repulsion. The increasing stability of the complex with decreasing charge states presumably allows other dissociation channels to make significant contributions. The fact that the loss of the small charged fragment was observed only from species that were reduced in charge prior to activation warrants the study of other complexes for charge state dependent fragmentation at charge states lower than those ordinarily provided via ESI. This work also illustrates the utility of the pulsed Q dissociation approach applied to entities as large as a protein–protein complex. In this case, it was able to provide a much more precise determination of the mass of the small cation lost from the complex than was available from the data collected under conventional ion trap CID conditions.

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