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**Dissociation of Large Gaseous Serine Clusters Produces Abundant Protonated Serine
Octamer**

Running Title: Dissociation of Large Gaseous Serine Clusters

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Abstract

Protonated serine octamer is especially abundant in spray ionization mass spectra of serine solutions under a wide range of conditions. Although serine octamer exists in low abundance in solution, abundant clusters, including octamer, can be formed by aggregation inside evaporating electrospray droplets. A minimum cluster size of 8 and 21 serine molecules was observed for doubly protonated and triply protonated clusters, respectively, formed by electrospray ionization of a 10 mM serine solution. Dissociation of these clusters results in charge separation to produce predominantly protonated serine dimer and some trimer and the complimentary charged ion. Dissociation of clusters significantly larger than the minimum cluster size occurs by sequential loss of serine molecules. Dissociation of all large clusters investigated leads to protonated octamer as the second most abundant cluster (protonated dimer is most abundant) at optimized collision energies. All larger clusters dissociate through a combination of charge separation and neutral serine loss to form small doubly protonated clusters, and the vast majority of protonated octamer is produced by dissociation of the doubly protonated decamer by charge separation. Protonated octamer abundance is optimized at a uniform energy per degrees of freedom for all clusters indicating that simultaneous dissociation of all large clusters will lead to abundant protonated octamer at an optimum ion temperature. These results provide evidence for another route to formation of abundant protonated octamer in spray ionization or other methods that promote formation and subsequent dissociation of large clusters.

Introduction

Protonated serine octamer has generated significant interest since the discovery of its unusually large abundance in electrospray ionization (ESI) mass spectra.¹⁻³ It has an unusually high homochiral preference when produced from solution using spray ionization methods¹⁻⁷ and even when the cluster is sublimed directly from heated solid serine.^{8,9} The high homochiral preference has led to the suggestion that this complex may have played a role in homochirogenesis.^{1,3,5,8-15} The octamer is also formed as a neutral structure from spraying solutions and it can also be assembled by condensation of serine molecules in the gas phase.^{5,16} The structure of this and related structures have been extensively investigated.^{1-3,7,12-15,17-28}

Protonated octamer is almost always a significant cluster in the mass spectrum under a wide variety of conditions leading to debates about the extent to which serine octamer exists in solution.^{1-6,8-11,14,17,26,29,30} Serine octamer may be formed in evaporating droplets, possibly enhanced at the liquid-gas interface.²⁶ Results from both NMR and IR studies indicate that the octamer does not exist in solution,²⁹ but more recent experiments show that serine octamer does exist in solution, albeit at relative low concentrations.³⁰ Nanoelectrospray emitters with small tips can be used to form sufficiently small droplets that only one or zero analyte molecules are likely to be present at a given solution concentration. Under these conditions, protonated octamer as well as larger clusters are observed indicating that these species exist in solution.³⁰ The use of larger tips leads to increased protonated octamer and other large clusters indicating that the additional ion signal is a result of aggregation inside the larger evaporating droplets that contain multiple analyte species.

Formation of clusters with up to 600 serine molecules have been reported, and under these conditions, protonated octamer is a minor species.⁷ Dissociation of large serine clusters in

the gas phase has also been reported to produce protonated octamer.^{25,26} Spencer et al. suggested that the octamer produced by dissociation of larger clusters by collision induced dissociation (CID) does not have homochiral specificity, but the precursor ions were not identified.²⁶ Dissociation of $(\text{Ser}_{15} + \text{H} + \text{Na})^{2+}$ at 50 kV collision energies results in the formation of smaller doubly and singly charged clusters formed by a combination of neutral serine loss and charge separation pathways.²⁵ The octamer formed in these experiments was exclusively sodiated. The dissociation of the octameric serine cluster occurs by neutral serine loss.

Dissociation of a variety of different multiply charged clusters has been investigated and can occur by neutral loss and/or charge separation pathways.³¹⁻³⁶ The extent to which clusters dissociate by these pathways depends on cluster size, charge state and subunit identity. For example, dissociation of $\text{La}^{3+}(\text{H}_2\text{O})_{18}$ occurs by loss of a neutral water molecule and by charge separation to produce protonated water clusters and the corresponding doubly charged hydrated metal hydroxide.³¹ Water loss occurs exclusively at larger cluster size whereas charge separation occurs for smaller clusters. The competition between charge separation and neutral molecule loss has been investigated for a wide variety of clusters.³¹⁻³⁶ For doubly charged metal ion clusters, the transition between charge separation reactions and neutral metal atom loss occurs at critical ratios of z^2/n , where z is the charge of the cluster and n the number of subunits in the cluster, that depends on the metal ion identity.³² Values of 0.39 to 0.97 were reported for a variety of different metal ions, and values for other multiply charged clusters of inorganic^{33,37-39} and multiply protonated peptide clusters³⁵ fall within this range.

Here, dissociation of multiply protonated serine clusters is investigated with the aim of understanding the dissociation pathways as a function of cluster size and charge state. An important finding is that protonated octamer can be formed from all of the doubly and triply

protonated clusters investigated and is the second most abundant cluster ion at an optimized collision energy. The dissociation pathways for these clusters are determined and the primary precursor for formation of protonated octamer from larger multiply charged clusters is identified.

Methods

Borosilicate capillaries (1.0 mm outer diameter, 0.78 mm inner diameter, Sutter Instruments, Novato, CA) were pulled using a Flaming/Brown P-87 micropipette puller (Sutter Instruments, Novato, CA) to produce nanoelectrospray ionization (nESI) emitter tips that have an inner diameter of $1.2 \pm 0.1 \mu\text{m}$. The inner diameter of the emitters was measured using a Hitachi TM-1000 scanning electron microscope (Schaumburg, IL) in the Electron Microscopy Laboratory at the University of California, Berkeley. Four replicate tips were pulled in order to measure the standard deviation of the tip diameters.

Ions were formed by nESI using 10 mM L-serine solutions (Sigma Aldrich, St. Louis, MI) in 49.95:49.95:0.1 water:methanol:acetic acid (Sigma Aldrich) and mass spectral data were acquired using a Waters Q-TOF Premier quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA). Ion formation is initiated by applying a voltage of 0.9 – 1.0 kV to a 0.127 mm diameter platinum wire that is inserted into the capillary and in contact with the solution. The voltage was increased until ion signal was stable and the spray was allowed to equilibrate for 1 min. before data acquisition. Instrument conditions were optimized to produce abundant protonated octamer. The source temperature was 80 °C and the extraction cone, sampling cone, and ion guide voltages were 2.0 V, 20 V, and 2.0 V respectively. The entrance and exit potentials of the collision cell were 0 V and -10 V, respectively with a collision gas (Argon) flow rate of 0.35 mL/min, resulting in a pressure of $\sim 4.4 \times 10^{-3}$ mbar in the collision cell.

Mass selection for collision induced dissociation experiments were done with a $m/z = 4$ isolation window. The collision voltage was varied from 0 V to 60 V. Spectra were averaged for 30 s. The abundance of protonated serine octamer produced by CID was measured as a function of collision voltage in 1.0 V increments to determine the value at which maximum octamer is produced. The charge and composition of peaks consisting of overlapping clusters with different charge states was determined from the isotopic distributions. Protonated serine is the most abundant ion that is formed, but sodium and potassium adducted serine is also produced at ~14% and 2% relative abundance, respectively. Much larger clusters with one or more sodium or potassium ions attached are also formed in low abundance. In some cases, these ions overlap in m/z with purely protonated clusters but can be distinguished based on their isotopic spacing owing to their non-overlapping charge states.

Results and Discussion

Fragmentation pathways of protonated octamer and octameric metaclusters.

Electrospray ionization of a 10 mM serine solution results in abundant ions at $m/z = 841$ consisting of mostly protonated serine octamer (93%) and minor overlapping contributions from octameric metaclusters corresponding to doubly protonated 16-mer (4%) and triply protonated 24-mer (3%) (Figure 1). The abundances of the octameric metaclusters are sensitive to instrument parameters and gaseous collisions.^{1,10,15,24} The slightly higher abundance of the 24-mer compared to that reported previously may be due to the relatively soft ion introduction conditions used here.^{1,15,30} With zero volts collision energy, there is sufficient ion energy to induce all three precursor ions at $m/z = 841$ to dissociate as a result of the relatively high Ar collision gas pressure that was used throughout all experiments, including those with additional collision

energy. The triply protonated 24-mer (24^{3+}) dissociates by loss of neutral serine molecules to form smaller triply charged clusters. Dissociation of this ion also results in doubly charged ions at higher m/z that are formed through a charge separation process to produce two complimentary charged fragment ions. Both monomer loss and charge separation reactions have been reported for several multiply charged serine cluster ions.^{25,26} Doubly charged ions at lower m/z are formed by dissociation of the 16^{2+} by neutral serine loss. The singly charged ions are formed primarily by dissociation of the protonated octamer as well as possible minor contributions from charge separation of the smaller doubly charged ions. The low abundance of the protonated heptamer compared to that of the protonated hexamer is indicative of the relative stabilities of these ions.^{1,3,14–16,19}

Minimum cluster size and charge separation. The smallest doubly and triply protonated cluster ions that are formed directly by electrospray ionization are 8^{2+} and 21^{3+} , respectively. These minimum cluster sizes are similar to the values of 9^{2+} and 22^{3+} reported by Concina et al.²⁵ Charge separation and neutral loss pathways typically have different entropies so that the minimum observed cluster size can depend on ion energies.^{33,35,39} The transition between charge separation and monomer loss depends on both cluster size and charge state. The approximate cluster sizes where this transition occurs for serine correspond to z^2/n values of 0.44 and 0.39 for the doubly and triply protonated clusters, respectively. These values are similar to those reported for many multiply charged clusters and for doubly protonated peptide clusters that undergo a similar charge separation pathway.³⁵

Dissociation of the doubly protonated octamer results in formation of a series of singly protonated ions up to the hexamer (Figure 2a). These ions must be formed by charge separation

in which both fragment ions from a dissociation pathway are charged. The charge separation pathways are more readily identified for larger clusters. This is illustrated for dissociation of 23^{3+} where neutral serine loss results in formation of triply protonated clusters as small as 21^{3+} (Figure 2b). Abundant singly protonated dimer as well as monomer and trimer are also formed. These results indicate that protonated dimer and to a much lesser extent protonated trimer are the main charge separation pathways. Charge separation by protonated monomer formation may also occur but this ion can also be formed by neutral serine loss from the protonated dimer. The absence of protonated heptamer in the dissociation spectrum of the doubly protonated octamer may indicate that the loss of protonated monomer is not a significant pathway. However, subsequent dissociation of the unstable protonated heptamer may obscure direct observation of this complimentary fragmentation product ion. Dissociation of 9^{2+} results in some protonated heptamer but no protonated octamer, again indicating that charge separation by formation of protonated monomer is not favored.

Charge separation upon dissociation of small doubly charged serine clusters has been reported previously.²⁵ Dissociation of 15^{2+} charged with one proton and one sodium ion with 50 kV collisions resulted in formation of octamer that was exclusively sodiated. Protonated serine clusters as large as the tetramer were observed. This suggests that protonated tetramer may also be a charge separation pathway at these high collision energies, but this ion may be formed from larger protonated clusters that subsequently undergo neutral loss. Charge separation has also been observed for two multiply protonated groups of metaclusters dissociated at low energy, but the charge separation pathways were not identified.^{3,25} The observation of protonated serine dimer as the main charge separation pathway is consistent with results from dissociation of

multiply protonated peptide clusters in which formation of protonated peptide dimers was the main charge separation pathway observed.³⁵

Charge separation versus neutral monomer loss pathways. For doubly and triply protonated clusters that are substantially above the minimum cluster size, loss of neutral serine molecules is favored. For example, dissociation of 29^{3+} at a collision voltage of 0 V results in predominantly the loss of neutral serine molecules to produce triply protonated ions as small as 21^{3+} (Figure 3a). Charge separation to produce singly protonated monomer and dimer is a minor dissociation pathway. Doubly charged ions as large as 25^{2+} are formed by charge separation at 5 V collisions (Figure 3b). Because formation of protonated dimer is the dominant charge separation pathway, this result indicates that the onset of charge separation as a minor reaction pathway starts at 27^{3+} and is the only process for 21^{3+} . At 28 V, doubly protonated ions as small as 8^{2+} and singly protonated ions up to the octamer are formed through a combination of both dissociation processes (Figure 3c). At this collision voltage, protonated octamer is the second most abundant cluster ion in the dissociation spectrum, as it is when these clusters ions are formed directly from solution. Protonated octamer was reported to be formed by CID of larger clusters but the minimum cluster sizes and dissociation pathways were not identified.²⁶ These results suggest an alternative pathway to formation of protonated octamer that is widely observed to be a highly abundant “magic number” cluster when solutions of serine are ionized using various spray ionization methods

Formation of protonated octamer from large multiply charged clusters. Abundant protonated octamer can be produced by dissociation of each of the large multiply charged clusters investigated. For example, dissociation of 37^{3+} at 0 V results in exclusively neutral

serine loss (Figure 4a). The appearance of protonated monomer and dimer in this spectrum originates from dissociation of 41^{4+} that is triply protonated with a potassium adduct at overlapping m/z . This ion undergoes neutral charge loss and charge separation, which produces singly protonated serine monomer, dimer, trimer and the corresponding $3+$ ions that retain the potassium adduct. With 15 V collisions, smaller triply protonated clusters formed by loss of neutral serine molecules are observed, and singly protonated and corresponding doubly protonated ions are also formed by charge separation (Figure 4b). With 30 V collisions, only doubly and small singly charged ions are observed (Figure 4c). Doubly charged ions as small as 11^{2+} are formed, but no protonated octamer is observed. With 47 V collisions, the protonated octamer is the second most abundant cluster, and doubly protonated ions as small as the octamer are formed (Figure 4d). The protonated octamer abundance is maximized at this collision voltage. This voltage is higher than that required to produce an optimum abundance of protonated octamer from 29^{3+} (Figure 3c). The sequential dissociation pathways for dissociation of 37^{3+} are summarized in Scheme 1.

The optimum collision voltage for production of abundant protonated octamer from both doubly and triply protonated clusters was identified by varying the collision voltage in 1 V increments, and these data are shown in Figure 5a. Abundant protonated octamer is produced over a relatively broad range of collision voltages (Figure 5a, inset). The optimum voltage increases linearly with cluster size for both the $+2$ and $+3$ clusters but these two ion series show different trend lines. Converting the collision voltages into center-of-mass collision energies for each cluster results in a linear relationship with cluster size independent of the precursor ion charge state (Figure 5b). The 11^{2+} is entirely dissociated into smaller clusters at 0 V collision energy, so these data are not included in Figure 5. It is important to note that the center-of-mass

collision energies correspond to single collisions at the initial energy induced by the applied collision voltage and do not account for multiple collisions that occur in these experiments. Thus, these values are more an indicator of relative energies transferred into the different clusters. These results indicate that formation of the octamer from gas-phase dissociation of larger clusters depends on the number of degrees of freedom in the cluster. The linearity of these data show that the protonated octamer abundance is optimized when the energy deposited per degree of freedom is the same for all the clusters. This result indicates that there is an optimum cluster temperature for producing protonated octamer by dissociation of larger clusters.

It is interesting to note that protonated octamer is not formed directly as a dissociation product from the much larger triply protonated clusters under the conditions investigated. Loss of neutral serine molecules occurs at large cluster size but charge separation to produce complimentary doubly charged clusters and corresponding singly protonated serine dimer, trimer and possibly monomer occurs at smaller cluster size. Thus, protonated octamer is only produced from doubly charged precursors. With 0 V collisions, charge separation and production of protonated octamer was observed for all of the doubly protonated clusters (11^{2+} , 13^{2+} , 15^{2+} , 17^{2+}). The observation of 11^{2+} with 30 V collisions of the 37^{3+} indicates that 12^{2+} undergoes neutral loss and not charge separation; charge separation from this ion would produce primarily singly protonated decamer, which is not observed (Figure 4c). Thus, the majority of protonated octamer must be formed directly by dissociation of doubly protonated serine clusters with 10 or 11 serine molecules, with the major contribution coming from 10^{2+} by charge separation and formation of protonated dimer.

Even dissociation of octameric metaclusters does not lead to direct formation of protonated octamer. The main dissociation pathway for these clusters is loss of neutral serine

molecules, not ejection of protonated octamer. The more abundant 15^{2+} than 14^{2+} from dissociation of 16^{2+} (Figure 1) suggests that this ion does not likely consist of two minimally interacting protonated octamers with the same structures as those formed either directly from solution or by dissociation of larger clusters. Loss of a serine molecule from one of the octamers would lead to an unstable heptamer, which would quickly dissociate to favor production of 14^{2+} over 15^{2+} . These results indicate that, unlike the neutral serine octamer that has been reported to be introduced directly into the gas phase by sublimation of crystalline serine,^{8,9} the protonated serine octamer is not “sublimed” directly from large gaseous cluster ions but rather formed through sequential charge separation and neutral serine loss pathways to ultimately form doubly protonated decamers that are responsible for direct formation of protonated octamer through charge separation and formation of the complimentary protonated serine dimer.

Conclusions

The dissociation of large multiply protonated serine clusters occurs by two pathways that depend on cluster size. Clusters substantially larger than the minimum cluster size dissociate by sequential loss of neutral serine molecules whereas charge separation to produce primarily protonated serine dimer and the complimentary ion occurs for clusters close to the minimum cluster size. Protonated octamer is formed by charge separation primarily from doubly protonated decamer to form the complimentary protonated serine dimer. This dissociation pathway can be accessed for any of the large doubly and triply charged clusters investigated. At an optimized collision energy, the singly protonated octamer can be the second most abundant cluster ion (with the protonated dimer the most abundant cluster) in the dissociation mass spectra of all of the clusters investigated. A significant finding is that optimum production of protonated

octamer that is formed by dissociation of larger clusters occurs at a uniform energy per degrees of freedom for the large clusters. This result indicates that the protonated octamer should be the second most abundant cluster ion formed by dissociation of higher order clusters at an optimized *temperature*. Thus, these results indicate another mechanism for formation of highly abundant protonated serine octamer for ions produced from serine solutions by various spray ionization methods.

Recent results indicate that serine octamer exists in solution albeit at low abundance and that significant protonated octamer can be formed by aggregation inside droplets as solvent evaporation occurs and the concentration of serine increases prior to gaseous ion formation.³⁰ Aggregation inside droplets can be minimized or even eliminated by using ESI emitters with tiny tips (100's of nm in diameter) or by reducing analyte concentration with the goal of producing initial droplets that contain either one or no analyte molecule per droplet. Under conditions commonly employed in many previous studies of serine octamer, aggregation inside the electrospray droplet likely contributes substantially to formation of abundant protonated octamer.

Much larger clusters can also be formed by aggregation. Sonic spray using large diameter emitters and soft source conditions can result in the formation of clusters consisting of over 600 serine molecules.⁷ Under these conditions, the protonated octamer comprises only ~0.3% of the total ion population. Our results indicate that with an optimized source temperature, these large clusters would all dissociate to form protonated serine octamer as the second most abundant cluster ion. Such a temperature could be achieved in a heated metal capillary ion introduction system, and this may explain results from other spray experiments where the protonated octamer is the most abundant ion in the mass spectra.^{1-4,6,14,23,25,26} Variability in the magic number character of the protonated octamer observed in previous

studies^{1,4-14,16,17,23,27-29} may also be explained by different effective temperatures of the ion populations as a result of different source conditions or ionization processes.

Conflicts of Interest

There are no conflicts to declare.

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Figures

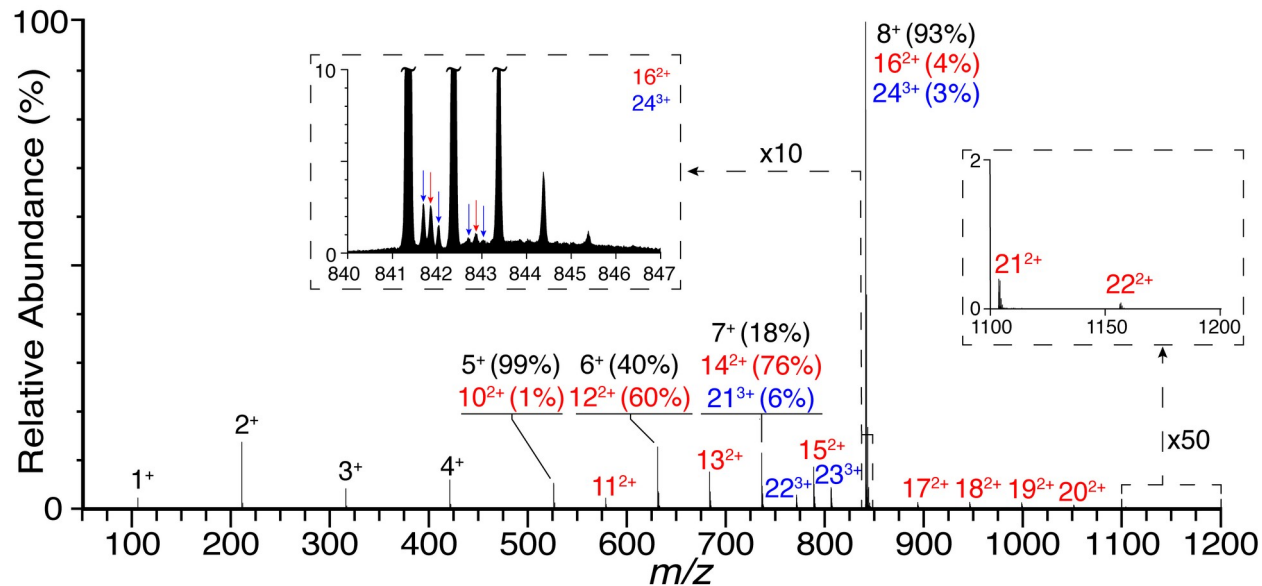


Figure 1. Collision induced dissociation mass spectrum of protonated serine cluster ions at $m/z = 841$ corresponding to protonated octamer and multiply protonated metaclusters measured with Ar collision gas in the cell but with 0 V additional collision energy. The percentage at each cluster indicates the abundance of each ion at overlapping m/z values.

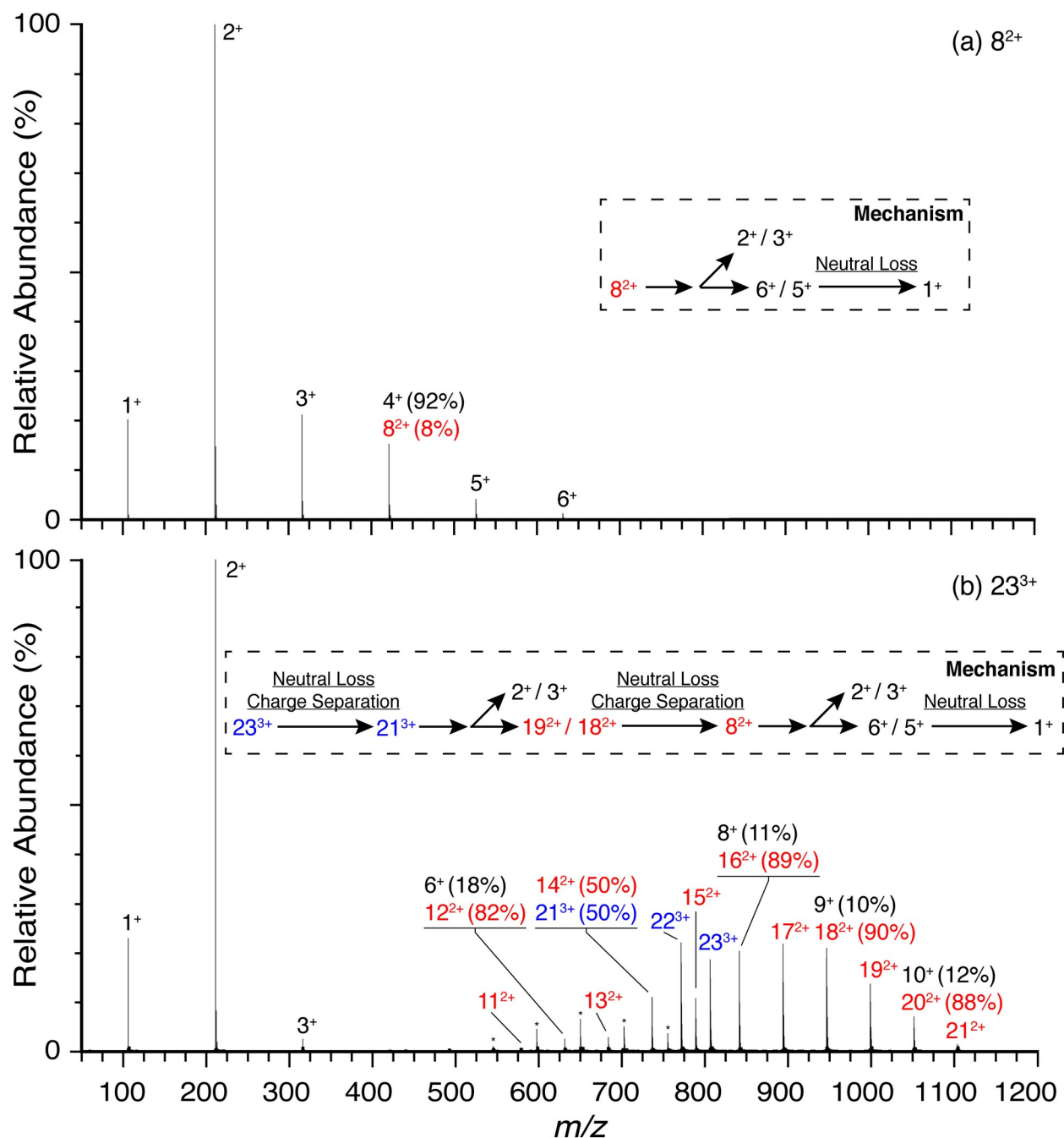


Figure 2. Collision induced dissociation mass spectra of (a) doubly protonated serine octamer (8^{2+}) and (b) triply protonated cluster with 23 serine molecules (23^{3+}) with Ar collision gas in the cell and 0 V additional collision energy. Peaks denoted with an asterisk are potassium adducted

fragment ions produced from $(\text{Ser}_{15} + \text{H} + \text{K})^{2+}$ that overlaps in m/z with the 23^{3+} . These low abundance ions are formed as a result of low levels of potassium present in solution.

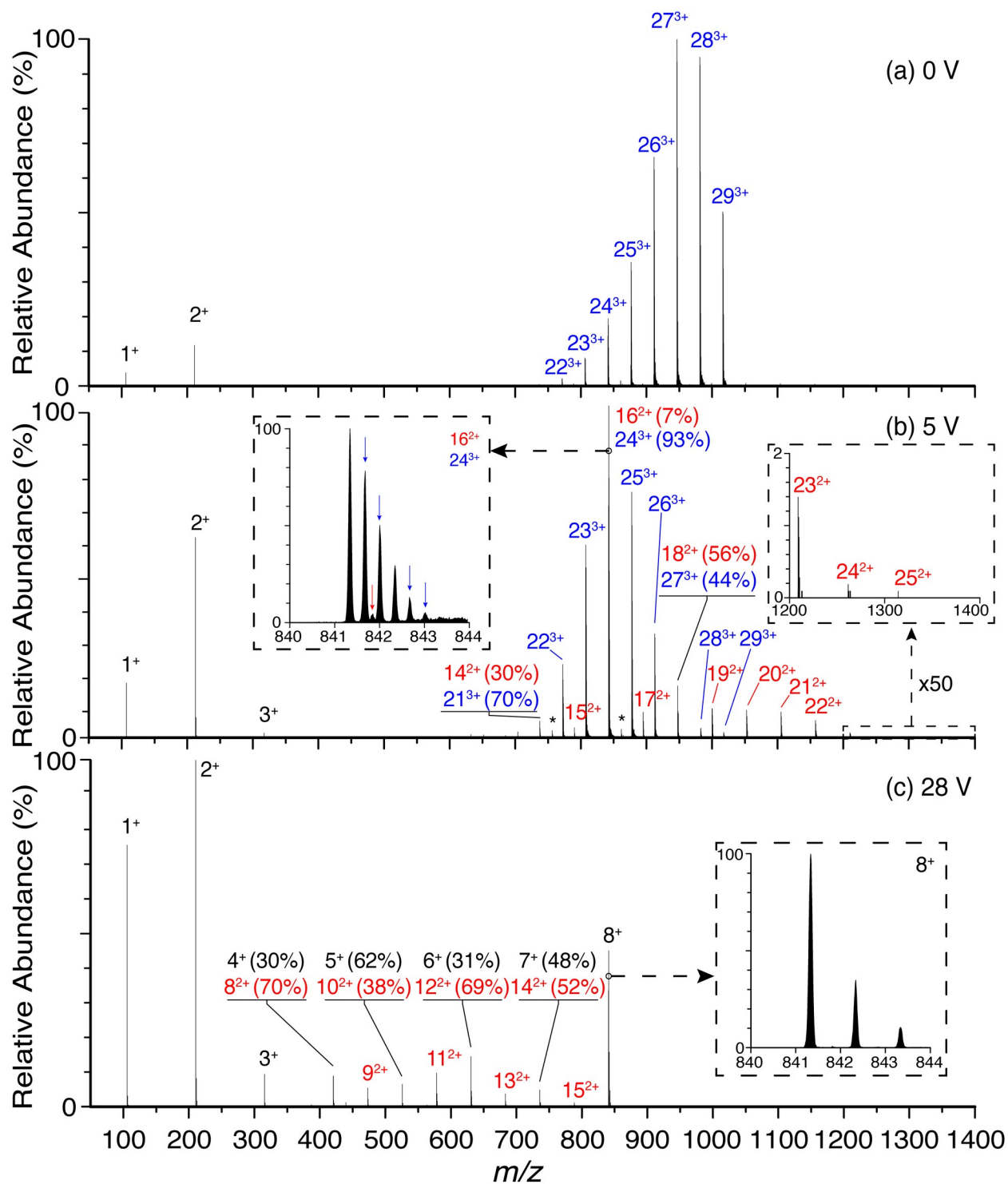


Figure 3. Collision induced dissociation spectra of 29^{3+} at collision voltages of (a) 0 V, (b) 5 V and (c) 28 V. Protonated octamer abundance is a maximum at 28 V collision voltage. Peaks marked with an asterisk are potassium adducted fragment ions formed by $(\text{Ser}_{19} + \text{H} + \text{K})^{2+}$ that overlaps in m/z with the 29^{3+} .

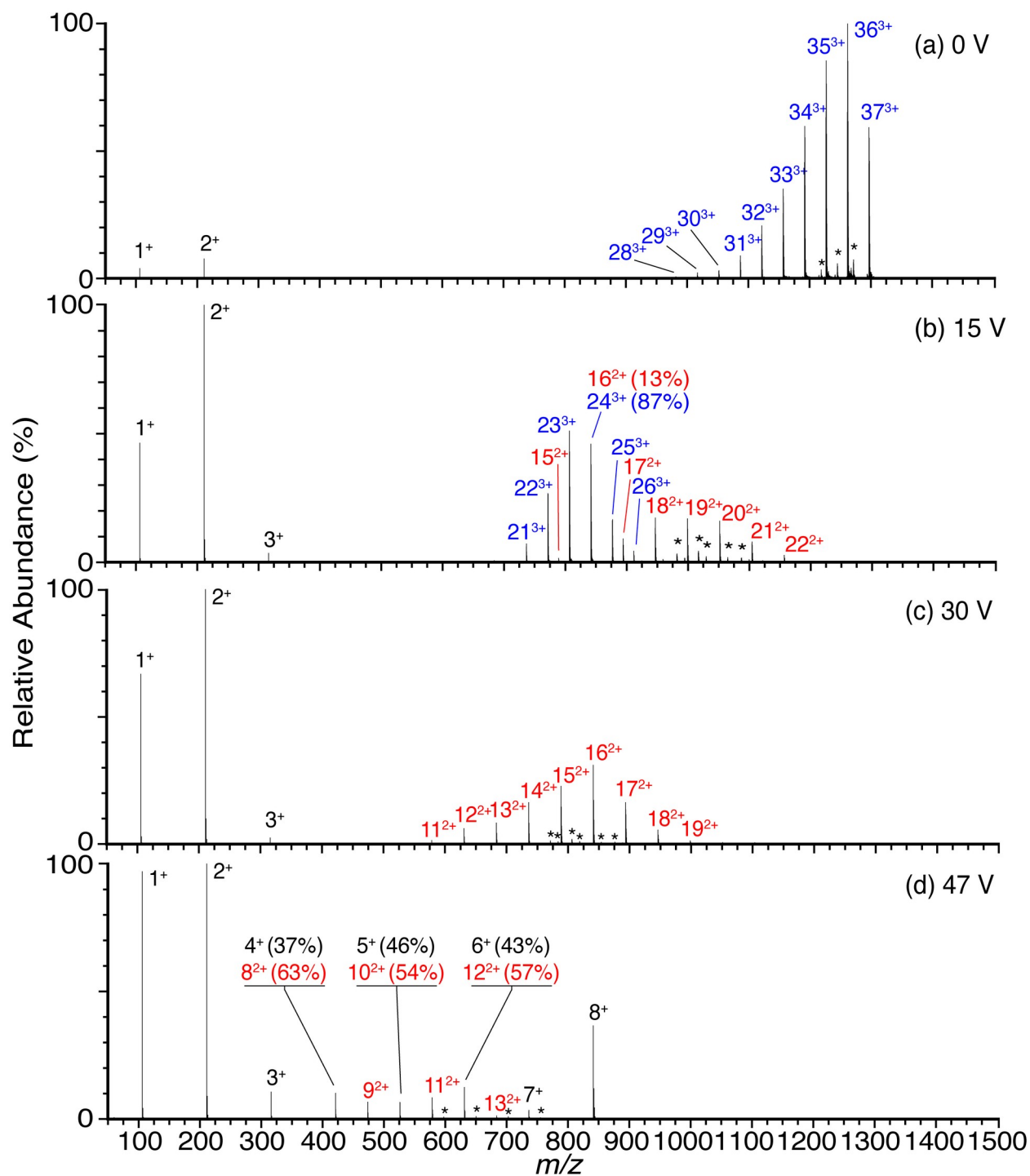


Figure 4. Collision induced dissociation of serine 37^{3+} at (a) 0 V, (b) 15 V, (c) 30 V and (d) 47 V collisional voltage. Dissociation by loss of neutral serine molecules occurs when the cluster sizes are significantly above the minimum cluster size whereas charge separation occurs when

the clusters approach this size. The protonated octamer abundance is a maximum at 47 V. Peaks marked with an asterisk are potassium adducted fragments produced from $(\text{Ser}_{49} + 3\text{H} + \text{K})^{4+}$ that overlaps in m/z with the 37^{3+} cluster.

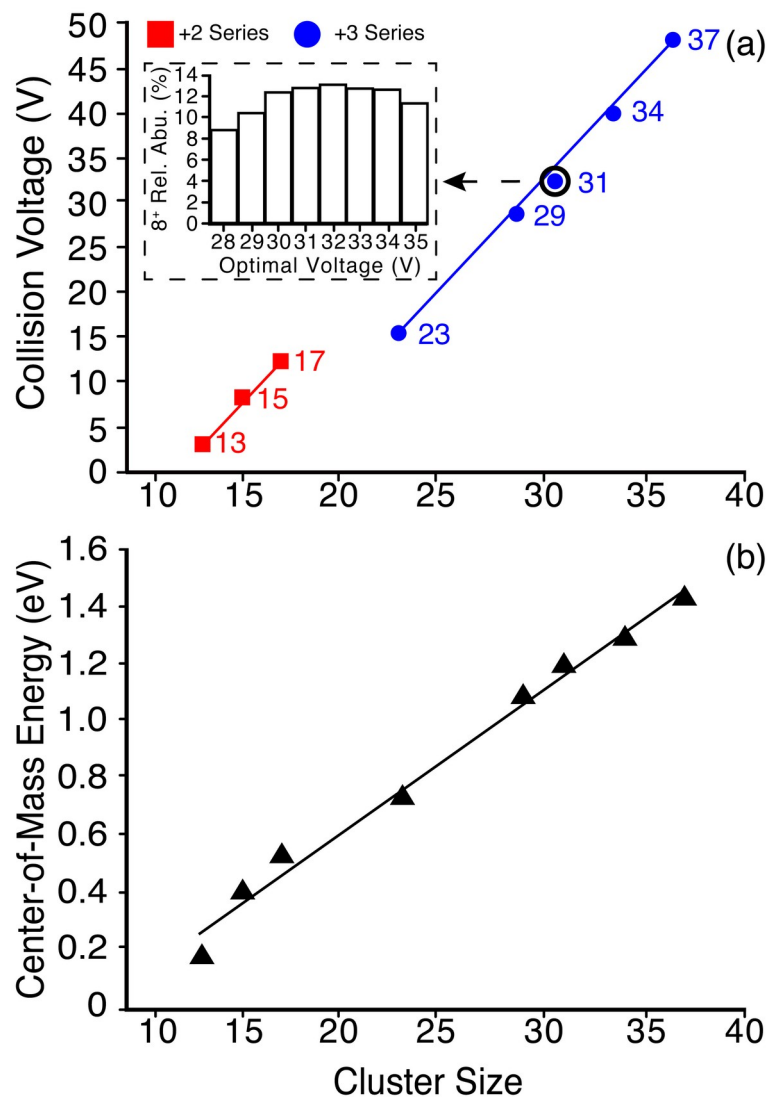
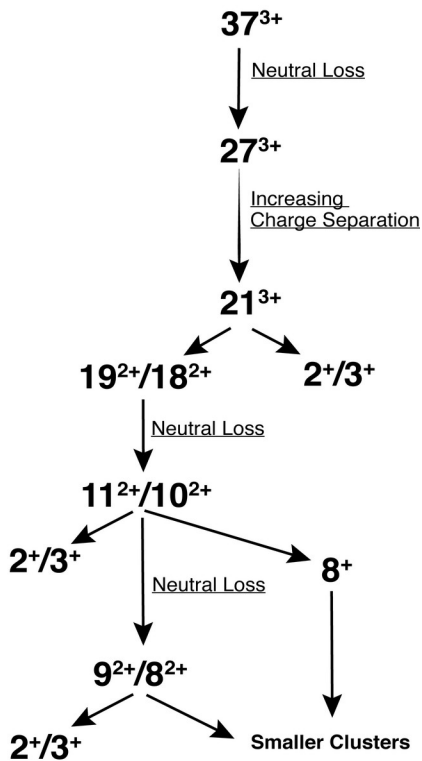
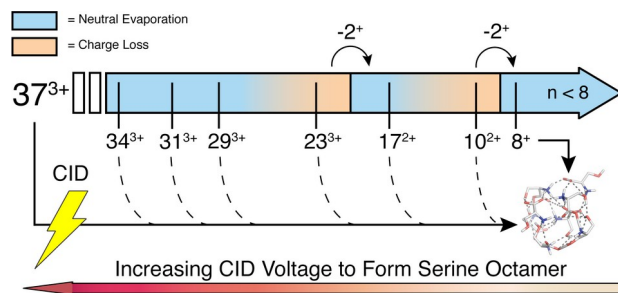


Figure 5. Optimum (a) collision voltage and (b) center-of-mass collision energy as a function of cluster size and charge state for maximum abundance of protonated octamer (relative to total ion abundance), which is the second most abundant cluster ion (the protonated dimer is most abundant) produced by CID of each cluster.



Scheme 1. Fragmentation pathways for serine 37^{3+} .

TOC Graphic



Dissociation of large multiply protonated serine clusters produces abundant protonated octamer at an optimum ion temperature predominantly through a doubly protonated decamer intermediate.