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Dissociation of Multiple Protein Ion Charge States Following a Single Gas-Phase Purification and Concentration Procedure

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The formation of a range of precursor ion charge states from a single concentrated and purified charge state, followed by activation of each charge state, is introduced as a means to obtain more protein structural information than is available from dissociation of a single charge state alone. This approach is illustrated using off-resonance collisional activation of the $[M + 8H]^{8+}$ to $[M + 6H]^{6+}$ precursor ions of the bacteriophage MS2 viral coat protein following concentration and purification of the $[M + 8H]^{8+}$ charge state. This range of charge states was selected on the basis of an ion trap collisional activation study of the effects of precursor ion charge state on the dissociation of the $[M + 12H]^{12+}$ to $[M + 5H]^{5+}$ ions. Gas-phase ion/ion proton-transfer reactions and the ion parking technique were applied to purify and concentrate selected precursor ion charge states as well as to simplify the product ion spectra. The high-charge-state ions fragment preferentially at the N-terminal side of proline residues while the product ion spectra of the lowest charge states investigated are dominated by C-terminal aspartic acid cleavages. Maximum structural information is obtained by fragmentation of the intermediate-charge states.

Several ionization methods capable of producing multiply charged ions from large polyatomic molecules are now available to characterize and identify proteins.[1−5] Principal among these is electrospray ionization (ESI).[1−3] The ramifications of the multiple charging phenomenon associated with biopolymers, such as proteins, are manifold. For example, it leads to the ability to measure the molecular masses significantly greater than the upper mass-to-charge limit of a mass analyzer. Furthermore, the formation of multiple charge states of the same molecule can also enhance the structural information available from tandem mass spectrometry experiments due to the fact that the identities and abundances of product ions can be highly sensitive to parent ion charge state (see below). However, the multiple charging phenomenon can have negative implications in some analytical scenarios, such as complex mixture analysis, wherein extensive overlap in mass-to-charge ratio of ions of different mass and charge can complicate mass analysis or parent ion isolation for tandem mass spectrometry. It is therefore often desirable to be able to manipulate ion charge states initially formed by ESI.

In the case of multiply protonated proteins, ion charge state manipulation has been shown to be particularly effective using negatively charged ions. Ion/ion reactions have been demonstrated both prior to sampling ions into a mass spectrometer[6−9] and after ions have been accumulated in a quadrupole ion trap.[10−14] The former approach enjoys the advantage of simplicity, in that it can be implemented with virtually any type of mass analyzer. However, from a practical standpoint, the approach is limited to use with mass analyzers with relatively high upper mass-to-charge limits (e.g., $m/z > 5000$) for analyses of high-mass biopolymers. The latter approach is attractive in that the ion/ion reaction period(s) can occur at arbitrary times during the course of an ion trap experiment (i.e., within the context of an MS^n experiment[15]). A number of distinct examples of the use of ion/ion proton-transfer reactions involving protein ions in a quadrupole ion trap for analytically useful measurements have been demonstrated. These include, for example, the use of ion/ion reactions to simplify mass spectra of protein mixtures[11−13] and to simplify protein product ion spectra derived from multiply charged parent ions.[16−24] A study employing ion/ion reactions both to simplify

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(1) Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64−71.

(2) Gaskell, S. J. *J. Mass Spectrom.* **1997**, *32*, 677−688.

(3) Cole, R. B. *Electrospray Ionization Mass Spectrometry*; Wiley-Interscience: New York, 1997.

(4) Mahoney, J. F.; Perel, J.; Ruatta, S. A.; Martino, P. A.; Husain, S.; Lee, T. D. *Rapid Commun. Mass Spectrom.* **1991**, *5*, 441−445.

(5) Karas, M.; Hillenkamp, F. *Anal. Chem.* **1988**, *60*, 2299−2301.

(6) Ogorozalek-Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Phys. Chem.* **1991**, *95*, 6412−6415.

(7) Scalf, M.; Westphall, M. S.; Krause, J.; Kaufman, S. L.; Smith, L. M. *Science* **1999**, *283*, 194−197.

(8) Scalf, M.; Westphall, M. S.; Smith, L. M. *Anal. Chem.* **2000**, *72*, 52−60.

(9) Ogorozalek-Loo, R. R.; Udseth, H. R.; Smith, R. D. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 695−705.

(10) Stephenson, J. L., Jr.; McLuckey, S. A. *J. Am. Chem. Soc.* **1996**, *118*, 7390−7397.

(11) Stephenson, J. L., Jr.; McLuckey, S. A. *Anal. Chem.* **1996**, *68*, 4026−4032.

(12) Stephenson, J. L., Jr.; McLuckey, S. A. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 585−596.

(13) Stephenson, J. L., Jr.; McLuckey, S. A. *J. Mass Spectrom.* **1998**, *33*, 664−672.

(14) Stephenson, J. L., Jr.; McLuckey, S. A. *Anal. Chem.* **1998**, *70*, 3533−3544.

(15) Louris, J. N.; Brodbelt-Lustig, J. S.; Cooks, R. G.; Glish, G. L.; Van Berkel, G. J.; McLuckey, S. A. *Int. J. Mass Spectrom. Ion Processes* **1990**, *96*, 117−137.

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the mass spectrum of a protein mixture, a protein lysate from *Escherichia coli* containing bacteriophage MS2, and to simplify the product ion spectra of a limited number of charge states of a targeted protein within the mixture, the bacteriophage MS2 coat protein, has been reported.[24]

It has recently been demonstrated that ion/ion reaction rates in an electrodynamic ion trap can be selectively inhibited, giving rise to a technique referred to as ion parking.[25] This technique relies on the mass-to-charge-dependent acceleration of ions stored in the presence of oppositely charged ions. The ion/ion reaction rates for the accelerated ion population are significantly reduced relative to other ions of the same polarity that are not accelerated. Therefore, it is possible to accumulate a large fraction of the initial ESI-generated ion population, which is dispersed over many charge states, into a single charge state, provided the parked ion is lower in charge than most of the initial ESI-generated ion population. In this circumstance, protein ions are "concentrated" into a single charge state. The ion parking technique inhibits the reaction rates of ions within a window of mass-to-charge values. Therefore, in the case of complex mixtures, it is possible that several proteins of different mass can be parked at the same mass-to-charge value, provided one of the possible protein charge states falls within the relevant m/z window. Product ion spectra derived from the parked parent ion population would then represent a combination of products from the various protein ions in the parked population. It has been demonstrated that this situation can be largely avoided by use of a double ion isolation experiment, which employs an ion parking period between isolation steps. This process can "charge-state purify" the isolated ion population. Hence, ion parking can facilitate both the concentration and charge-state purification of protein ions in the gas phase for subsequent ion activation. These procedures have recently been demonstrated with a protein fraction derived from *E. coli* for several of the protein components present in the mixture.[26]

An important use of protein ion concentration and purification via ion parking is the preparation of a parent ion population for subsequent dissociation. Based on charge-state-dependent fragmentation behavior in the ion trap noted to date,[16−24] the structural information available from a product ion spectrum can be highly dependent upon parent ion charge. For example, a general tendency favoring cleavages N-terminal to proline residues at relatively high protein ion charge states and C-terminal to aspartic acid residues at relatively low charge states has been noted. Frequently, intermediate-charge states tend to yield the greatest diversity of fragmentation channels. The factors governing fragmentation of protein ions are the subjects of ongoing study. However, it is clear that the probability for accurate identification of a protein from product ion spectra of protein ions is enhanced when multiple parent ion charge states are interrogated.[16,20,22,23] Ordinarily, this would require a distinct MS^n procedure for the isolation and activation of each parent ion charge state of interest. However, MS^n strategies that take advantage of ion/ion proton-transfer reactions can provide the option of obtaining fragmentation information from several parent ion charge states following a single ion accumulation period and a single protein ion concentration and purification procedure.

One of the areas where this approach could be applicable is in the detection of viruses present in a complex biological matrix. Bacteriophage MS2 is an icosahedral virus that is specific to bacteria that contain the F plasmid. The coat protein of bacteriophage MS2 is present at 180 copies/virion (MW = 13 728)[27] and could be used as a marker for the detection of this virus in a complex bacterial lysate. Direct analysis of this viral capsid protein was performed previously via MALDI[28] and ESI mass spectrometry.[29,30] Identification of the bacteriophage MS2 viral coat protein expressed in *E. coli* was also performed previously by analysis of the product ions formed by dissociation of the intact protein ions.[24] However, only a small number of precursor ion charge states (+7 to +9) were examined. The spectra of these charge states were dominated by preferential cleavage N-terminal to proline residues, yielding mainly b_{92}/y_{37}, b_{116}/y_{13}, and b_{118}/y_{11} ions. In this report, we summarize the charge-state-dependent fragmentation behavior of the coat protein of bacteriophage MS2 over a wider range of charge states (+12 to +5) and use this protein to illustrate a novel approach to obtaining fragmentation behavior from multiple parent ion charge states after concentration and purification of the protein ions in the gas phase.

EXPERIMENTAL SECTION

Bacteriophage MS2 Coat Protein. The host bacterial strain *E. coli* (ATCC 15597) and bacteriophage MS2 (15597-B1) were obtained from American Type Culture Collection (Rockville, MD). The freeze-dried *E. coli* was reactivated on agar plates at 37 °C for 24 h under sterile conditions. The aqueous media used to prepare the agar plates and grow the *E. coli* was composed of 10 mL of 10% glucose (Sigma, St. Louis, MO), 2.0 mL of 1 M $CaCl_2$ (Sigma, St. Louis, MO), 1.0 mL of 10 mg/mL thiamine (Sigma), 10.0 g of tryptone (Fisher Scientific, Pittsburgh, PA), 1.0 g of yeast extract (Fisher Scientific), and 8.0 g of NaCl (Sigma) on a per liter basis. The plates contained the same ingredients plus 10 g/L agar (Difco, Sparks, MD).

We found that it was necessary to screen the reactivated *E. coli* to select bacteria that could be infected by MS2. Single *E.*

(16) Schaaff, T. G.; Cargile, B. J.; Stephenson, J. L., Jr.; McLuckey, S. A. *Anal. Chem.* **2000**, *72*, 899−907.
(17) Stephenson, J. L., Jr.; Cargile, B. J.; McLuckey, S. A. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 2040−2048.
(18) Wells, J. M.; Stephenson, J. L., Jr.; McLuckey, S. A. *Int. J. Mass Spectrom.* **2000**, *203*, A1−A9.
(19) Wells, J. M.; Reid, G. E.; Engel, B. J.; Pan, P.; McLuckey, S. A. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 873−876.
(20) Newton, K. A.; Chrisman, P. A.; Reid, G. E.; Wells, J. M.; McLuckey, S. A. *Int. J. Mass Spectrom.* **2001**, *212*, 359−376.
(21) Reid, G. E.; Stephenson, J. L., Jr.; McLuckey, S. A. *Anal. Chem.* **2002**, *74*, 577−583.
(22) Engel, B. J.; Pan, P.; Reid, G. E.; Wells, J. M.; McLuckey, S. A. *Int. J. Mass Spectrom.* **2002**, *219*, 171−187.
(23) Reid, G. E.; Wu, J.; Chrisman, P. A.; Wells, J. M.; McLuckey, S. A. *Anal. Chem.* **2001**, *73*, 3274−3281.
(24) Cargile, B. J.; McLuckey, S. A.; Stephenson, J. L., Jr. *Anal. Chem.* **2001**, *73*, 1277−1285.
(25) McLuckey, S. A.; Reid, G. E.; Wells, J. M. *Anal. Chem.* **2002**, *74*, 336−346.
(26) Reid, G. E.; Shang, H.; Hogan, J. M.; Lee, G. U.; McLuckey, S. A. *J. Am. Chem. Soc.* **2002**, *124*, 7353−7362.
(27) Brock, T. D.; Madigan, M. T.; Markinko, J. M.; Parker, J. *Biology of Microorganisms*, 7th ed.; Prentice Hall: Englewood Cliffs, NJ, 1994; Chapter 6.
(28) Thomas, J. J.; Falk, B.; Fenselau, C.; Jackman, J.; Ezzell, J. *Anal. Chem.* **1998**, *70*, 3863−3867.
(29) Tito, M. A.; Tars, K.; Valegard, K.; Hajdu, J.; Robinson, C. V. *J. Am. Chem. Soc.* **2000**, *122*, 3550−3551.
(30) Xiang, F.; Anderson, G. A.; Veenstra, T. D.; Lipton, M. S.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 2475−2481.

coli colonies were suspended in 2 mL of growth media and grown to 0.2 OD at 600 nm as determined with a dual-channel UV spectrophotometer (PerkinElmer, Wellesley, MA). A fraction of each culture was infected with 100 μL of MS2 phage, and the infected culture was incubated for 3 h to allow for phage growth. MS2 growth was signaled when the dark yellow, turbid *E. coli* culture was transformed to a clear amber. Only MS2-sensitive *E. coli* was used for further growth.

The MS2-sensitive *E. coli* was grown to 0.2 OD at 600 nm in 100 mL of medium. The *E. coli* culture was inoculated with 2 mL of MS2 phage solution and incubated until MS2 growth was completed. The culture was centrifuged at 5000*g* in a J2-MC centrifuge (Beckman Coulter, Fullerton, CA) for 20 min to remove unlysed *E. coli* and bacteria debris. The crude MS2 solution was stirred overnight at 4 °C with 29.2 g of NaCl (Sigma) and 100 g of PEG-8000 (Sigma) per liter of crude MS2. This solution was centrifuged at 8000*g* in a J2-MC centrifuge (Beckman Coulter) for 30 min, and the pellet was dissolved in 5 mL of pH 7.4 10 mM Tris (Pharmacia Biotech AB, Uppsala, Sweden), 100 mM NaCl (Sigma), 0.1 mM $MgSO_4$ (Sigma), and 0.01 mM EDTA (Sigma). It was necessary to repeatedly draw the pellet through a pipet to break up the cellular protein and DNA debris. The MS2 was separated from the other materials in the pellet by ultracentrifugation. About 0.6 g of CsCl (Sigma) was added per gram of solution to reach the density of 1.40 g/mL. This solution was then subjected to 192000*g* with a Ti50.2 rotor in a L7-55 ultracentrifuge (Beckman Coulter) for 24 h. The MS2 formed a whitish blue band that was clearly visible in the middle of the centrifuge tube. This band was carefully removed with a sterile syringe with a 25-gauge $^5/_8$-in. needle and dialyzed overnight against water using 10-kDa MWCO dialysis cassette (Pierce, Rockford, IL).

Prior to mass spectrometry experiments, the MS2 solutions were diluted 20-fold in 65% (v/v) acetic acid aqueous solution to dissolve the coat protein by breaking the hydrophobic regions in it.

Mass Spectrometry. All experiments were performed with a homemade electrospray source coupled to a Finnigan-MAT (San Jose, CA) ion trap mass spectrometer[31] that was modified for the injection of negatively charged perfluoro-1,3-dimethylcyclohexane (PDCH) ions through a hole in the ring electrode. All experiments were controlled by ICMS software.[32] The spectra recorded after ion/ion reactions were used to reduce ion charge states are referred to as post-ion/ion mass spectra. Each mass spectrum presented herein is the average of 300−500 scans. A detailed description of the typical instrumental setup for ion/ion reactions can be found in the literature.[33] Briefly, in a typical experiment (~3 s/scan), an electrospray ion accumulation period of several hundred milliseconds was employed, followed by isolation of ions corresponding to a specific charge state, using multiple resonance ejection ramps to sequentially eject ions of m/z higher and lower than that of interest. In this study, the ion parking technique was employed to concentrate and purify the precursor charge states, whereby a resonance excitation voltage applied to the end-cap electrodes of the ion trap was tuned to inhibit the ion/ion reaction rate of a specific range of ion m/z ratios. With this technique, it is possible to concentrate a large majority of the ion population originally formed with a distribution of charge states into a single lower charge state. Ion parking was achieved using a short period of anion injection time (1−10 ms), followed by a relatively long cation/anion mutual storage time (~300 ms) with a resonance excitation voltage of about 600−900 mV_{p-p} applied close to the z-dimension secular frequency of the m/z to be parked. Then, following isolation of the parked ion and further charge-state reduction, a selected precursor ion charge state could be subsequently purified for dissociation by further parking of a lower charge-state ion of interest. The purified precursor ion population could then be subjected directly to collisional activation. Alternatively, the purified precursor ion population could be subjected to an additional short ion/ion reaction period, resulting in a distribution of lower charge states that could then be sequentially activated by collisional activation. The details of the scan functions for individual experiments can be found in the following section. A collisional activation period of ~300 ms was typically used with a resonance excitation voltage of ~200−800 mV_{p-p} applied to the end caps. After the collisional activation period, anions were injected into the ion trap for ~20 ms followed by another cation/anion mutual storage period of ~100−200 ms. During this period, the majority of the product ions were reduced to the +1 charge state.[34] Mass analysis by resonance ejection for these spectra (termed herein as post-ion/ion MS/MS spectra) was performed at either 15 000 Hz and 1.5 V_{p-p}, to give an upper mass-to-charge limit of 14 450, or 89 202 Hz and 10.5 V_{p-p}, to give an upper mass-to-charge limit of 2600.

To represent the relative abundances of the product ions formed by cleavage at each amide bond along the protein backbone, the complementary b- and y-type product ion abundances for a given charge state were summed and expressed as a fraction of the total product ion abundance and plotted versus the amino acid sequence. It was noted that the detection sensitivity of product ion spectra collected at the lower mass range was ~1.5 times that of those collected at high-mass range. Therefore, the abundances of the ions acquired at the low-mass range were divided by a factor of 1.5 prior to summing their abundances with those from the high-mass range.

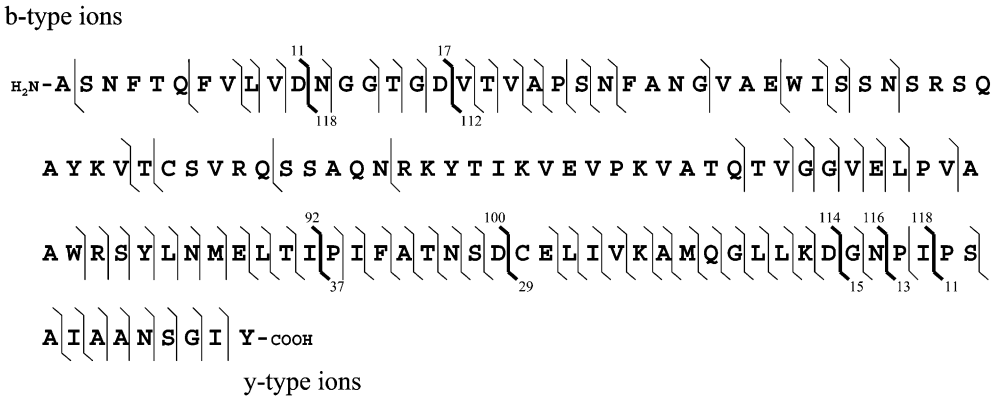
RESULTS AND DISCUSSION

Charge-State-Dependent Fragmentation of the Bacteriophage MS2 Coat Protein. To better characterize the fragmentation behavior of the bacteriophage MS2 viral coat protein, we have studied a wider range of individual charge states than those previously examined, including those below +7, where favored fragmentation C-terminal to aspartic acid residues is expected to be observed.[35,36] As shown in Chart 1, there are four aspartic acid residues (Asp11, Asp17, Asp100, Asp114) in the amino acid sequence.

With the instrumental conditions and spray solution composition used in this work, the highest charged species observed was

(31) Van Berkel, G. J.; Glish, G. L.; McLuckey, S. A. *Anal. Chem.* **1990**, *62*, 1284−1295.
(32) ICMS software provided by N. Yates and the University of Florida.
(33) Stephenson, J. L., Jr.; McLuckey, S. A. *Int. J. Mass Spectrom. Ion Processes* **1997**, *162*, 89−106.
(34) McLuckey, S. A.; Stephenson, J. L., Jr.; Asano, K. G. *Anal. Chem.* **1998**, *70*, 1198−1202.
(35) Tsaprailis, G.; Nair, H.; Somogyi, A.; Wysocki, V. H.; Zhong, W. Q.; Futrell, J. H.; Summerfield, S. G.; Gaskell, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 5142−5154.
(36) Tsaprailis, G.; Somogyi, A.; Nikolaev, E. N.; Wysocki, V. H. *Int. J. Mass Spectrom.* **2000**, 195/*196*, 467−479.

Chart 1



the $[M + 13H]^{13+}$ ion. However, the $[M + 12H]^{12+}$ ion constituted the most highly charged ion for which it was feasible to perform the tandem MS ion/ion reaction experiment. The $[M + 7H]^{7+}$ ion was the lowest charge state formed directly by electrospray. However, product ion spectra for charge states of the bacteriophage MS2 coat protein ranging from $[M + 12H]^{12+}$ to $[M + 5H]^{5+}$ were collected with the benefit of ion/ion proton-transfer reactions for the formation of lower charge-state precursors than those formed directly by ESI. The efficiency for collisionally activating low-charge states of high-mass ions in quadrupole ion traps is complicated by competition between ion activation and ion ejection, due to the relatively shallow well depths within which high mass-to-charge ratio ions are stored. Furthermore, limited structural information is generally obtained from the low-charge states as small molecule losses including NH_3 and H_2O dominate the product ion spectrum.[16,23] Therefore, charge states lower than the $[M + 5H]^{5+}$ ion were not examined here. In the case of the $[M + 12H]^{12+}$charge state, the ion was simply isolated and subjected to collisional activation due to the fact that it was not feasible to perform double isolation from the next highest charge state. Therefore, some low-level chemical noise was observed in the post-ion/ion spectra of this charge state. For other parent charge states, two ion parking periods were used to "charge-state purify" the precursor ions prior to collisional activation, thereby resulting in significantly lower background chemical noise. In this process, the first ion parking period was used to park ions of one charge state greater than that of the parent ion of interest. This ion population was isolated and then shifted to the next lower charge state by a second ion parking period. Next, this newly parked precursor ion charge state was isolated and then collisionally activated. After reduction of the resultant multiply charged product ion population to predominantly the +1 charge state by further ion/ion reactions, the post-ion/ion reaction product ion spectrum was collected. Under the on-resonance collisional activation conditions employed here, first-generation product ions tend to be favored so that each cleavage leads to both an N-terminal b-type product and the complementary C-terminal y-type product. Both products can be detected provided that they each carry charge and are within the mass scan range. Due to the limits of the observable mass-to-charge range at the low resonance ejection frequencies employed here, low-m/z ions do not appear under the same acquisition conditions as high-m/z ions, which consequently results in the loss of some of the fragmentation information. Therefore, in this study, by varying the acquisition conditions, product ion spectra at both high-m/z range, covering m/z 2450−14450, and low-m/z range, covering 640−2600, were recorded.

Figure 1 is an illustrative case whereby the $[M + 8H]^{8+}$ ion of bacteriophage MS2 coat protein was dissociated in the ion trap and spectra were acquired at both high- and low-m/z range (Figure 1A and B, respectively). Each spectrum was normalized according to its most abundant product ion peak. The majority of high-m/z range fragments corresponded to b-type ions wherein charge resides on the N-terminal side of the cleavage while the low-m/z range fragments corresponded to the complementary y-type ions. As noted previously,[24] at this relatively high charge state, cleavages at the N-terminal of proline residues, resulting in the products b_{118}/y_{11} (Ile^{118}−Pro^{119}), b_{116}/y_{13} (Asn^{116}−Pro^{117}), and b_{92}/y_{37} (Ile^{92n}−Pro^{93}), were the dominant fragmentation channels. The CID spectra of the $[M + 8H]^{8+}$ charge state of bacteriophage MS2 coat protein exhibit significant similarities in terms of the number and relative abundances of the observed fragmentation

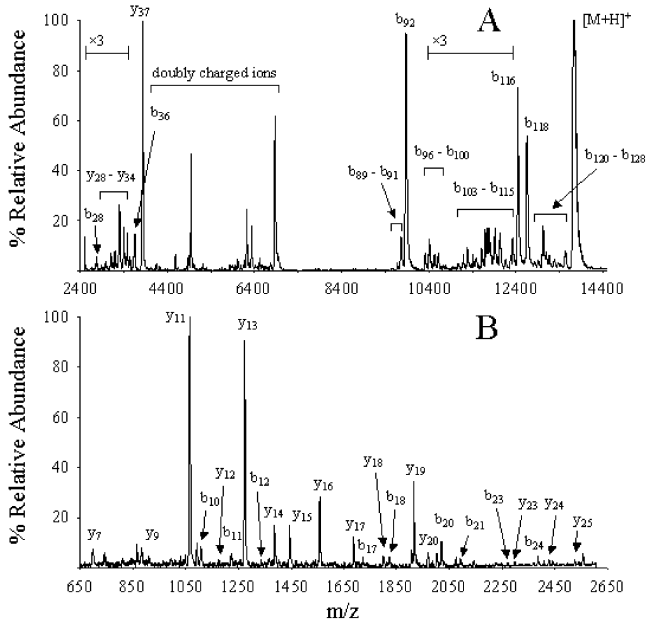


Figure 1. Post-ion/ion reaction CID MS/MS product ion spectra of the $[M + 8H]^{8+}$ ion of bacteriophage MS2 coat protein acquired under resonance ejection conditions of (A) 15 000 Hz and 1.5 V_{p-p} and (B) 89 202 Hz and 10.5 V_{p-p}.

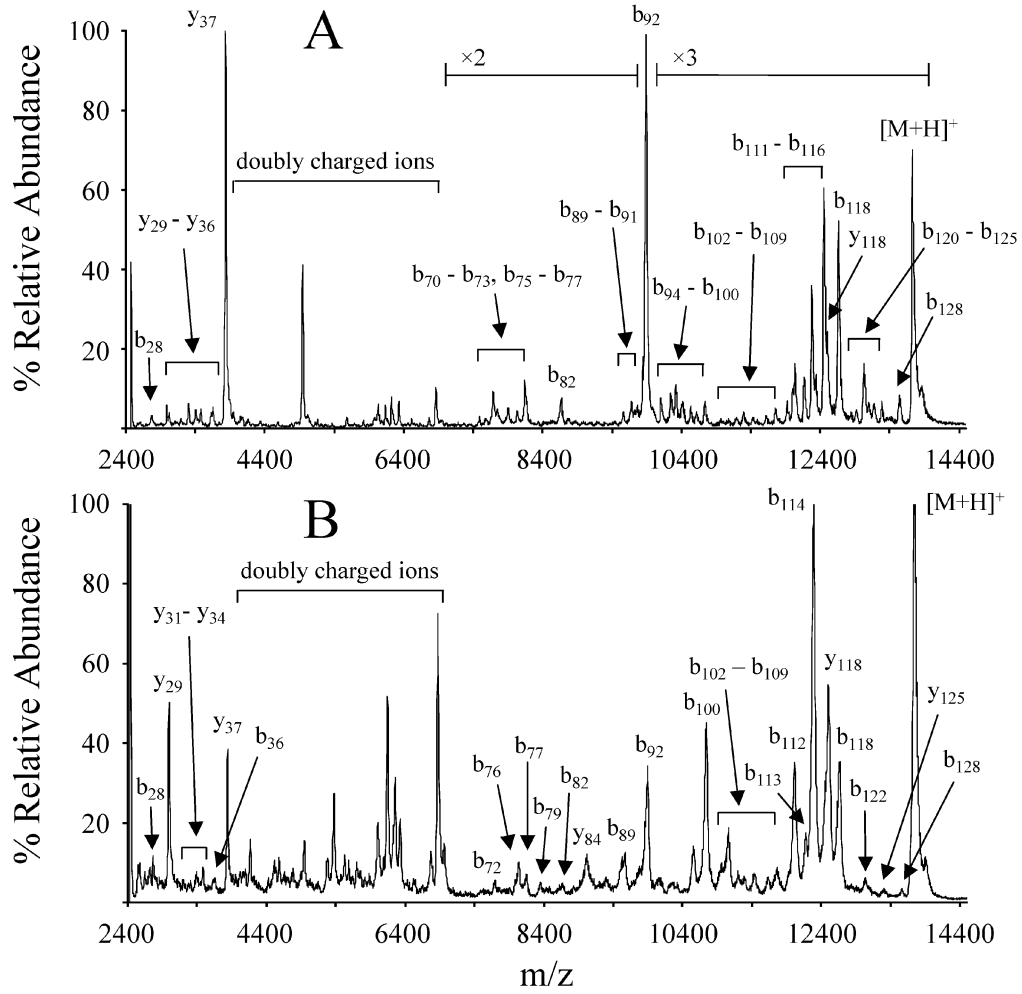


Figure 2. Post-ion/ion reaction CID MS/MS product ion spectra of (A) the $[M + 7H]^{7+}$ and (B) $[M + 6H]^{6+}$ ions of bacteriophage MS2 coat protein collected under resonance ejection conditions of 15 000 Hz and 1.5 V_{p-p}.

channels compared to previously reported results. However, the quality of the spectra shown here has been dramatically improved over previous results as a result of the ion parking procedure. Therefore, series of adjacent fragmentations, including those giving rise to $b_{89}-b_{92}$, $b_{96}-b_{100}$, $b_{103}-b_{116}$, and $b_{120}-b_{128}$ in the high-m/z range and $y_{11}-y_{20}$ in the low-m/z range could be easily observed and used as "sequence tags" for the identification of this protein, if necessary. This is an expansion in the sequence tag length compared to that reported previously, where only the $b_{104}-b_{115}$ and $b_{120}-b_{125}$ sequences were obtained from the same charge state. The expanded sequence information likely resulted from the increased precursor ion abundance afforded by the ion parking technique.

In the case of charge states ranging from $[M + 12H]^{12+}$ to $[M + 9H]^{9+}$, the product ion spectra were dominated by N-terminal proline cleavages. The b_{92}/y_{37} fragmentation channel constituted the most abundant cleavage in the product ion spectra of the $[M + 12H]^{12+}$ to $[M + 10H]^{10+}$ and the $[M + 8H]^{8+}$ ions. Interestingly, this fragmentation channel showed a reduced relative contribution in the product ion spectrum of the $[M + 9H]^{9+}$ ion. In this charge state, cleavage of the protein to form the b_{116}/y_{13} ion pair contributed most heavily to the spectrum.

As the charge states of the ions decreased, the major fragmentation channels switched from preferential proline cleavages to aspartic acid cleavages, as shown in Figure 2, where the post-ion/ion spectra of the $[M + 7H]^{7+}$ and $[M + 6H]^{6+}$ ions were displayed (high-m/z range for each). Although the proline cleavage at b_{92}/y_{37} dominated the product ion spectrum of the $[M + 7H]^{7+}$ ion, as it did for the $[M + 8H]^{8+}$ ion, the proline cleavages giving rise to the b_{116} and b_{118} ions made far smaller contributions. At the same time, aspartic acid cleavages giving rise to b_{114} and b_{100} ions made significant contributions. As the charge state decreased further, the product ion spectrum of the $[M + 6H]^{6+}$ ion was dominated by preferential cleavage C-terminal to the four aspartic acid residues (Asp11, Asp17, Asp100, Asp114) while the cleavages N-terminal to proline were diminished. Small molecule losses, corresponding to NH_3 or H_2O, in conjunction with amide bond cleavages, were also observed in the fragmentation of this charge state. This was especially obvious in the low-m/z range product ion spectrum (data not shown). A variety of nonspecific amide bond cleavages also contributed to the spectra of Figure 2. For the $[M + 5H]^{5+}$ ion, aspartic acid cleavages dominated the spectra and losses of small molecules (NH_3 or H_2O) were also observed, which was especially noteworthy in the low-m/z range.

To represent the relative abundances of the product ions formed by cleavage at each amide bond in the protein, each of the complementary b- and y-type product ion abundances for a given charge state were summed, expressed as a fraction of the

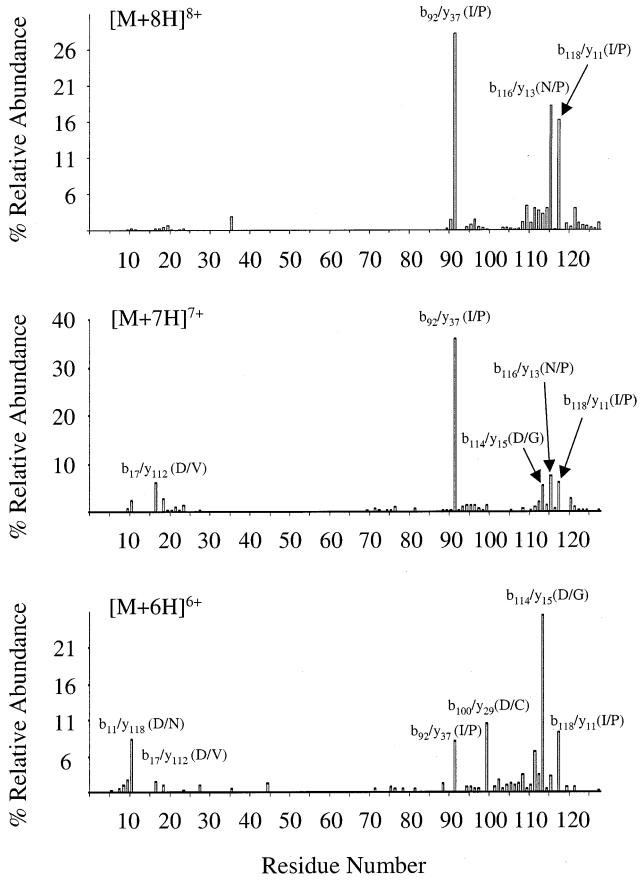


Figure 3. Relative abundances of the product ions plotted against the amino acid sequence. The complementary b- and y-type product ion abundances from the charge states (A) $[M + 8H]^{8+}$, (B) $[M + 7H]^{7+}$, and (C) $[M + 6H]^{6+}$ were summed and expressed as a fraction of the total product ion abundance.

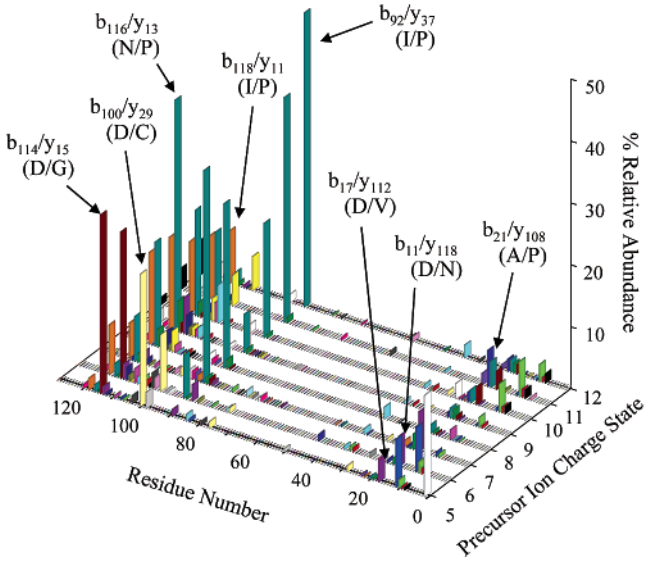


Figure 4. Dissociation map of bacteriophage MS2 coat protein as a function of precursor ion charge over the range of $[M + 5H]^{5+}$ to $[M + 12H]^{12+}$ precursor ion charge states. The *x*-axis represents the 129 consecutive amino acid residues within bacteriophage MS2 coat protein where, for example, residue 92 corresponds to a Pro residue and the b_{92}/y_{37} complementary ion pair. The *y*-axis represents the protein precursor ion charge states ranging from $[M + 5H]^{5+}$ to $[M + 12H]^{12+}$. The *z*-axis represents the sum of the ion abundances corresponding to the dissociation of a given amide bond normalized to the sum of the observed product ion abundances.

total product ion abundance, and plotted versus amino acid sequence (see Figure 3A–C for the +8, +7, and +6 charge states, respectively). This information was drawn from 500 scan averages for each charge state. For those ions acquired using different resonance ejection conditions, the abundances of the ions obtained from the low-m/z range were divided by a normalization factor of 1.5 to account for higher detection sensitivity compared to the high-m/z range product ions. Only signals with abundances equal to or greater than 0.1% of the total product ion abundance were included in this plot. The change in the fragmentation behavior with charge state can easily be observed by comparing the data obtained for the +8 and +7 charge states with the +6 charge state.

To summarize the precursor ion charge-state-dependent fragmentation for all of the charge states of bacteriophage MS2 coat protein studied here, the data from each of the charge states ranging from $[M + 5H]^{5+}$ to $[M + 12H]^{12+}$ have been presented in a format whereby the charge states and residue number are shown in the *x* and *y* dimensions, respectively, and the relative contribution of each dissociation channel are indicated in the *z* dimension (see Figure 4). This display provides a "dissociation map" for this protein. The dominant cleavages for the relatively high charge states (from +12 down to +8) occurred mostly N-terminal to proline residues. At intermediate charge states (+7, +6), proline cleavages were still observed while the relative abundances of fragmentations C-terminal to aspartic acid residues increased as the precursor ion charge state decreased. In addition, these precursor ions exhibited the highest degree of nonspecific fragmentation. A total of 80 out of 128 amide bond cleavages (63%) were observed when the data from all of the charge states examined were combined (summarized in Scheme 1). A total of 45 cleavages were observed from dissociation of the $[M + 7H]^{7+}$ ion, which was the single charge state giving the most informative structural information. The most comprehensive sequence coverage was obtained in the C-terminal region of the protein while less comprehensive coverage was observed in the N-terminal region of the protein. Little fragmentation was observed in the middle of the sequence. The overall tendencies for fragmentation of the bacteriophage MS2 coat protein as a function of precursor ion charge state share commonalities with the limited number of proteins thus far studied over a wide charge-state range.[20,22,23] Cleavages at the N-terminal side of proline residues are often observed at high-charge states whereas fragmentations C-terminal to aspartic acid residues are often observed at moderately low charge states. Intermediate charge states tend to show the greatest degree of nonspecific fragmentation. In this case, the +7 and +6 charge states showed the most extensive nonspecific fragmentations. This overall behavior has been interpreted on the basis of proton mobility. At high-charge states, proton mobility is limited by the Coulomb field. Cleavages N-terminal to proline are favored on the basis of the relatively high basicity of the secondary nitrogen at this site. At lower charge states, proton mobility is limited by the fact that the protons are situated at the most basic sites. Cleavages C-terminal to aspartic acid residues dominate because this process does not require a mobile proton to catalyze the cleavage.[35,37] At intermediate-charge states, proton mobility

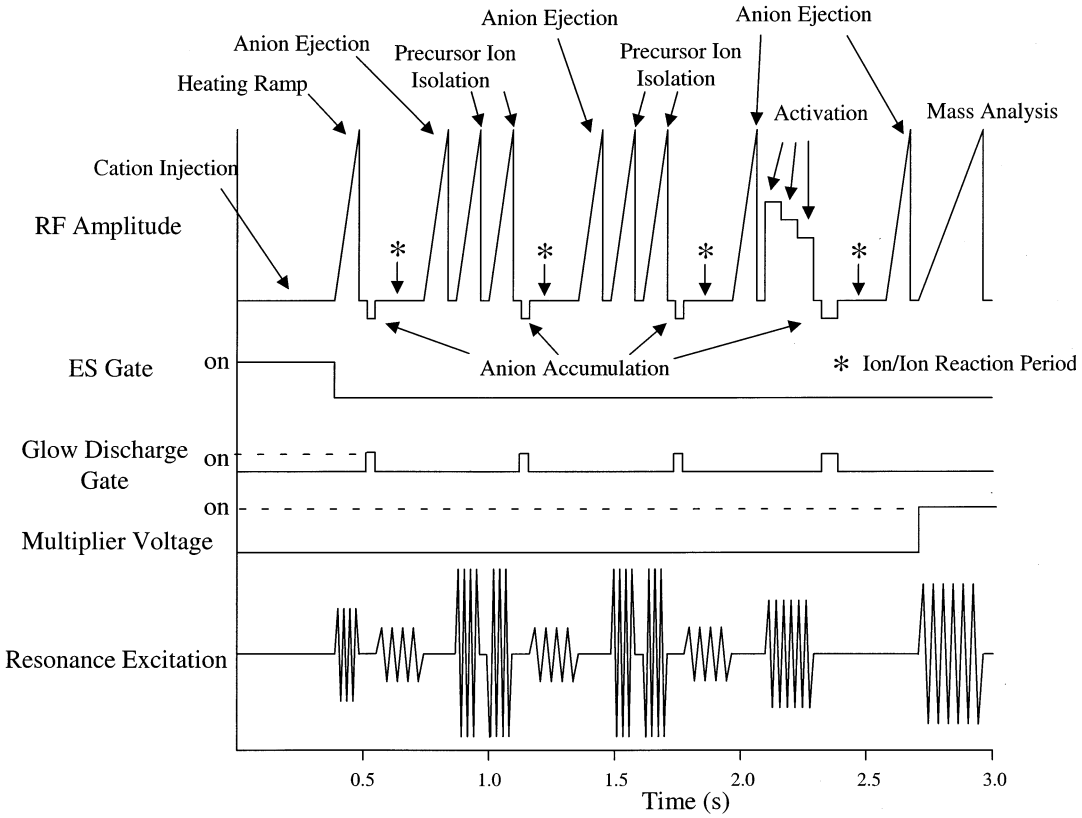


Figure 5. Schematic diagram of the experimental scan function used for sequential fragmentation of multiple precursor ion charge states.

tends to be greatest because the Coulomb field is lower than that at high-charge states but the number of protons exceeds the number of arginine residues in the sequence, allowing migration of "mobile" protons along the protein amide backbone. At very low charge states, losses of small molecules dominate presumably due to a high degree of intramolecular charge solvation of the protons located on the most basic sites.[38]

While the overall dissociation behavior of the protein is largely consistent with other unmodified proteins studied to date, several specific observations are unique to this protein. For example, the relative lack of fragmentation in the middle of the sequence stands out. Another unexplained observation is the marked decrease in the b_{92}/y_{37} cleavage channel in the +9 charge state, which is accompanied by an increase in the abundance of b_{116}/y_{13} cleavage channel. Similar behavior was noted in the previous study of this protein. Neither of the multiply charged product ions corresponding to this cleavage fall within the m/z region associated with the resonance excitation frequency used for this charge state and would therefore not be expected to be ejected or further fragmented, thus depleting their ion signals. It is therefore unclear why this channel, which is dominant for the +8 and +10 charge states, makes a relatively small contribution to the +9 ion.

Product Ion Information from Multiple-Parent Ion Charge States Following Gas-Phase Protein Ion Purification and Concentration. For the purpose of rapid protein identification and characterization, it is often desirable to obtain product ion spectra from multiple-parent ion charge states. Doing so increases the probability that sufficient structural information will be forthcoming for confident identification. However, the acquisition of product ion spectra on individual charge states using separate ion concentration/purification procedures for each parent ion is not likely to be the most efficient approach for protein identification. We have therefore evaluated an approach for obtaining product ion fragmentation data from multiple-parent ion charge states following a single ion concentration/purification process.

The procedure involved the sequential ion parking steps ordinarily used to concentrate and purify a single-parent ion charge state for subsequent collisional activation. However, before ion activation was effected, a short ion/ion reaction period was used to produce a distribution of parent ion charge states from the original purified charge state. After producing this range of charges states, resonance excitation of two or more of the parent ion charge states was applied sequentially. Two different ion acceleration methods for collision activation were evaluated, including on-resonance and off-resonance excitation. In on-resonance excitation, a relatively low voltage (~0.3−1.6 V_{p-p}) is applied at a frequency corresponding to the center of the parent ion m/z ratio. In this scenario, the excitation frequency is within the range of fundamental z-dimension secular frequencies of the precursor ion population. In off-resonance excitation, originally described and referred to as "red-shifted off-resonance large amplitude excitation",[39] a much higher voltage (~4.0−8.0 V_{p-p}) is usually applied at ~1 kHz below the frequency of the on-resonance excitation. One of the major advantages of on-resonance

(37) Yu, W.; Vath, J. E.; Huberty, M. C.; Martin, S. A. *Anal. Chem.* **1993**, *65*, 3015−3023.

(38) Schnier, P. D.; Price, W. D.; Jockusch, R. A.; Williams, E. R. *J. Am. Chem. Soc.* **1996**, *118*, 7178−7189.

(39) Qin, J.; Chait, B. T. *Anal. Chem.* **1996**, *68*, 2108−2112.

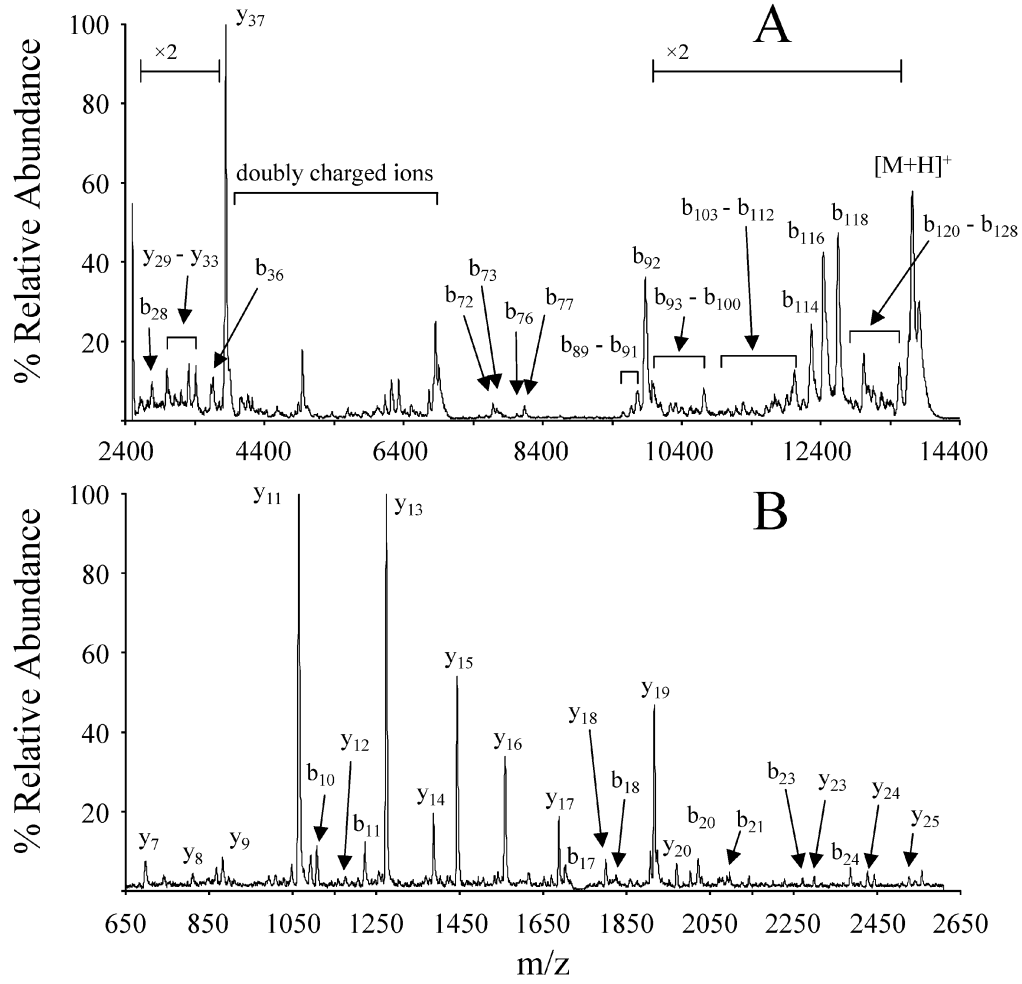


Figure 6. Composite post-ion/ion reaction CID MS/MS spectra from the sequential fragmentation experiment involving the $[M + 8H]^{8+}$, $[M + 7H]^{7+}$, and $[M + 6H]^{6+}$ charge states acquired under resonance ejection conditions of (A) 15 000 Hz and 1.5 V_{p-p} and (B) 89 202 Hz and 10.5 V_{p-p}.

excitation for multiply charged parent ions is that the potential for ejection of product ions is reduced. Under off-resonance activation conditions, due to the ejection or further fragmentation of any product ions that fall within the fairly large m/z window affected by the excitation signal, a region of the product ion spectrum devoid of any signal can be observed.[20] Furthermore, it has been noted that, compared to on-resonance excitation, off-resonance excitation appears to be less effective in inducing fragmentation as the parent ion m/z increases, due to increasing ion ejection from the trap with higher activation amplitudes.[20] Therefore, in previous studies on the dissociation of intact proteins, on-resonance excitation has been more commonly applied. However, the effectiveness of on-resonance excitation is more highly sensitive to tuning conditions than off-resonance excitation. This is due to relatively narrow effective ion acceleration bandwidth and its sensitivity to space charge effects. Tuning for on-resonance excitation was also found to be problematic when multiple precursor ion charge states were present. Due to normal fluctuations in ion signals from one scan to the next, it was difficult to find on-resonance excitation conditions that were consistently effective for all parent ion charge states.

Off-resonance excitation samples a much larger proportion of an isolated parent ion population due to a relatively broad effective ion acceleration bandwidth. It was found that this bandwidth could accommodate scan-to-scan ion number variations. Therefore, in this study, off-resonance excitation conditions were employed in which three parent ion charge states (from +8 to +6) of bacteriophage MS2 coat protein were fragmented following a single ion accumulation period and a single protein ion concentration and purification procedure. These three charge states were chosen because, as discussed above, the activation of parent charge states ranging from +8 to +6 yielded most of the characteristic fragmentation channels representative of both high- and low-charge states.

The scan function for the multiple precursor ion sequential activation experiment is illustrated in Figure 5. Following an ionization period of 100−300 ms, a "heating ramp" was employed to remove any adducts attached to the initial precursor ion population, by applying a low-amplitude single-frequency resonance excitation voltage to the end caps while simultaneously sweeping the amplitude of the rf applied to the ring electrode. Ion parking and isolation steps were then applied to concentrate ion signals into the +9 charge state. The isolated +9 ion population was then further reduced to the +8 charge state by a second ion parking and isolation process. These two ion parking and isolation periods purified the bacteriophage MS2 coat protein ion signal from possible overlapping m/z values of contaminants associated with the initially isolated m/z range. A short ion/ion

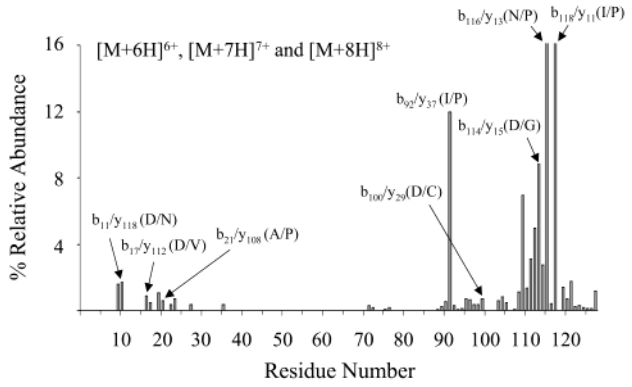


Figure 7. Percent relative abundance of the summed b- and y-type product ions for dissociation of the $[M + 8H]^{8+}$, $[M + 7H]^{7+}$, and $[M + 6H]^{6+}$ ions of bacteriophage MS2 coat protein, formed after gas-phase concentration and purification of the $[M + 8H]^{8+}$ precursor ion.

reaction step was then used to distribute the ion signal into a range of charge states from +8 to +6, which were then subjected to CID under off-resonance activation conditions. The products of these CID steps were reduced to +1 charge state with a final ion/ion reaction period.

The post-ion/ion product ion spectra at both high- and low-m/z ranges for these three contiguous charge states are shown in Figure 6A and B, respectively. The relative abundances of the product ions formed by specific cleavages at each amide bond throughout the amino acid sequences, expressed as a fraction of the total product ion abundance, are shown in Figure 7.

Under on-resonance conditions, activation of the individual +6 to +8 charge states resulted in 62 cleavages along the amino acid sequence. In contrast, 49 fragmentation channels were observed from the data obtained via the off-resonance sequential activation experiment of these three charge states. Although the number of cleavages obtained from the sequential activation experiment was less than that observed from the three individual charge states, it was more than what could be obtained from any single charge state. All of the preferential C-terminal aspartic acid and N-terminal proline cleavages observed in the on-resonance CID spectra of the individual charge states were also observed in the off-resonance spectra. Therefore, the sequential activation strategy provided an approach to obtain more comprehensive fragmentation information than could be obtained from a single charge state alone. As can be seen in Figure 7, cleavage N-terminal to proline at three sites accounted for 46% of the product ion abundance, while C-terminal aspartic cleavage contributed 13% to the total signal. Nonspecific fragmentations along the amino acid backbone summed to 41% of the total cleavages observed. Fragmentation favoring the proline cleavage over aspartic acid cleavage is likely due to the greater relative abundance of the +8 charge state compared to the +6 charge state in the initial ion population. By varying the time of the third ion/ion reaction period, the relative abundance of the three charge states could be varied and consequently the relative abundances of the product ions could also be manipulated (data not shown). Another source of product ion discrimination occurs when product ions fall within the fairly large m/z window affected by the excitation signals applied under off-resonance conditions and undergo ejection or further fragmentation.

CONCLUSIONS

The charge-state-dependent fragmentation behavior of protonated bacteriophage MS2 coat protein ions ranging from $[M + 12H]^{12+}$ to $[M + 5H]^{5+}$ has been examined by tandem mass spectrometry using electrospray ionization coupled to a quadrupole ion trap mass spectrometer. Gas-phase ion/ion proton-transfer reactions combined with ion parking of selected charge states have been used to purify and concentrate parent ion charge states as well as to simplify interpretation of product ion spectra. The overall charge-state-dependent dissociation behavior of the bacteriophage MS2 coat protein parallels that of other proteins studied to date. At high-charge states, cleavage N-terminal to proline residues dominates the product spectra, while at low-charge states, the dominant fragmentation channels involve preferential cleavage C-terminal to aspartic acid residues. The intermediate-charge states tend to yield the maximum structural information. Sequential activation of multiple charge states in a single experiment has been shown to be a feasible method for obtaining fragmentation from multiple parent ion charge states after concentration and purification of the proteins ions in the gas phase, thereby yielding structural information from both high and low parent ion charge states in a single experiment.

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