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Gaseous apomyoglobin ion dissociation in a quadrupole ion trap: $[M + 2H]^{2+}$–$[M + 21H]^{21+}$

Kelly A. Newton, Paul A. Chrisman, Gavin E. Reid, J. Mitchell Wells, Scott A. McLuckey*

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

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Abstract

The dissociation of the multiply protonated ions of apomyoglobin ranging in charge from $[M + 2H]^{2+}$ to $[M + 21H]^{21+}$ have been studied using collisional activation and ion/ion reactions in a quadrupole ion trap. A variety of collisional activation conditions were explored for each charge state to determine optimal conditions for yielding the highest quality product ion spectra. Product ion spectra for charge states greater than $[M + 6H]^{6+}$ were acquired using both on-resonance and off-resonance collisional activation, with on-resonance activation conditions providing the highest quality spectra. Product ion spectra for the lowest charge states could only be acquired using on-resonance collisional activation. The lowest charge states show a high propensity for losses of small molecules, as well as a number of favored amide bond cleavages, such as fragmentation C-terminal to aspartic acid residues. A novel, dominant cleavage between adjacent lysine-histidine residues was also observed, particularly for charge states higher than $[M + 6H]^{6+}$. The largest number of structurally informative fragments, corresponding to b-type or y-type product ions, were produced from the intermediate charge states of $[M + 10H]^{10+}$ to $[M + 14H]^{14+}$. The product ion spectra for the charge states of $[M + 15H]^{15+}$ and higher were dominated by the y_{151} ion, which appeared to be related to protonation of the N-terminus and, possibly, a secondary structure effect. The overall charge state dependent fragmentation behavior of apomyoglobin ions parallels that of other protein ions studied to date using a quadrupole ion trap in that the most extensive structural information is yielded by parent ions of intermediate charge states. This behavior is consistent with these intermediate charge states either being comprised of a diversity of parent ion structures, having a relatively high degree of proton mobility, or a combination of both. (Int J Mass Spectrom 212 (2001) 359–376)

Keywords: Apomyoglobin ions; Electrospray ionization; Quadrupole ion trap; Collisional activation; Ion/ion reactions

1. Introduction

The speed, specificity, and sensitivity of tandem mass spectrometry, along with the structural information it can provide via fragmentation reactions, makes it especially attractive for use in strategies requiring rapid protein identification. The strengths of tandem mass spectrometry can be applied in the biological arena due to the advent of ionization techniques such as electrospray ionization (ESI) [1–5] and matrix assisted laser desorption ionization (MALDI) [6–8], which allow for the ionization of, for example, pep-

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

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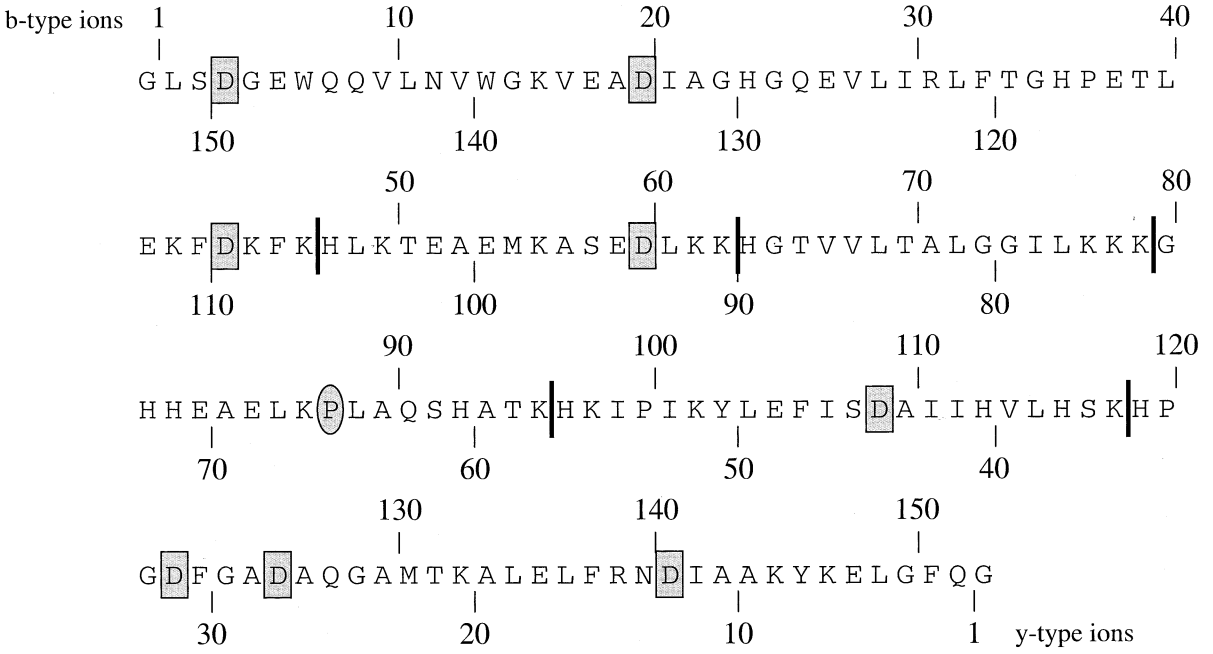
tides, proteins, and oligonucleotides. In addition to providing a method for ionizing these species, the multiple charging phenomenon associated with ESI facilitates the fragmentation of high mass ions as well as the analysis of these ions using mass analyzers of modest mass-to-charge range.

Obtaining protein sequence information has long been of interest, but only in the last ten years has mass spectrometry played a major role. Prior to this, conventional methods for determining the primary sequence of a protein employed Edman degradation of intact proteins or peptides resulting from chemical degradation or enzymatic digestion [9]. Tandem mass spectrometry has advantages in speed and sensitivity over condensed-phase peptide sequencing approaches and, as a result, has seen increased use as a tool in protein science.

A commonly employed strategy for protein identification involves proteolytic digestion of the protein of interest followed by mass analysis of the peptide fragments. The masses of these peptides are characteristic of the protein, and provide a protein "mass fingerprint" which can be used in database searches to identify the protein [10–14]. A more comprehensive approach is to subject each of the proteolytically derived peptides to tandem mass spectrometry [15–17]. Subsequent identification of each peptide may be performed either by protein sequence database analysis of the uninterpreted product ion spectra (e.g. SEQUEST [18–20]) or, provided that sufficient fragmentation occurs between adjacent residues of the peptide ion to allow the generation of "sequence tags," through database searching of a partially derived amino acid sequence [21].

An alternative approach to protein identification is to obtain primary sequence information directly from dissociation of the intact protein (i.e. without prior recourse to digestion). In this scenario, intact protein parent ions are fragmented in the gas-phase via one or more dissociation techniques tandem mass spectrometry (MS/MS) and, if necessary, the resulting product ions are subjected to further dissociation (MS^n) until sufficient fragmentation occurs to allow for the identification of the protein by the sequence tag strategy [22,23] or through determination of the complete amino acid sequence [24–29]. This is referred to as "top-down" sequencing [30]. The top-down sequencing approach has significant potential advantages in speed over alternative sequencing methods. However, inducing fragmentation between every residue of an intact protein can be challenging, even with the use of MS^n, such that sequence coverage is often incomplete. Therefore, as the fragmentation of peptide and protein ions plays a key role in several strategies for protein identification, studying the dissociation of peptide and protein ions under a variety of conditions is warranted in order to maximize the structural information that can be obtained. Although the fragmentation of peptides has been extensively studied in a variety of tandem mass spectrometers and under a wide range of conditions [31–40], the fragmentation of whole protein ions has received far less attention. Early studies of whole proteins using tandem mass spectrometry were performed using triple quadrupole instrumentation [41–48]. More recently, Smith and coworkers [49–52], as well as McLafferty and coworkers [53–66], have emphasized whole protein ion fragmentation using high magnetic field strength Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometry. The high resolving power and mass accuracy of FT-ICR allows for the interpretation of the complicated product ion spectra produced by fragmentation of multiply charged protein ions. A variety of activation techniques have been examined in conjunction with FT-ICR including several collisional activation methods [53,54], photodissociation [56,67,68], surface-induced dissociation [55], and electron-capture dissociation [26,28,29,58–62].

Effort has also been extended toward the use of the quadrupole ion trap for collisional activation of whole protein ions. A major limitation of this approach is that quadrupole ion traps typically operate at resolving powers too low to resolve the isotope spacings of product ions greater in mass than a few kDa. Therefore, a key element in the use of quadrupole ion traps for the identification of proteins by dissociation of their intact multiply charged ions is the application of ion/ion proton transfer reactions to reduce the multiply charged product ions largely to the +1 charge



Scheme 1. The amino acid sequence of horse heart apomyoglobin. The sites where preferential cleavage was observed are indicated as follows: C-terminal to aspartic acid (shaded boxes), N-terminal to proline (shaded ovals), between adjacent lysine - histidine and in a region containing a number of adjacent lysine residues (bold lines).

state [69]. In addition, a wider range of precursor ions may be generated by performing ion/ion reactions prior to dissociation, compared to those formed by ESI alone. Results for a broad range of multiply charged ions of ubiquitin [70] ($[M + 4H]^{4+}$ to $[M + 12H]^{12+}$), insulin [71] ($[M + 1H]^{+}$ to $[M + 5H]^{5+}$), and human hemoglobin β-chain [72] ($[M + 2H]^{2+}$ to $[M + 17H]^{17+}$) have been reported. A more detailed study of the ubiquitin system, including the $[M + 1H]^{+}$ to $[M + 12H]^{12+}$ charge states, has recently been completed [73]. In general, many of the "preferred cleavages" commonly observed with peptide ions have also been seen in multiply charged protein ions [38,46,74–78]. These include cleavage C-terminal to aspartic acid and, to a lesser extent, glutamic acid, as well as cleavage N-terminal to proline residues. However, these preferred cleavages are not always observed and other types of cleavages can sometimes be competitive, depending on a number of variables that can play a role in directing protein ion fragmentation. These variables are expected to include activation conditions, parent ion charge state, protein ion primary structure, secondary structure, and perhaps, tertiary structure, as well as the presence of post-translational modifications such as disulfide linkages [79]. If gas-phase protein ion fragmentation is to be used as a general tool for protein identification, an exploration of these variables is required.

In the work presented here, ion trap collisional activation and ion/ion proton transfer reactions were applied to a systematic study of the protonated ions of gaseous apomyoglobin (Scheme 1). Myoglobin constitutes a "standard" protein in mass spectrometry and the multiply charged ions derived there from have been studied by a variety of other techniques. The dissociation behavior of apomyoglobin ions, however, has not been extensively studied [80–82]. Product ion spectra of apomyoglobin parent ions ranging in charge state from $[M + 2H]^{2+}$ to $[M + 21H]^{21+}$ have been collected over a range of ion trap collisional activation conditions. The data presented here demonstrate the variety of fragmentation information

that can be obtained using ion trap collisional activation and ion/ion chemistry. It also provides a new contribution to the current "database" of fragmentation behavior of multiply charged intact protein ions.

2. Experimental

Horse heart holomyoglobin was obtained from Sigma (St. Louis, MO). Perfluoro-1,3 dimethylcyclohexane (PDCH) was purchased from Aldrich (Milwaukee, WI). Apomyoglobin samples for electrospray were prepared by dissolving holomyoglobin in either methanol/H_2O/acetic acid (90:9:1) or 1% acetic acid/H_2O solutions at concentrations of ~5 μM. The methanol/H_2O/acetic acid solution was delivered to the mass spectrometer via a syringe pump at a flow rate of 1 μL/min using a stainless steel electrospray needle. Typically, the voltage applied to the electrospray needle ranged from +3.7–4.0 kV. The 1% acetic acid/H_2O solution was introduced to the mass spectrometer by nanoelectrospray ionization using a voltage of approximately +1.1 kV.

All experiments were performed using a home-built electrospray source coupled to a Finnigan ion trap mass spectrometer (ITMS) (San Jose, CA) [83] modified to allow the addition of negatively charged PDCH ions through a hole in the ring electrode [84]. A typical scan function used in this study featured positive ion accumulation (600 ms), isolation of selected multiply charged precursor ions (180 ms), collisional activation (300 ms), anion injection (45 ms), mutual cation/anion storage [ion/ion reaction (90 ms)], followed by mass analysis using resonance ejection. Apomyoglobin charge states $[M + 21H]^{21+}$ to $[M + 9H]^{9+}$ were accessible directly from the charge state distribution produced by electrospray ionization. Lower charge states were formed via ion/ion proton transfer reactions with negative PDCH ions [85]. Thus, negative ion accumulation and ion/ion reaction periods were inserted into the scan function prior to precursor ion isolation for charge states $[M + 8H]^{8+}$ and lower. Variation of the collisional activation frequency and amplitude was performed to explore the effects of these variables on the resulting fragmentation. Both on-resonance [a low amplitude (~300–600mV) frequency corresponding to the z-dimension fundamental secular frequency of the parent ion of interest] and off-resonance (~1000 Hz lower than the z-dimension fundamental secular frequency of the ion, with an amplitude of ~3.0 V) collisional activation was used to yield abundant product ions for the high to medium charge states. Product ion spectra for the lowest charge states could only be acquired using on-resonance collisional activation conditions. The off-resonance technique was originally termed "red-shifted off-resonance large-amplitude excitation" [86] by Qin and Chait in the study of the dissociation of singly charged peptides in the quadrupole ion trap using MALDI. In order to simplify the appearance of the product ion spectra for subsequent sequence analysis, the multiply charged product ions resulting from collisional activation were subjected to ion/ion proton transfer reactions, thereby reducing the charge states to predominantly $[M + H]^+$ [70]. The spectra recorded after these ion/ion reactions are referred to herein as post-ion/ion product ion spectra. Resonance ejection for these post-ion/ion spectra was performed at 12 202 Hz and 1.72 $V_{p\text{-}p}$. The spectra shown for each charge state are the average of 500–1000 individual post-ion/ion scans.

Post-ion/ion product ion spectra were calibrated using the $[M + H]^+$ and $[M + 2H]^{2+}$ charge states of apomyoglobin formed by ion/ion reactions in the absence of collisional activation. The mass accuracy associated with these measurements is roughly 300 ppm and is limited by peak under sampling by the Finnigan ITMS data system when the mass-to-charge range is extended. Product ion assignments have been restricted to b-type and y-type ions, which are the commonly observed fragment types from even-electron peptide and protein ion dissociations. In some cases, both b-type and y-type ions fall within the mass uncertainty of a particular product ion. Both possibilities are listed for some of the product ion peaks, whereas only the most likely product assignment is listed for others. Criteria used to establish the relative likelihood of the identity for product ion signals included factors such as fragmentation at a favored site (e.g. an aspartic acid, proline, or lysine residue) or

the appearance of the ion in a series of peaks suggestive of fragmentation along an adjacent series of residues.

3. Results and discussion

The appearance of a product ion spectrum is determined in part by the ion activation conditions. In the case of the quadrupole ion trap, the major variables are the amplitude of the resonance excitation waveform, how closely the excitation frequency matches that of the z-dimension secular frequency of the parent ion, bath gas pressure and identity (for example, helium versus argon), and the excitation time. The amplitude and frequency of the excitation waveform and the bath gas conditions determine the parent ion steady-state internal energy distribution during activation [87]. The excitation time defines the range of dissociation rates that can contribute to the product ion spectrum. An exhaustive systematic exploration of collisional activation conditions was beyond the scope of this study. However, a variety of conditions were explored in order to determine the sensitivity of the product ion spectra over the range of conditions that produce good product ion yields. Spectra were then recorded using conditions found to provide the best product ion signal-to-noise ratios. Results observed from a study of the collisional activation conditions are summarized first followed by an overview of the product ion spectra obtained as a function of parent ion charge.

3.1. Collisional activation conditions

3.1.1. Effect of on- versus off-resonance activation

Two different ion acceleration methods for collisional activation were used; viz., on-resonance excitation and off-resonance excitation. In on-resonance excitation, a relatively low voltage (~300–600 mV) was applied at a frequency corresponding to the center of the parent ion peak. Under this condition, the excitation frequency is very near the range of fundamental z-dimension secular frequencies associated with the parent ions oscillating in an ion trap with roughly 10% octopolar character [88,89]. In off-resonance excitation, a much higher voltage (~3.0 V) is applied at about 1 kHz below the frequency of the on-resonance excitation. The essential elements of the latter approach were first described by Qin and Chait [86]. A major disadvantage of off-resonance excitation for multiply charged parent ions is that it can result in a region of the product ion spectrum being devoid of any signal, as illustrated in Fig. 1. Here, a comparison is made between the pre-ion/ion product ion spectra for the $[M + 11H]^{11+}$ ion of apomyoglobin, obtained using off- and on-resonance collisional activation [Figs. 1(a) and 1(b), respectively]. The off-resonance collisional activation of the $[M + 11H]^{11+}$ ion shows a mass-to-charge region spanning roughly *m/z* 1540–1588 in which no ions are observed. This is due primarily to ejection, or further fragmentation, of any product ions that fall within the fairly large *m/z* window affected by the excitation signal. Off-resonance excitation, therefore, can potentially lead to a loss of information. The use of an excitation frequency that is lower than that of the parent ion results in discrimination against product ions of mass-to-charge greater than that of the parent ion. The formation of such ions only occurs with multiply charged parent ions. An advantage of off-resonance excitation is that it samples a large proportion of the isolated parent ion population as a result of the relatively large effective ion acceleration bandwidth employed. Off-resonance excitation was found to give rise to product ion spectra that were weighted more heavily toward a limited number of channels compared to on-resonance excitation. On the other hand, off-resonance excitation appeared to be less effective than on-resonance excitation at inducing fragmentation as the parent ion mass-to-charge ratio increased (i.e. as parent ion charge decreased) because of increasing ion ejection from the trap with higher activation amplitudes.

In contrast to off-resonance excitation, on-resonance excitation suffers much less from the potential for ejecting product ions. Fig. 2 compares the post-ion/ion product ion spectra produced by off-resonance [Fig. 2(a)] and on-resonance [Fig. 2(b)] excitation for the $[M + 11H]^{11+}$ charge state of apomyoglobin.

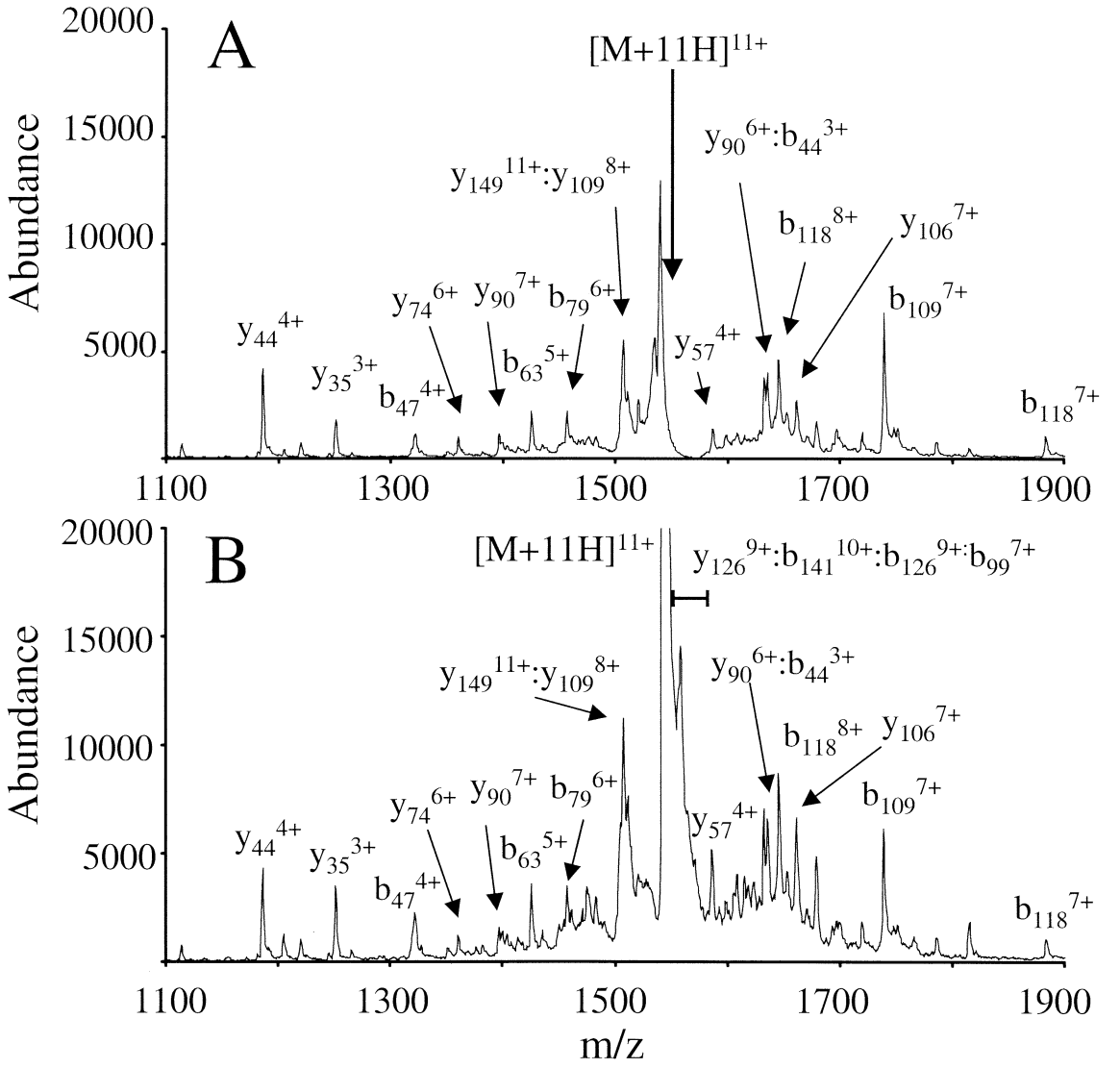


Fig. 1. Pre-ion/ion reaction MS/MS product ion spectra of the $[M + 11H]^{11+}$ apomyoglobin ion obtained using (A) off-resonance (87 470 Hz, 4016 mV, 300 ms) and (B) on-resonance (88 470 Hz, 678 mV, 300 ms) collisional activation.

Prominent, well-defined product ion signals are observed in both spectra. However, the loss of product ion information using off-resonance excitation is evident by the presence of a number of additional product ions in the on-resonance excitation spectrum, including b^+_{141}, y^+_{126}, b^+_{126}, b^+_{99}, and b^+_{98}, which are not observed in the off-resonance excitation spectrum. The multiply charged versions of these product ions likely correspond to the region of *m/z* 1540–1588 which were ejected from the trap or further fragmented during the off-resonance excitation. The on-resonance excitation spectrum exhibits an increased level of chemical noise compared to the off-resonance spectrum, most likely due to contributions from low abundance fragmentations of the protein backbone. Note that a number of product ions resolved in the post-ion/ion spectra could not be unambiguously assigned in the pre-ion/ion data. In particular, a resolv-

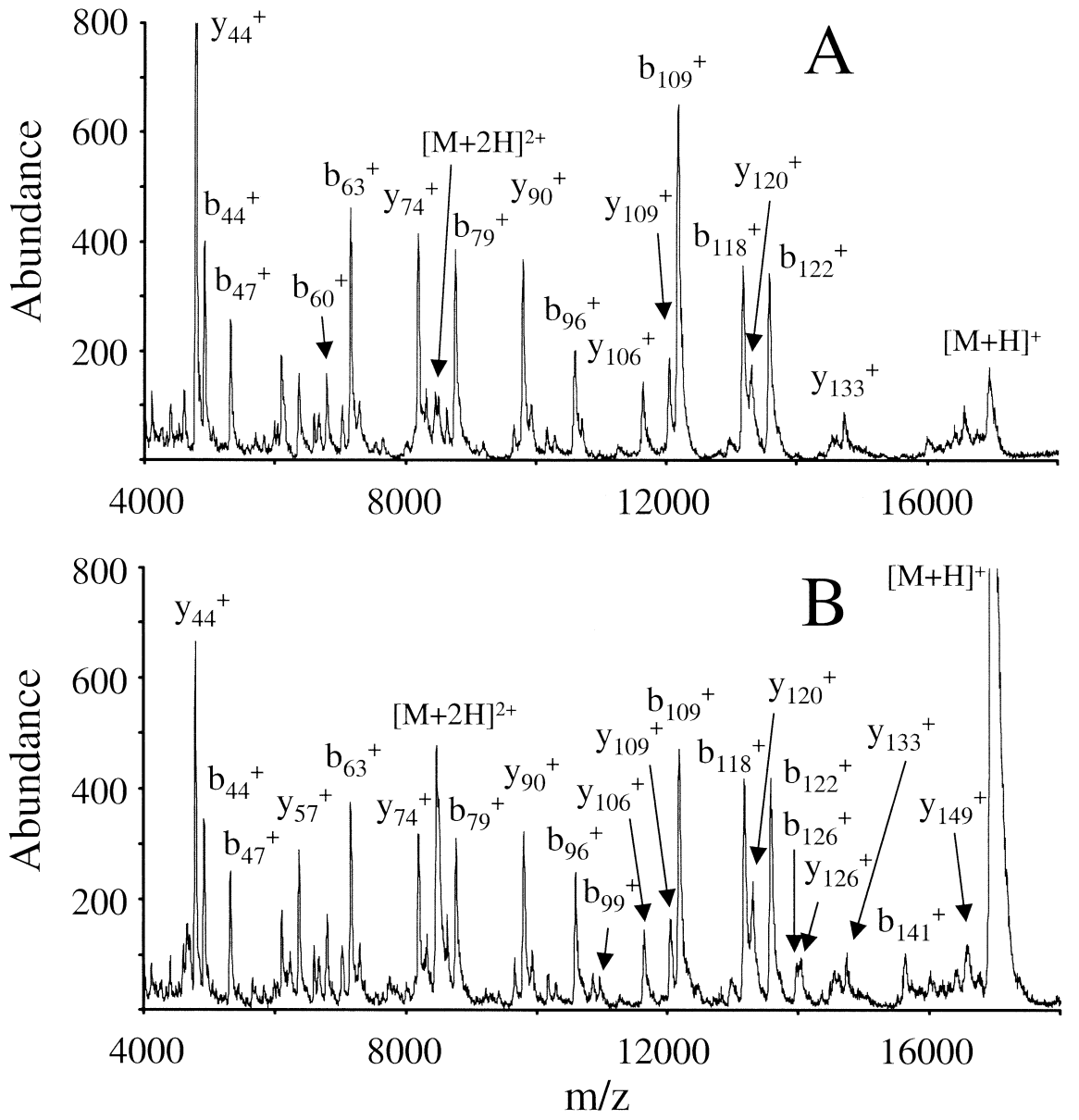


Fig. 2. Post-ion/ion reaction MS/MS product ion spectra of the $[M + 11H]^{11+}$ apomyoglobin ion obtained using (A) off-resonance (88 470 Hz, 4016 mV, 300 ms) and (B) on-resonance (88 470 Hz, 678 mV, 300 ms) collisional activation.

ing power of 16 000 would be required to unambiguously assign the y_{90}^{6+} (*m/z* 1635.39) and b_{44}^{3+} (*m/z* 1635.49) ions (see Fig. 1), whose multiply charged pre-ion/ion products differ by only 0.1 Da. These ions are easily resolved following ion/ion reactions (Fig. 2).

The effect of on-resonance activation frequency using a constant activation amplitude was also examined. The activation frequency range included frequencies corresponding to the low mass and high mass sides of the parent ion peak. Based on the declining parent ion abundance and increasing product ion abundance, it was obvious when the frequency

of activation was near resonance. The absolute abundances of all product ions increased with decreasing parent ion signal, as expected, but no clear changes in the relative product ion abundances were observed as the on-resonance activation frequency was varied.

3.1.2. Effect of activation time

The duration of the collisional activation period was systematically varied from 10 to 300 ms to determine a suitable activation time applicable to all of the parent ion charge states. As mentioned earlier, for a given parent ion, the ion acceleration parameters of activation amplitude, frequency, and bath gas conditions determine the dissociation rates for each ion. The activation time defines the range of dissociation rates that can be sampled during the ion trap collisional activation experiment. The use of relatively short activation times requires the use of relatively high excitation amplitudes to drive dissociation rates at values sufficient for good product ion yields. Both competitive and consecutive dissociation reactions can contribute to the spectrum with the latter being most likely at high activation amplitudes/high dissociation rates. Although it is of interest to study protein ion dissociation behavior over a wide range of activation conditions, complex spectra comprised of contributions from extensive consecutive fragmentation, which often involve the losses of small molecules such as water and/or ammonia, are difficult to interpret with the resolving power of the ion trap used in these studies. Therefore, activation conditions were sought that provide good peak definition to facilitate spectral interpretation. In general, after an initial period of about 20 ms, during which little fragmentation was observed, the abundance of the parent ion population decreased exponentially with increasing activation times and in conjunction with the growth in product ion signals. The short delay before exponential loss of the parent ion arises both from the finite turn-on time of the activation signal and the time required for the parent ion population to make the transition from the initial parent ion internal energy distribution to the final steady-state parent ion internal energy distribution [90,91]. In general, most fragmentation was observed to be complete within a time period of 20–100 ms (dissociation rates $>10\ s^{-1}$). An activation period of 300 ms was chosen for all of the parent ion charge states studied here to allow for dissociation rates as low as $3\ s^{-1}$ to contribute to the product ion spectra.

3.1.3. Effect of activation amplitude

The amplitude of the activation waveform is an important variable in determining the parent ion internal energy distribution in ion trap collisional activation. Thus, the effect of activation amplitude on apomyoglobin ion fragmentation was studied by varying the on-resonance excitation activation amplitude for a particular charge state. Shown in Fig. 3 are product ion spectra for the $[M + 12H]^{12+}$ charge state of apomyoglobin collected at various activation amplitudes. Using a 543 mV activation amplitude [Fig. 3(a)], the dissociation rate is relatively low, as reflected by the significant residual parent ion signal. Several distinct fragment ions are observed under these conditions and are assigned as b^+_{122}, b^+_{118}, b^+_{109}, y^+_{109}, y^+_{106}, b^+_{96}, y^+_{90}, b^+_{79}, y^+_{74}, b^+_{63} b^+_{44}, and y^+_{44} products. Using a 678 mV activation amplitude [Fig. 3(b)], the parent ion has been depleted much further reflecting a greater dissociation rate than that observed at the 543 mV activation amplitude. A dramatic increase in the array of product ions was also observed. Hence, whereas the fragmentation channels observed in the lower amplitude dissociation spectra are still observed, a number of new peaks, corresponding to additional fragmentation channels, also appear. Furthermore, there are changes in product ion abundances such that product ion peaks are better resolved in the higher amplitude spectrum [e.g. compare the y^+_{120} ions in Figs. 3(a) and 3(b)]. The apparent increase in baseline noise in the spectrum acquired using 678 mV activation conditions is almost exclusively chemical in nature and originates from the contribution of many low probability competitive fragmentations of the protein backbone. In order to facilitate the identification of the most favored dissociation pathways of protein ions, the spectra reported below were subsequently acquired under activation amplitude conditions that optimize the appearance of dominant fragmentation channels.

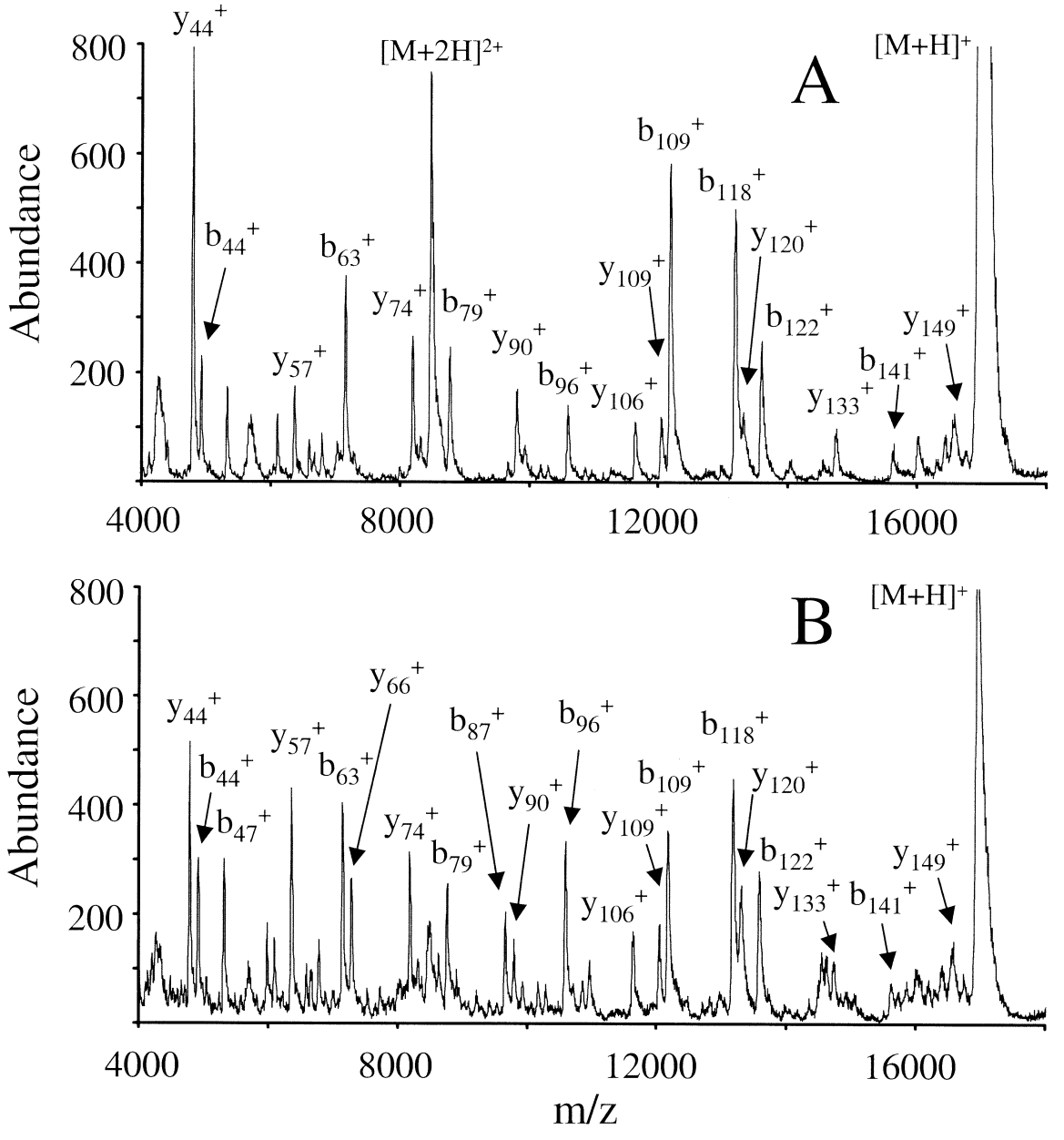


Fig. 3. Post-ion/ion product ion spectra of the $[M + 12H]^{12+}$ apomyoglobin ion obtained using on-resonance (88 540 Hz, 300 ms) collisional activation at amplitudes of (A) 543 and (B) 678 mV.

3.2. Collision induced dissociation product ion spectra of the $[M + 2H]^{2+}$ to $[M + 21H]^{21+}$ charge states of apomyoglobin

Parent ion charge state has long been known to strongly influence the fragmentation of peptide ions [92,93]. The relatively limited data set for charge state dependent fragmentation of protein ions also demonstrates that parent ion charge is an important variable in determining the appearance of the product ion spectra. In the case of ion trap collisional activation, data presented for ubiquitin [70,73], hemoglobin

β-chain ions [72] and insulin ions [71], have all demonstrated that parent ion charge state is a major factor in determining the favored dissociation channels of the ions. In addition, elements of secondary and tertiary structure may also influence the number and distribution of charge sites throughout the protein ion, and hence the fragmentation channels accessible from dissociation experiment. In order to characterize the apomyoglobin system, therefore, it is desirable to investigate the fragmentation reactions for the largest charge state range possible. Since apomyoglobin charge states lower than $[M + 9H]^{9+}$ were not directly accessible from the initial electrospray ion distribution, ion/ion proton transfer reactions with negative ions derived from PDCH were used to form the lower charge states. Although the quality of the mass measurement of the low charge parent ions is insufficient to conclude that proton transfer reactions are the exclusive mechanism for charge manipulation, in every case in which the fragmentation of a parent ion charge state formed directly from the electrospray distribution was compared to that of a parent ion of the same charge state formed by proton transfer ion/ion reactions, the spectra were indistinguishable within experimental error. Such experiments suggest that the activated parent ion populations were essentially the same whether they were formed directly via electrospray or indirectly via an ion/ion proton transfer reaction. This observation is in accordance with previous studies of ion/ion reactions involving multiply-protonated proteins and negative ions derived from PDCH [85].

By varying the electrospray ionization interface conditions and using ion/ion reactions, apomyoglobin charge states $[M + 2H]^{2+}$ to $[M + 21H]^{21+}$ were formed and subjected to ion trap collisional activation. For the purpose of summarizing the results obtained, the data are categorized by parent ion charge into low, intermediate, and high charge states.

3.2.1. Collision induced dissociation of the low ($[M + 2H]^{2+}$ to $[M + 10H]^{10+}$) charge states of apomyoglobin

The efficiency with which product ion spectra for the low charge states of high mass ions using ion trap collisional activation can be acquired is complicated by the competition between ion activation and ion ejection from the ion trap, due to the relatively shallow well depths within which high mass-to-charge ions are stored. Compounding this further is the possibility that parent ions can become increasingly stable as charges are removed. This can arise both from the reduction in internal Coulombic repulsion as charge decreases and through the stabilization of remaining charge(s) by location at highly basic sites (e.g. arginine residues) and by intramolecular charge solvation. Therefore, in order to minimize parent ion ejection whereas maximizing the formation of product ions, relatively low activation amplitudes and long activation times must be used. In the case of insulin ions, which contain several disulfide linkages, a change in the dominant dissociation mechanism was observed as charge state was reduced [70]. In the cases of ubiquitin and human hemoglobin β-chain ions, product ion spectra of low charge states yielded poorly defined product ion peaks reflecting the contributions of consecutive fragmentations involving the loss of small neutral molecules of ammonia and/or water [72,73]. To date, the parent ion of highest mass-to-charge ratio for which a product ion spectrum has been obtained is that of singly-protonated ubiquitin (*m/z* 8565.8) [73].

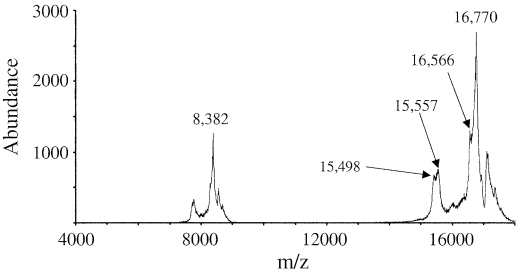


Fig. 4. Post-ion/ion product ion spectrum derived from the $[M + 2H]^{2+}$ apomyoglobin parent ion acquired using on-resonance 27 000 Hz collisional activation at 865 mV for 300 ms.

The lowest charge state of apomyoglobin for which product ion spectra could be obtained was the $[M + 2H]^{2+}$ ion (Fig. 4). The product ions are formed with relatively low efficiency, implying that many of the parent ions are ejected from the ion trap.

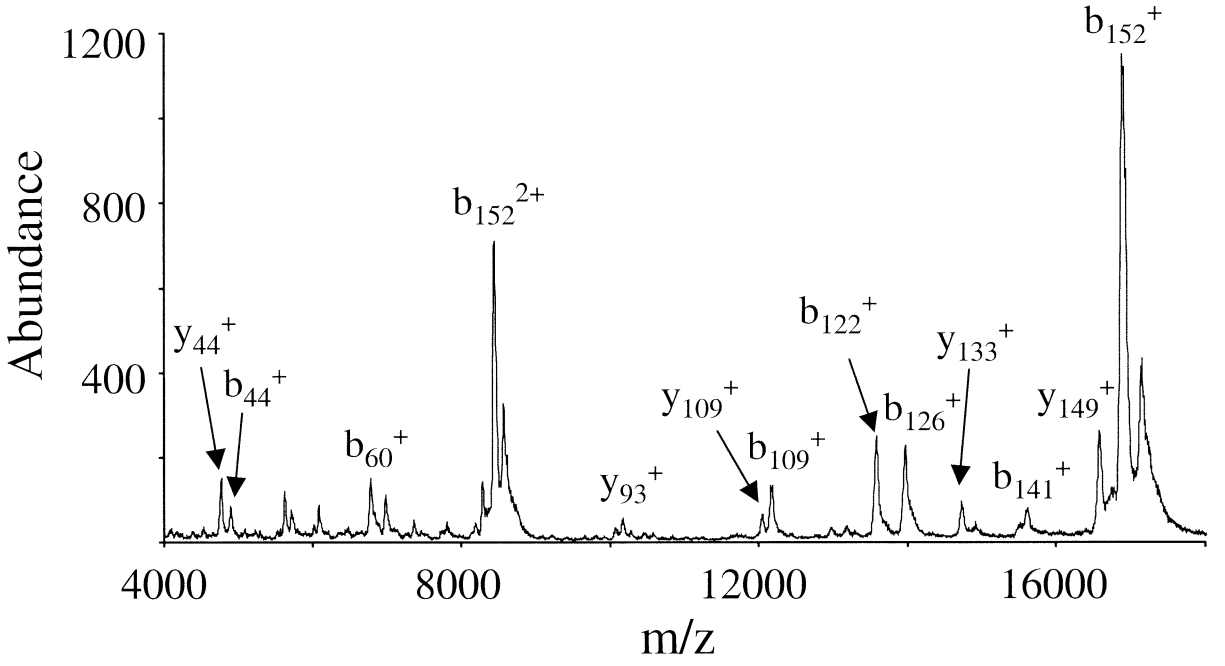


Fig. 5. Post-ion/ion product ion spectrum derived from the $[M + 6H]^{6+}$ apomyoglobin parent ion acquired using on-resonance 65 040 Hz collisional activation at 961 mV for 300 ms.

In addition, the product ions formed do not correspond closely to expected b- or y-type fragments. Rather, they appear to be formed in conjunction with the loss of one or more small molecules, presumably ammonia and/or water. Several of the signals correspond to prominent cleavages observed in the collision-induced dissociation (CID) spectra of higher charge states, except that the peaks are shifted to lower mass. For example, the signal with a peak maximum at *m/z* 16 566 probably corresponds to ions related to the y_{149}^{+} (*m/z* 16 580), a fragment observed to be prominent in the product ion spectra of other relatively low charge parent ions (see Figs. 5 and 6, for example). The overlapping peaks at *m/z* 15 557 and 15 498 are not representative of b or y ions. The product ion which is seen for lower charge states in this mass range is b_{141}^{+} (*m/z* 15 627). The broad nature of these peaks may suggest further fragmentation of the product ions.

As a general rule, the low charge state parent ions, classified herein as +10 and below, feature less diverse fragmentation and also exhibit distinctly different fragmentation channels than those of the intermediate and high charge states (see below). The majority of the product ions arise from cleavages C-terminal to aspartic acid residues. For example, in the case of the $[M + 6H]^{6+}$ ion (Fig. 5), prominent product ions at *m/z* values consistent with C-terminal aspartic acid cleavages include the following: y_{149}^{+}, b_{141}^{+}, y_{133}^{+}, b_{126}^{+}, b_{122}^{+}, b_{109}^{+}, y_{109}^{+}, y_{93}^{+}, b_{60}^{+}, b_{44}^{+}, and y_{44}^{+}. The most abundant signal in the product ion spectrum however, appears at a relatively high mass-to-charge ratio (*m/z* 16 881) and corresponds to the loss of 70 Da from the parent ion. This ion is consistent with formation of the b_{152}^{+} ion, although the neutral loss of ammonia or water, plus the loss of glycine from the N-terminus would also give this mass. It is not clear why either of these ions would be formed from this charge state as neither corresponds to known preferential fragmentation sites. Fig. 6 shows the post-ion/ion product ion spectrum of the $[M + 9H]^{9+}$ parent ion, which exhibits many of the same prominent dissociation channels observed with the $[M + 6H]^{6+}$ parent ion (i.e., fragmentations occur largely C-termi-

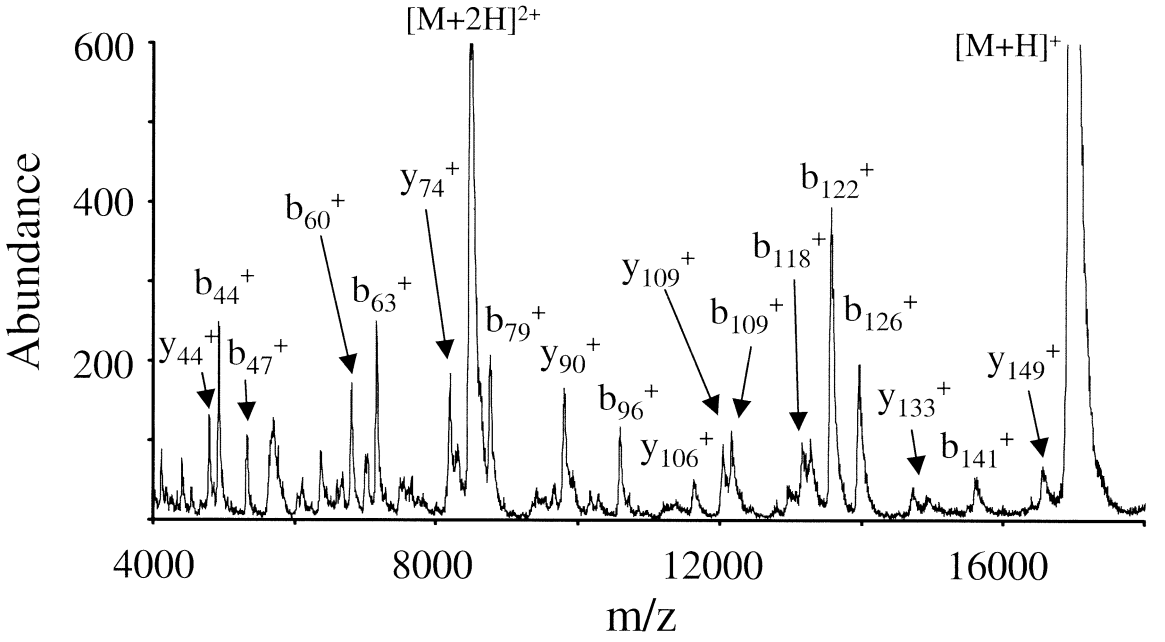


Fig. 6. Post-ion/ion product ion spectrum derived from the $[M + 9H]^{9+}$ apomyoglobin parent ion acquired using on-resonance 88 670 Hz collisional activation at 678 mV for 300 ms.

nal to aspartic acid residues). However, this product ion spectrum also contains several new prominent channels, including those giving rise to the b_{118}^+, y_{106}^+, b_{96}^+, y_{90}^+, b_{79}^+, y_{74}^+, b_{63}^+, and b_{47}^+ ions. Except for the complementary b_{79}^+ and y_{74}^+ ions, which arise from cleavage C-terminal to a lysine in a region containing several basic residues (viz., KKK-GHH), all of these product ions arise from cleavage between adjacent lysine-histidine residues. Several of the parent ion charge states of apomyoglobin, particularly in the range of $[M + 8H]^{8+}$ to $[M + 15H]^{15+}$, show an apparent tendency to fragment at sites between adjacent lysine-histidine residues.

3.2.2. Collision induced dissociation of the intermediate ($[M + 11H]^{11+}$ to $[M + 14H]^{14+}$) charge states of apomyoglobin

The intermediate charge states, $[M + 11H]^{11+}$ to $[M + 14H]^{14+}$, of apomyoglobin are not dominated by any single type of fragmentation. The distinguishing characteristic of the product ion spectra of these intermediate charge states is that they tend to show more extensive nonspecific fragmentation than either the high or low charge states. That is, in addition to the product ions from the favored reaction channels discussed previously for the low charge states, they also exhibit minor contributions from a wide range of "nonspecific" dissociation channels throughout the protein. For example, Fig. 3(b) shows the post-ion/ion reaction CID spectrum of the $[M + 12H]^{12+}$ ion of apomyoglobin. An examination of this spectrum reveals that product ions corresponding to fragmentation of 86 of the 153 amide linkages were observed, providing a sequence coverage of 56%. Complementary fragments (i.e. both b- and y-type ions) are seen for 30 of the cleavages, out of the approximately 50 that can be observed within the mass range collected.

3.2.3. Collision induced dissociation of the high ($[M + 15H]^{15+}$ to $[M + 21H]^{21+}$) charge states of apomyoglobin

The high charge state parent ions, $[M + 15H]^{15+}$ and higher, showed unique behavior in that they tended to fragment largely by a single dissociation

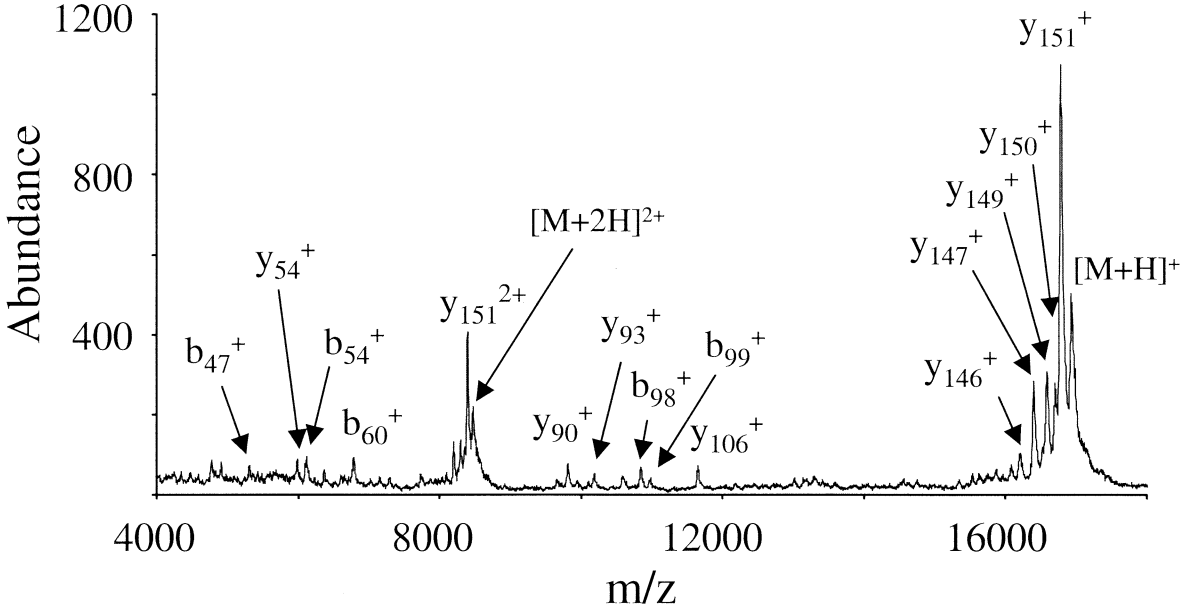


Fig. 7. Post-ion/ion product ion spectrum derived from the $[M + 18H]^{18+}$ apomyoglobin parent ion acquired using on-resonance 88 360 Hz collisional activation at 339 mV for 300 ms.

channel giving rise to the y_{151}^+ product ion. This single fragmentation accounts for as much as 70% of the product ion abundance for these charge states. The y_{151} product has been identified previously in early protein dissociation studies [80–82]. Fig. 7, which shows the post-ion/ion product ion spectrum of the $[M + 18H]^{18+}$ ion, provides an example. In addition to the y_{151}^+ product, other low abundance fragments are also observed, such as y_{149}^+-y_{146}^+ ions corresponding to fragmentation near the N-terminal end of the protein, and y_{106}^+, b_{98}^+, b_{99}^+, and y_{90}^+ ions corresponding to fragmentation near basic residues and/or N-terminal to proline. Formation of the y_{151}^+ fragment appears to be related to protonation of the N-terminus. This conclusion is based upon the observation that the y_{151}^+ fragment, whenever it is observed, is always seen as an $(n - 1)^+$ product in the pre-ion/ion product ion spectra (data not shown). Presumably, the singly charged complementary b_2^+ fragment is also formed but it is not observed in these experiments as its mass-to-charge ratio falls below the low mass cut-off used during the parent ion activation period. As the parent ion charge state increases, it becomes increasingly likely that the N-terminus of the protein carries a charge. This may be a prerequisite for observing the dissociation channel leading to the y_{151} product. The y_{151} product results from cleavage between Leu2 and Ser3 residues not normally associated with preferential cleavage based on previously reported peptide ion dissociation studies. However, it is interesting to speculate that a secondary structure effect, caused by the beginning of a α-helix at the residues in the known crystal structure of apomyoglobin, perhaps has a role in this facile cleavage. Evidence for maintaining protein ion secondary structure in the gas phase has been implicated in previous polypeptide dissociation studies [94–96].

Fig. 8 summarizes the fragmentation of apomyoglobin as a function of the charge states $[M + 6H]^{6+}$ to $[M + 21H]^{20+}$. These data were generated by summing the abundances of the complementary b- and y-type product ions corresponding to amide bond cleavage from each charge state, and then expressing these abundances as a normalized fraction of the total ion abundance with respect to the amino acid sequence. This information was drawn from 200 scan

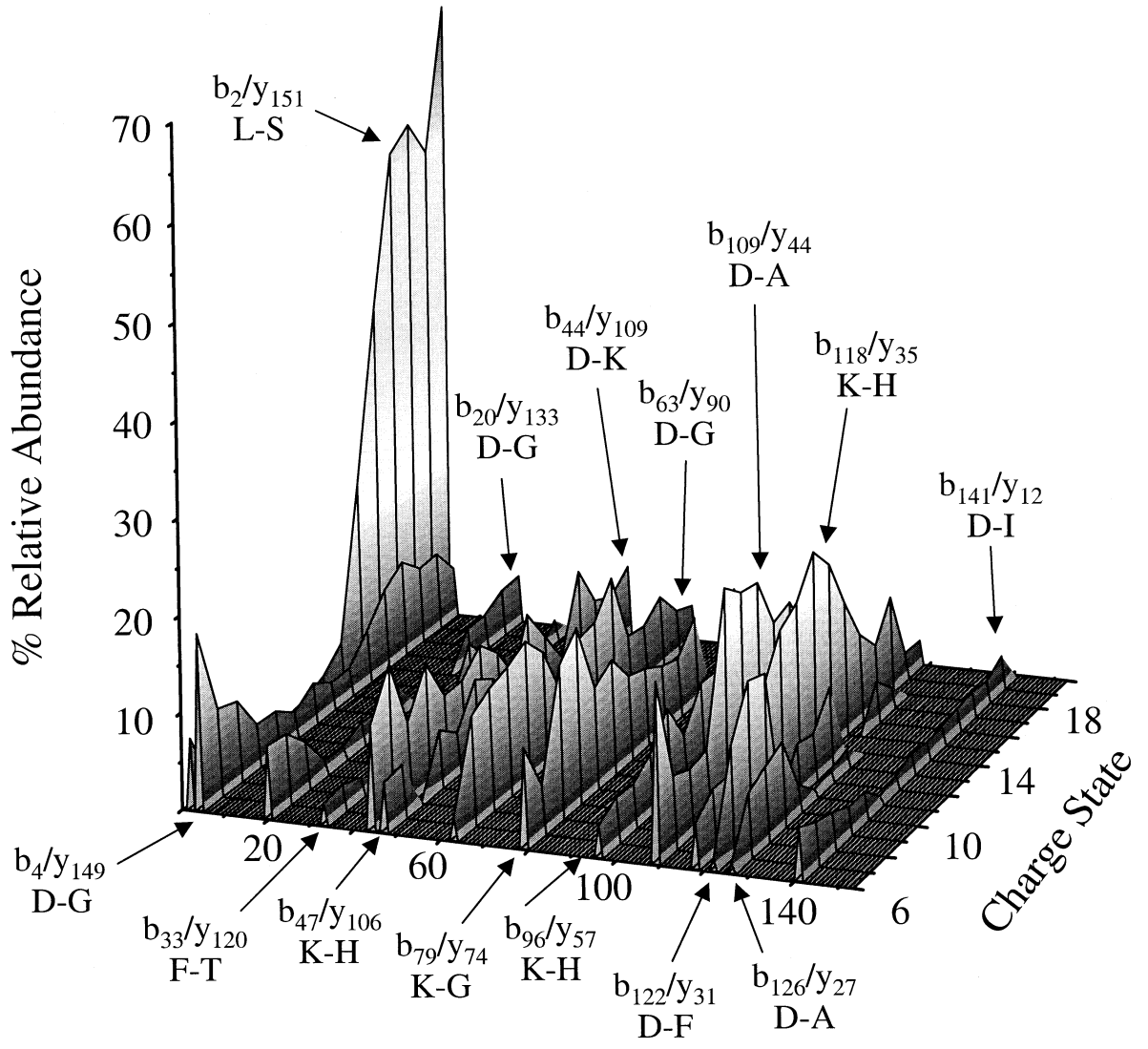
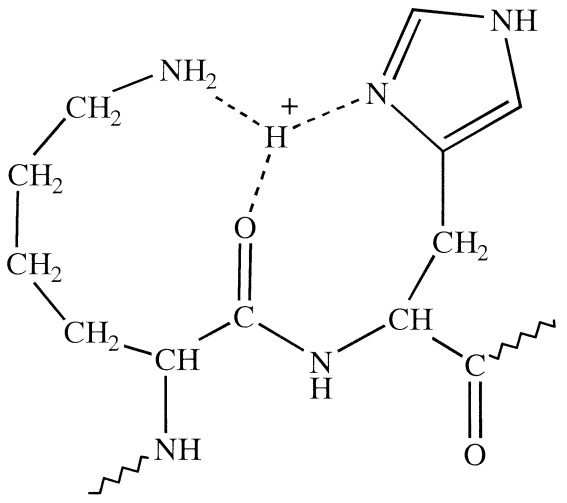


Fig. 8. Summary of the major fragmentation channels observed for ion trap collisional activation of positive apomyoglobin ions as a function of parent ion charge state.

averages from each charge state. Displaying the data in this format makes it difficult to see the contributions from the fragmentation channels that make small but observable contributions to the product ion spectra. However, summarizing the data in this way is useful for observing the charge state dependence of the major fragmentation channels. As indicated previously, the dominant cleavages in the medium to low charge states occur mostly C-terminal to aspartic acid residues and between adjacent lysine-histidine residues.

Cleavage C-terminal to aspartic acid residues and N-terminal to proline are known to be facile fragmentations for large polypeptide and protein chains [39,46], but the preference for lysine-histidine cleavage, particularly at low and intermediate charge states is a new observation. This may possibly be due to initial localization of a proton at these sites by charge



Scheme 2. Stabilization of the protonation site at adjacent lysine - histidine residues by charge solvation.

solvation through the carbonyl oxygen as well as the ϵ-amino and imidazole nitrogens of lysine and histidine respectively (Scheme 2). Subsequently, side chain attack from the lysine leads to charge directed cleavage of the amide bond C-terminal to the lysine residue. Gas-phase studies of collision cross sections have shown solvation of the charge near basic sites and carbonyl oxygens in small peptides [97]. Fragmentation studies of small peptides containing lysine and histidine have demonstrated the role of the basic side chains in sequestering charge and directing fragmentation through the amide bond C-terminal to the lysine [98,99] or histidine [39,99]. It is reasonable to assume that this novel preferential cleavage has not been previously noted in studies of peptide fragmentation because the most widely employed method for generating peptides for sequence analysis, tryptic digestion, results in peptides having lysine or arginine residues at their C-terminus, thereby precluding the presence of adjacent lysine-histidine residues. Although they are less abundant, product ions corresponding to cleavages N-terminal to proline are also observed for intermediate and high charge states. Note that whereas the b_{87}^{+}/y_{66}^{+} complementary product ion pair seen in Fig. 3 for the $[M + 12H]^{12+}$ ion corresponds to cleavage N-terminal to proline, this also corresponds to cleavage C-terminal to lysine and, as such, may be observed for similar reasons as those discussed previously for the lysine-histidine cleavage.

A decrease in the abundance of product ions corresponding to cleavage at residue 122 (C-terminal to aspartic acid) and an increase in the abundance of product ions corresponding to cleavage at residue 118 (between adjacent lysine-histidine residues) marks the change from low to intermediate charge states. Thus, the lysine-histidine fragmentation tendency appears to be most important at intermediate charge states. The highest charge states are dominated by the fragmentation channel leading to the y_{151} fragment. As indicated previously, this cleavage may be related to protein secondary structure and may require protonation at the N-terminus in order to compete effectively with the other fragmentation channels.

The apomyoglobin system shows behavior that, in some respects, parallels the charge state dependent fragmentation behavior already noted for ubiquitin and the β-chain of hemoglobin. The general trend is that low charge states give relatively less structural information than intermediate charge states. Dominant cleavages at low charge states tend to be cleavages C-terminal to aspartic acid residues along with a relatively high tendency for the loss of small molecules. The intermediate charge states tend to give the most extensive primary structure information in that many peptide cleavages contribute to the overall product ion spectrum. Interestingly, the highest charge states tend to show less extensive fragmentation and, hence, provide less primary structure information than the intermediate charge states. In the case of apomyoglobin, it is the dominance of the channel leading to the y_{151} product ion that inhibits the formation of other structurally informative products.

Although it is, perhaps, premature to draw general conclusions about the charge state dependence on the fragmentation reactions of protein ions from the limited data set discussed herein, a consistent picture seems to be emerging from the ion trap collisional activation data collected to date. The diversity of peptide bond cleavage channels that contribute to product ion spectra might be correlated with either proton mobility [33] or the diversity of parent ion structures associated with each charge state. If it can

be assumed that most peptide bond cleavages are charge catalyzed, the availability of charge can play an important role in determining the extent of fragmentation of a polypeptide system. In the case of low charge state ions, it might be assumed that the relatively few charges on the ion are sequestered at the most basic residues and can be further stabilized by intramolecular charge solvation. In such a scenario, proton mobility might be expected to be low and fragmentation might be expected to be dominated by processes that do not require mobilization of the charge. Some small molecule losses might fall into this category. Furthermore, cleavages at aspartic acid residues have been shown to be most preferred when the number of ionizing protons associated with a peptide ion is less than or equal to the number of arginine residues in the peptide (i.e. when the protons are not mobile) [77]. The high charge states may show less extensive fragmentation than intermediate charge states due to limited proton mobility as well, but for different reasons than those giving rise to limited charge mobility in the low charge state ions. In the case of the relatively highly charged ions, many of the basic residues may be protonated, but the protons are confined because of the relatively large Coulombic repulsion associated with high charge states. Ion mobility [100] and collision cross-section [101–103] measurements have indicated that the Coulomb field associated with multiple charging can affect the three-dimensional structures of gaseous apomyoglobin ions such that the highest charge states assume an elongated shape. This change in shape takes place to minimize Coulombic repulsion within the ion. An alternative to a picture in which proton mobility determines the diversity of fragmentation is one in which the diversity of activated parent ion structures (as opposed to thermal energy parent ion structures) determines the range of dissociation channels that contribute to the product ion spectrum. In this picture, a variety of activated parent ion structures, which do not rapidly interconvert, can contribute to the product ion spectrum. The data presented here cannot distinguish between these two extreme pictures (i.e., a variety of activated parent ion structures that can freely interconvert (proton mobility) versus a variety of activated parent ion structures that do not freely interconvert). Which picture comes closest to describing the actual situation has important implications. The former picture implies that the method of preparation of the ions (e.g. solution conditions, electrospray interface conditions, etc.) should have no influence on the product ion spectrum whereas the latter picture implies that the method of ion preparation may play a significant role in determining the relative contributions of the fragmenting parent ion structures. For the apomyoglobin ions studied here, the product ion spectra showed no clear sensitivity to solution or electrospray interface conditions used to form the ions. This observation appears to support the rapid parent ion interconversion picture, but it is hardly conclusive as there are no independent means to determine whether the structures of the initial parent ion population varied over the range of conditions used to form the ions. This important issue is a topic of further investigation.

4. Conclusions

Of the variables that affect the fragmentation behavior of gaseous positive apomyoglobin ions (viz., charge state and collisional activation conditions), charge state is clearly the dominant factor. The overall charge state dependence on the dissociation behavior of apomyoglobin ions parallels that of other proteins in that the intermediate charge states tend to yield the maximum structural information. This behavior is consistent with there being either a greater degree of proton mobility in intermediate charge state ions, a greater diversity of parent ion structures within the population of ions that constitute a charge state, or both. The preferential fragmentation between adjacent lysine-histidine residues in apomyoglobin ions, observed for the first time, may have implications for protein sequence analysis. The fragmentation behavior of a number of other proteins containing numerous lysine and adjacent lysine-histidine residues is currently being investigated in order to determine the generality of this observation, and to determine the potential use of this preferential cleavage in protein identification strategies.

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