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Charge dependence of protonated insulin decompositions

J. Mitchell Wells[a], James L. Stephenson Jr.[b], Scott A. McLuckey[a,*]

[a]*Department of Chemistry, 1393 Brown Laboratory, Purdue University, West Lafayette, IN 47907-1393, USA*
[b]*Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6365, USA*

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Abstract

Ion trap collisional activation is used to study the effects of charge state on protonated insulin decompositions for three forms of insulin: bovine, porcine, and human. Tandem mass spectrometry data are presented for ions with one to five protons dissociated under identical resonance excitation conditions. The $(M+5H)^{5+}$ and $(M+4H)^{4+}$ ions fragment exclusively by peptide bond cleavage of bonds outside the cycles formed by the disulfide linkages present in the insulin molecule, whereas the $(M+3H)^{3+}$ and $(M+2H)^{2+}$ ions appear to show a mixture of peptide bond cleavage and fragments arising from mechanisms associated with disulfide bond cleavage. The $(M+H)^{+}$ ion fragments almost exclusively by way of disulfide bond cleavage, with the only major exception being cleavage on the C-terminal side of glutamic acid residues external to the cycles formed by the disulfide linkages. (Int J Mass Spectrom 203 (2000) A1–A9) © 2000 Elsevier Science B.V.

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1. Introduction

The unimolecular decomposition behavior of gaseous polypeptide ions has become particularly relevant in biological research due to the utility of this behavior for the identification of proteins [1]. A common strategy is to use chemical or enzymatic digestion of proteins followed by tandem mass spectrometry (MS/MS) of the ions derived from the relatively small peptide digestion products to obtain sufficient primary sequence information for identification. The decomposition behavior of peptide ions has therefore been the subject of extensive research [2–7]. An alternate but less well-developed strategy is to subject whole protein ions to tandem mass spectrometry without prior recourse to enzymatic digestion. Early work along these lines was conducted using triple quadrupole tandem mass spectrometry [8–12]. This form of instrumentation, however, is limited in its ability to yield readily interpretable data due to ambiguities in assigning product ion charge states. The use of high resolving power Fourier transform ion cyclotron resonance (FTICR) tandem mass spectrometry, on the other hand, allows for product ion charge state determination by the measurement of the isotope spacing [13]. McLafferty and co-workers have been particularly prominent in pio-

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

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neering the tandem mass spectrometry of whole protein ions using FTICR instrumentation [14–16]. An alternate strategy to the measurement of product ion isotope spacing for the determination of product ion charge states is to convert product ions largely to singly charged ions by way of ion/ion proton transfer reactions [17]. The use of ion/ion chemistry in this way enables the decomposition behavior of multiply charged protein ions to be studied using quadrupole ion trap tandem mass spectrometers which typically operate at resolving powers too low to resolve the isotope spacing of product ions greater in mass than a few kilodaltons. Further, ion/ion proton transfer reactions allow for a wider range of parent ion charge states to be studied [18].

Protein ion dissociation behavior is currently less well understood than peptide ion dissociation in part because the body of observations associated with protein ion dissociation is still relatively small. Further, protein ions present a higher degree of dimensionality than peptide ions. This higher degree of dimensionality arises from the diversity in higher order structures that proteins can assume, a multiplicity of post-translational modifications that can be present in a protein, as well as the wide range of charge states that can be accessed for protein ions. In this communication, we describe the charge state dependence of positive insulin ion decompositions. Aside from the well-known physiological importance of insulin, the ions derived therefore are of interest from the protein ion dissociation point of view due to the presence of three disulfide linkages. In particular, the A and B chains that comprise insulin are attached by way of two such disulfide linkages. It has recently been shown that electron capture by multiply charged protein ions, including insulin ions, leads to preferential cleavage at disulfide linkages if they are present [19]. Such a tendency has not been observed for even-electron multiply charged protein ions containing disulfide linkages, at least under FTICR [19] and ion trap [20] collisional activation conditions; however, a triply charged polypeptide (17 residues) containing two disulfide linkages has been observed to fragment by disulfide bond cleavage upon collisional activation in a quadrupole ion trap [21]. In this communication, we show a dramatic dependence of the preferred dissociation channels on parent ion charge for insulin ions. The most striking observation is that almost all of the fragmentation associated with the singly charged ions arises from disulfide linkage cleavages whereas highly charged ions show little or no evidence for cleavages at disulfide linkages. Further, sequential fragmentation following cleavages between carbon and sulfur atoms of disulfide linkages is noted. Selective cleavage at disulfide linkages has previously been observed from singly charged ions derived from fast atom bombardment and sector-based tandem mass spectrometry [22,23], from matrix-assisted laser desorption ionization (MALDI) combined with time-of-flight mass spectrometry, in both in-source [21,24] and postsource decay modes [21,24–27], and from MALDI combined with ion trap tandem mass spectrometry [28]. However, conclusions regarding observed differences in the fragmentation behavior arising from singly charged ions versus multiply charged ions have been difficult to draw due to data having been collected on different instrument types with significant differences in time scales and ion energies. The parent ion charge state manipulation afforded by ion/ion proton transfer chemistry has allowed for the study of singly and multiply charged insulin ions to be conducted under essentially identical collisional activation conditions.

2. Experiment

Bovine, porcine, and human insulin were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. The proteins were dissolved in methanol with 2% acetic acid to concentrations of approximately 10 μM, and were electrosprayed from a 100 μm inner-diameter stainless steel needle to which a potential of approximately +3000 V was applied. The solution flow rate was 1.0 μL/min. The electrospray interface [29] and instrumental setup for ion/ion reactions [30] in the ion trap have been described previously. The typical experimental procedure entailed an electrospray ion accumulation period of some hundreds of milliseconds, followed by the isolation of ions corresponding to a

specific charge state of insulin. Ion isolation was effected with multiple resonance ejection ramps to sequentially eject ions of *m/z* higher and lower than those of interest [31]. Ions with charge states of +5 and +4 were isolated directly from the starting electrospray ion population. For the +3, +2, and +1 charge states, the initial electrospray ion population was subjected to proton transfer reactions with anions derived from glow discharge ionization [32] of perfluoro-1,3-dimethylcyclohexane (PDCH) in order to reduce the distribution of charge states to form sufficient numbers of the charge state of interest for subsequent isolation and collisional activation. Collisional activation of the +5 to +1 charge states was effected at a q_z value of 0.09. The amplitude of the resonance excitation voltage applied to the end caps ranged from 130 to 920 mV_{pp}. The collisional activation period ranged from 100 to 500 ms.

To assist in interpreting the product ion spectra derived from multiply charged parent ions, PDCH anions were injected into the ion trap for approximately 20 ms directly after the ion activation period followed by a cation/anion mutual storage period of 60–130 ms. During this reaction period, most product ions were converted to singly charged ions. A 10–20 ms ramp of the rf amplitude was used to eject all ions below *m/z* 650 prior to mass analysis. This step was performed to eject the residual PDCH anions so that they would not lead to deleterious effects on mass analysis [33]. Mass analysis was effected by resonance ejection [34] of the ions at q_z=0.088 (ejection frequency of 34.202 kHz) using a resonance ejection amplitude of 2.1 V_{pp}. The spectra shown are typically the average of 100–200 individual mass analysis scans. Mass calibration of pre-ion/ion product ion spectra was achieved by using the electrospray mass spectrum of bovine insulin as the calibration standard. The charge states of the parent ions were used to calibrate the high-mass region for post-ion/ion product ion spectra.

3. Results and discussion

Fig. 1 shows the product ion spectra obtained from the $(M+5H)^{5+}$ to the $(M+H)^{+}$ precursor ions derived from bovine insulin after the initially formed product ions were subjected to ion/ion proton transfer reactions to yield largely singly charged product ions. These types of spectra are referred to herein as post-ion/ion reaction product ion spectra. Product ion spectra derived from multiply charged ions (i.e. "pre-ion/ion" product ion spectra) are typically comprised of ions with charges ranging from unity up to the charge of the parent ion. The fact that product ion charge is not known a priori can complicate the interpretation of product ion spectra derived from multiply charged precursor ions. Further, detection may show a significant dependence upon product ion charge and can therefore provide misleading information regarding the relative contributions of competing dissociation channels if they lead to different combinations of product charge states and if no measures are taken to correct for the charge dependence of the detector. Also, the optimum conditions for mass analysis of product ions derived from different precursor ion charge states differ due to differences in product ion charges and the mass-to-charge range over which they are formed. These complications can be avoided in large part by subjecting product ions to proton transfer reactions with oppositely charged ions to the extent that most of the product ions are singly charged. In this way, ambiguities about product ion charge are avoided, differential detector response as a function of charge state becomes less important, and mass analysis conditions for products formed from each precursor ion charge state can be held constant. The disadvantage in presenting the data in this way is that information regarding the initial charge states of the product ions is lost. In the case of the insulin ions studied here, the charge partitioning among the product ions as a function of charge state was unremarkable. For the multiply charged ions, some reaction channels leading to *b*-type and *y*-type [35] fragments showed competition between the loss of a neutral molecule versus the loss of the same nominal fragment as a singly charged ion. This competition did not appear to be strongly dependent upon charge state. Therefore, only the post-ion/ion reaction product ion spectra are shown here.

All of the data in Fig. 1 were collected under the

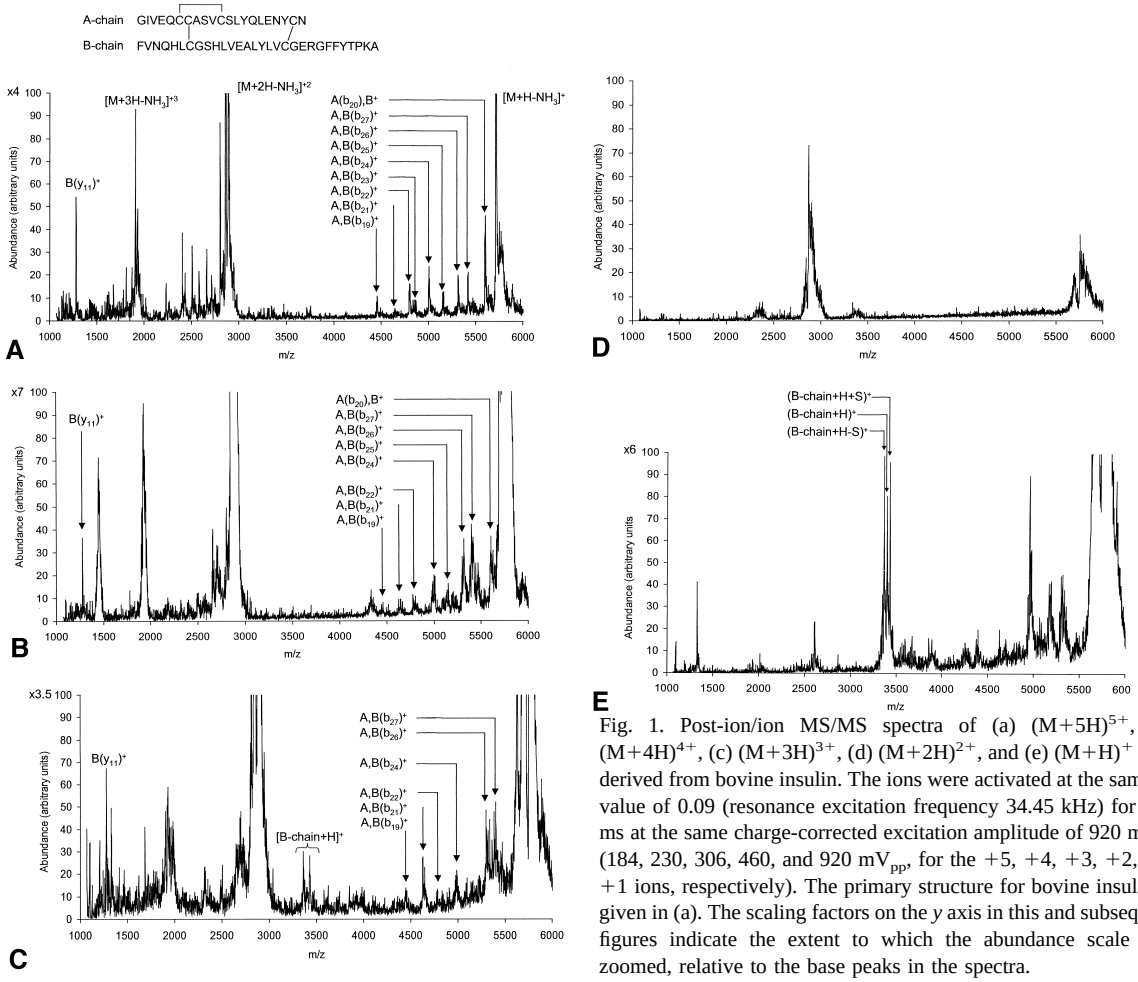


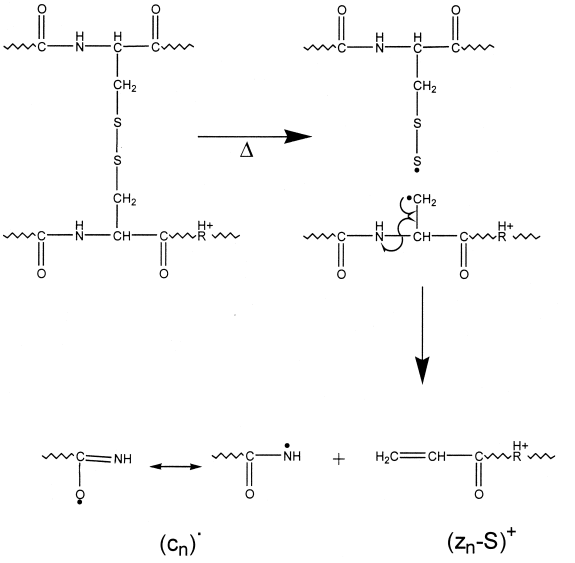
Fig. 1. Post-ion/ion MS/MS spectra of (a) $(M+5H)^{5+}$, (b) $(M+4H)^{4+}$, (c) $(M+3H)^{3+}$, (d) $(M+2H)^{2+}$, and (e) $(M+H)^{+}$ ions derived from bovine insulin. The ions were activated at the same q_z value of 0.09 (resonance excitation frequency 34.45 kHz) for 500 ms at the same charge-corrected excitation amplitude of 920 mV_{pp} (184, 230, 306, 460, and 920 mV_{pp}, for the +5, +4, +3, +2, and +1 ions, respectively). The primary structure for bovine insulin is given in (a). The scaling factors on the *y* axis in this and subsequent figures indicate the extent to which the abundance scale was zoomed, relative to the base peaks in the spectra.

same nominal conditions. That is, all precursor ions were activated at the same frequency (i.e., the same q_z value) and the same charge-normalized resonance excitation amplitude (i.e. the product of precursor charge and resonance excitation amplitude was held constant). Activating the ions in this way should elevate each precursor ion to the same internal energy, provided the collision cross sections of the ions are the same [36]. Although it is expected that there should be some variation in collision cross section with charge state, in the absence of quantitative information regarding differences in ion size the procedure used here is the most readily justifiable approach to subjecting ions derived from different precursor ion charge states to identical collisional activation conditions.

Relating the charge dependence of positive insulin ion decompositions under ion trap collisional activation conditions is, perhaps, best approached by comparing and contrasting the behavior of the $(M+5H)^{5+}$ and $(M+H)^{+}$ ions. These ions represent the two extremes in the data sets collected for bovine, human, and porcine insulin. All three forms showed essentially identical behavior in terms of the favored dissociation channels for each charge state. As has been shown previously, the $(M+5H)^{5+}$ ion of bovine insulin [Fig. 1(a)] fragments by water and/or ammonia loss and by cleavages at peptide linkages to form *b*-type and *y*-type fragments in regions external to the disulfide linkages [20]. There is no evidence for cleavages associated with the disulfide linkages in the data for any of the insulin $(M+5H)^{5+}$ precursor ions.

The assignments for the singly charged product ions derived from the $(M+5H)^{5+}$ precursor ion are indicated in Fig. 1(a). In stark contrast with the behavior of the $(M+5H)^{5+}$ ion, the $(M+H)^{+}$ ions yields numerous abundant fragments that cannot be assigned to any of the possible *b*-type and *y*-type ions. Further, several highly abundant products are observed at masses that can only arise from decompositions of the two disulfide linkages that bind the A and B chains. For example, a cluster of peaks that corresponds in mass to protonated B-chain molecules with varying numbers of sulfur atoms is indicated in Fig. 1(e). The most abundant fragments correspond to those with one, two, and three sulfur atoms, respectively. No evidence for protonated A-chain ions is observed, presumably due to the fact that the proton in the singly charged parent ion is expected to be situated on the lone arginine residue of the molecule (i.e. the B-22 residue). The product ions of mass lower than the masses associated with the B-chain ions are therefore expected to be, and are consistent with (see the following), fragments of the B chain.

Most of the major fragmentation channels of the $(M+H)^{+}$ ion can be rationalized on the basis of competing fragmentations at disulfide linkages followed by one or more consecutive fragmentations. Cleavage of a single disulfide linkage results in no change in mass such that the product of the first cleavage remains in resonance with the resonance excitation voltage thereby facilitating further dissociation. The resolving power $M/\Delta M_{\mathrm{FWHM}}=500–1000$) and mass accuracy (500 ppm) associated with the spectra collected for these ions does not allow for the delineation of hydrogen transfers that might take place in conjunction with the disulfide linkage cleavages. Therefore, we have included here a general mechanism for the major products, shown in Scheme 1, which is consistent with the measurements. This mechanism assumes that the single proton is located on the B-22 arginine residue of the B chain and does not necessarily involve charge-directed fragmentation. Further, it includes homolytic cleavages. However, the transfer of a hydrogen atom between the two chains during the course of the cleavage of the disulfide linkage (i.e. heterolytic cleavage) cannot be precluded on the basis of the data presented herein.



Scheme 1. Proposed disulfide cleavage fragmentation mechanism leading to $(z_n-S)^{+}$ ions.

Product ion spectra for the three forms of insulin $(M+H)^{+}$ ions are compared in Fig. 2, with the primary sequence of each form indicated in the insets. Most of the product ion signal from the $(M+H)^{+}$ precursors can be attributed to an initial cleavage at one of the disulfide linkages. Both sulfur-sulfur and carbon–sulfur bond cleavages are observed. To clarify the ensuing discussion, the nine sulfur–sulfur and carbon–sulfur bonds are numbered as shown in Scheme 2. The peaks associated with the B chain in Figs. 1(e) and 2 arise from cleavages at both interchain disulfide linkages. The major B-chain product ions are comprised of one, two, or three sulfur atoms. The product containing one sulfur atom arises from one -C-S- cleavage on the B-chain side (either bond 3 or 6) and one -S-S- cleavage (either bond 2 or 5). The product containing two sulfur atoms can arise from two -S-S- cleavages (bonds 2 and 5) or one -C-S- cleavage on the B-chain side (bonds 3 or 6) and one -C-S- cleavage on the A-chain side (bonds 1 or 4). The product containing three sulfur atoms arises from cleavage of either bond 1 or 4 and bond 2 or 5.

Product ions at *m/z* 1330, 2613, and 2645 are

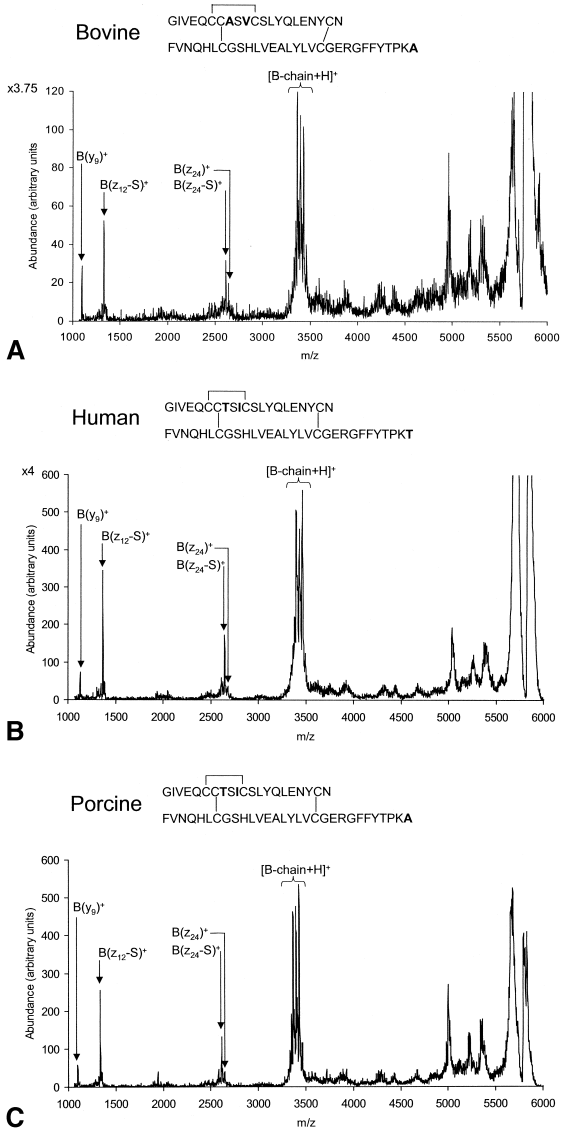
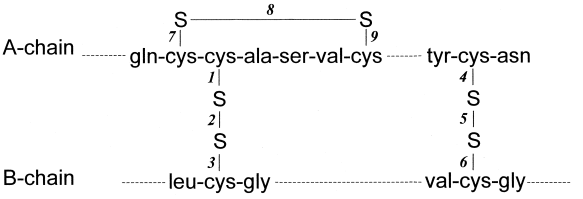


Fig. 2. Comparison of MS/MS spectra for three forms of insulin $(M+H)^+$ ions derived from (a) bovine, (b) human, and (c) porcine insulin. The insets give the primary structure of each form of insulin, with the sequence differences in bold. Note that all spectra were collected at a nominal excitation q_z value of 0.09, however, the excitation frequency, amplitude, and time varied for each spectrum, so that the ions cannot be assumed to have been at the same internal temperature.

abundant products in the tandem mass spectrometry spectra of the bovine and porcine insulin $(M+H)^+$ ions at moderate to high resonance excitation voltages. The analogous products observed for human



Scheme 2. Numbering system used to refer to disulfide linkage bonds in the text.

insulin $(M+H)^+$ show the expected differences in mass arising from the fact that the B-chain C-terminal residue in human insulin is threonine whereas it is alanine in bovine and porcine insulins. These product ions are presumed to arise from a mechanism that begins with heterolytic cleavage of bond 3 or bond 6 followed by loss of a radical *c*-type species to yield the complementary *z*-type fragment without the original cysteine sulfur (see Scheme 1). These ions are labeled herein as $B(z_n\text{-S})$ fragments. The *m/z* 1330 ion is interpreted to be the $B(z_{12}\text{-S})^+$ species, formed via cleavage of bond 6 and bonds 1, 2, or 3, followed by loss of a radical $B(c_{18})$ fragment. Note that this fragment ion could also be formed directly from the intact insulin ion without cleavage of bonds 1, 2, or 3 by way of the loss of $A,B(c_{18})$ radical. The *m/z* 2613 ion is interpreted to be the $B(z_{24}\text{-S})^+$ ion, formed by means of cleavage of bond 3 and bond 5 with subsequent loss of a radical $B(c_6)$ fragment. Note that this ion still contains one sulfur atom, which is expected to be located on the B-19 cysteine. The *m/z* 2645 ion derived from the B-chain ion with two sulfur atoms is also expected to be a z_{24} fragment but with two sulfur atoms attached to the B-19 cysteine. This ion must be formed by way of cleavage of bond 3 and bond 4, with subsequent loss of a radical $B(c_6)$ fragment. There is also evidence in the spectra of Fig. 2 for a peak corresponding to $B(z_{24}\text{-}2S)^+$, presumably resulting from cleavage of bonds 3 and 6, followed by loss of the $B(c_6)$ radical.

A series of experiments utilizing a third stage of tandem mass spectrometry (MS/MS/MS) were conducted in which the first generation B-chain ions with one, two, and three sulfur atoms were each isolated and activated (data not shown). The B-chain ion with

three sulfur atoms showed very poor fragmentation efficiency with no prominent second-generation products noted. This result is consistent with the fact that in this fragment, both cysteines must retain sulfurs, and hence the mechanism in Scheme 1 does not operate. The B-chain ion with two sulfur atoms showed more efficient fragmentation than the ion with three sulfur atoms. In the cases of the porcine and bovine insulin precursor ions, this ion yielded prominent products at nominal mass-to-charge values of *m/z* 1330 and *m/z* 2645. The B-chain ions from those precursors with one sulfur atom showed the highest efficiency of conversion from precursor to product ions and yielded second generation products at *m/z* 1330 and *m/z* 2613. These MS/MS/MS results are consistent with the interpretation given above for the identities of the product ions in the MS/MS spectra.

MS/MS data collected as a function of resonance excitation amplitude were useful in making assignments to the major product ions of mass greater than those of the B-chain ions. Fig. 3 shows MS/MS spectra of the bovine insulin $(M+H)^+$ ion at three resonance excitation amplitudes. As the amplitude of the resonance excitation amplitude is increased, the first two product ions to appear correspond to $A,B(z_{24})^+$ and $A(z_{16}),B^+$ ions at nominal *m/z* values of 4977 and 5189, respectively. The $A,B(z_{24})^+$ product is expected to arise from initial cleavage of bond 3 followed by loss of the B-chain c_6 fragment via the process indicated in Scheme 1. The $A(z_{16}),B^+$ product is expected to arise from cleavage of the intrachain disulfide linkage between A-6 and A-11 at bond 7 followed by loss of the A-chain c_5 fragment. As the resonance excitation voltage is increased further, the B-chain ions are observed along with the $(z_{12}\text{-S})^+$ and $(z_{24}\text{-S})^+$ products. The only other major product signals in the data for the $(M+H)^+$ ion are observed in the region of *m/z* 5340. These product ions begin to appear at resonance excitation amplitudes greater than those associated with the $A(z_{16}),B^+$ and $A,B(z_{24})^+$ products discussed previously. No processes involving an initial disulfide cleavage followed by a cleavage on the N-terminal side of a cysteine residue appear to be able to give rise to products in the vicinity of this mass. The product assignment that is

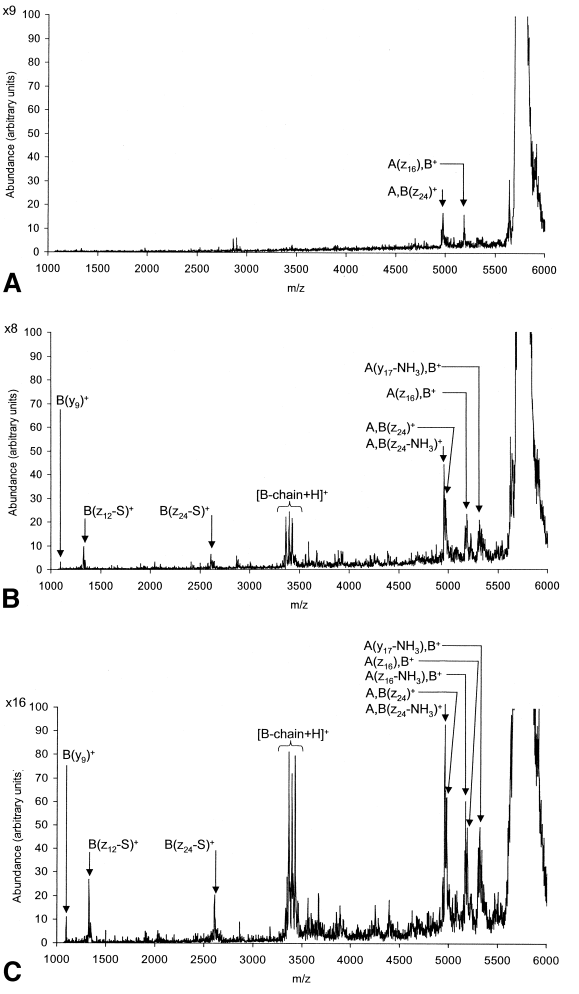


Fig. 3. MS/MS spectra for the $(M+H)^+$ of bovine insulin excited at a q_z value of 0.09 (resonance excitation frequency 34.45 kHz) for 500 ms at (a) 748 mV_{pp}, (b) 805 mV_{pp}, and (c) 863 mV_{pp}.

consistent with the data from the various forms of insulin for the nominal *m/z* 5340 product in Fig. 1(e) is the $A(y_{17}),B^+$ ion arising from cleavage at the C-terminal side of the A-4 glutamic acid residue. However, there are also several other peaks on the low *m/z* side of the *m/z* 5340 product that can arise from sequential fragmentation associated with the nominal $A(y_{17}),B^+$ ion (see the following) or may arise from competitive fragmentation channels. The $B(y_9)^+$ ion, which arises from cleavage at the C-terminal side of the B-21 glutamic acid, is the only

other clearly identifiable product arising from conventional amide bond cleavage.

As the resonance excitation amplitude increases, the contributions from sequential fragmentations involving losses of small molecules such as ammonia and water become increasingly apparent, especially at product ion masses greater than or equal to those of the B-chain ions. For example, the relative abundances of the fragments that correspond to the B-chain ions missing a water or ammonia molecule clearly increase with resonance excitation amplitude. Further, the signals associated with the larger fragments tend to become broader and shift to lower mass as resonance excitation increases. This behavior is expected to be particularly prominent with precursor ions that require the cleavage of at least two bonds before two separate fragments can be formed. Sequential fragmentations involving small molecules can complicate product ion assignment, particularly if MS/MS data is acquired only at relatively high resonance excitation amplitudes. This behavior was noted for all insulin precursor ion charge states. It is apparent for the $(M+H)^+$ ions in Fig. 3.

It is interesting to note that obtaining product ion spectra from the $(M+4H)^{4+}$, $(M+3H)^{3+}$, and $(M+2H)^{2+}$ precursor ions with sharp and clearly defined product ions under any conditions was far more challenging than with either the $(M+5H)^{5+}$ or $(M+H)^+$ ions. The $(M+2H)^{2+}$ ion proved to be particularly problematic. The reasons underlying these observations are open to speculation. The insulin molecules each nominally contain six potential protonation sites corresponding to the two N-termini, the B-22 arginine, the B-29 lysine, and the B-5 and B-10 histidines. In the case of the $(M+5H)^{5+}$ ion, at least one of the N-termini is expected to be protonated whereas this need not be the case with the other charge states. Aside from the N-terminus of the A chain, all nominal basic sites in insulin are present in the B chain. A possible reason for the difficulty in fragmenting the intermediate charge states is the stabilization of charge on the B chain by the presence of the A chain. The degree of such stabilization would be expected to be least for the singly charged ion protonated on arginine. The $(M+4H)^{4+}$ and $(M+3H)^{3+}$ precursors show many of the same *b*-type and *y*-type ions observed for the $(M+5H)^{5+}$ ion. The $(M+3H)^{3+}$ ion, however, also shows clear evidence for disulfide linkage dissociation from the appearance of B chain and $(z_{12}\text{-S})^+$ ions. The doubly protonated precursor also shows evidence for B-chain fragments but yields few other clearly identifiable fragments. Singly charged ion fragmentation, as discussed previously, is dominated by initial cleavage at the disulfide linkages with no dominant fragments arising from the channels that lead to the abundant fragments for the $(M+5H)^{5+}$ ion. The doubly charged ion may also fragment predominantly via the disulfide cleavage processes but these processes are apparently significantly less facile that with the singly charged ions.

4. Conclusions

The data presented here illustrate that the protein ion charge state selected for MS/MS analysis can greatly influence the quality and type of data obtained, even for a relatively small protein such as insulin. In this case, the presence of multiple disulfide linkages appears to make the influence of charge state on fragmentation particularly strong, with a major change in the preferred dissociation mechanisms between the $(M+5H)^{5+}$ and $(M+H)^+$ ions. The $(M+5H)^{5+}$ ions primarily show conventional "sequence" ions from regions outside the cycles defined by the disulfide linkages, while the $(M+H)^+$ ions almost exclusively show processes initiated by disulfide linkage cleavage. Cleavage of a single disulfide does not result in a mass change such that resonance excitation can drive sequential reactions. These include losses of small molecules, cleavage of a second disulfide linkage, and cleavage on the N-terminal side of cysteines that have lost the sulfur atom in disulfide linkage dissociation at a -C-S- bond. Fragmentation of ions of the charge states intermediate between 5+ and 1+ appear to be less facile that those for the two extremes. The 4+ ion shows fragmentation similar to that of the 5+ ion whereas the triply charged ions shows evidence for competition between cleavage of peptide linkages and disulfide linkages. The doubly

charged ions yield relatively poor quality MS/MS spectra but appear to show evidence for disulfide linkage cleavage. Cleavage at peptide linkages, however, cannot be precluded. With the notable exceptions of cleavages at glutamic acid residues outside the cysteine linkages, the singly charged ions yield products that require the cleavage of at least one disulfide linkage followed by a second cleavage either at another disulfide linkage or on the N-terminal side of a cysteine that has lost its sulfur. The observed behavior may have general implications for the dissociation reactions of ions derived from disulfide linked proteins that require at least two steps before a change in mass is observed when a disulfide linkage has been cleaved. Further studies with disulfide, linked proteins such as lysozyme and bovine pancreatic trypsin inhibitor are currently underway.

Acknowledgements

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