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Triply charged bradykinin and gramicidin radical cations: their formation and the selective enhancement of charge-directed cleavage processes

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Abstract

We report on the formation and collision-induced dissociation of hydrogen deficient peptide radical cations of the type $[M+nH]^{(n+1)+\cdot}$. These ions are formed from multiply protonated precursors (i.e. $[M+nH]^{n+}$) by collisional electron transfer in high energy collisions with dioxygen. Increasing the charge state of doubly protonated bradykinin ($[BK+2H]^{2+}$), results in a selective enhancement of b type ions with an especially dominant b_6 ion. Further, we observe a c_4 ion that is ascribed to the radical character of the triply charged ion. The charge reversal spectrum of deprotonated bradykinin $[BK–H]^-$ provides additional information with the appearance of z type ions, probably formed from the hydrogen-deficient $[BK–H]^{+\cdot}$ radical cation which is also observed. The fragmentation pattern of the triply charged gramicidin S radical ($[GS+2H]^{3+\cdot}$) also differs from those of the singly and doubly protonated peptide and reveals structure-indicative acylium ions such as VOLFPVOL and OLFP (O = Ornithine). (Int J Mass Spectrom 213 (2002) 225–235) © 2002 Elsevier Science B.V.

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1. Introduction

Several mass spectrometric techniques have been successfully used to elucidate the primary structure of peptides, including low- and high-energy collision induced dissociation (CID) [1], surface induced dissociation [2], laser infrared multiphoton dissociation [3], blackbody infrared dissociation [4], and electron capture dissociation (ECD) [5–7].

The vast majority of studies on gaseous protein and peptide cations concern even electron ions (i.e. closed shell ions such as $[M+nH]^{n+}$, M = neutral peptide). Radical cations exhibit a different reactivity than their even-electron counterparts, and the fragmentation of peptide radical cations may yield new sequence ions that are important for determination of the primary structure. The traditional way of forming organic radical cations is by electron ionization (EI) of vaporized molecules. However, peptides are nonvolatile compounds due to their ionic character and thus require derivatization in order to make them volatile and amenable to EI. To circumvent this problem, Wu and Fenselau [8] generated a beam of neutral peptides

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from CID of proton-bound dimers comprised of a peptide and a more basic molecule; subsequently the neutral peptides were collisionally ionized with dioxygen. Pérez et al. [9] used a more direct way to desorb intact neutral peptides by using laser-induced acoustic desorption. Grotemeyer and Schlag [10] have introduced another technique that involves laser evaporation of intact peptide molecules followed by multiphoton ionization. Chu et al. [11] recently reported a protocol based on CID of a Cu(dien)-peptide^{2+} complex (dien = diethylentriamine) thereby forming the peptide radical cation. Finally, in ECD experiments, instead of electron stripping of neutral peptides, multiply protonated peptides capture an electron thereby also becoming radical cations, $[M+nH]^{(n-1)+\cdot}$ [5–7]. These hypervalent species often dissociate since all molecular bonding orbitals of the even-electron precursor ion are occupied.

In our laboratory, an accelerator mass spectrometer has been equipped with an electrospray ion source, which allows us to study the fragmentation behavior of ionic biomolecules at very high collision energies; the acceleration voltage (50 kV) is a factor of five to ten higher than that of a typical commercial sector instrument. Previously, we showed that electron stripping of protonated amino acids (e.g. tyrosine) and multiply protonated proteins (e.g. lysozyme) is an especially efficient process at these high collision energies [12–14]. The present work concerns the fragmentation pathways of peptide radical cations of the type $[M+nH]^{(n+1)+\cdot}$ formed after collisional electron detachment. These ions carry an extra charge relative to the number of ionizing protons, *n*, and are denoted hydrogen-deficient ions because with an appropriate hydrogen atom donor, it should be possible to generate $[M+(n+1)H]^{(n+1)+}$ ions. The cleavage reactions of $[M+nH]^{(n+1)+\cdot}$ are governed by both the radical cation character and the high Coulomb repulsion in the peptide ion. We demonstrate the capability of the method by two case studies: a linear chain peptide (the nonapeptide Bradykinin = Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg abbreviated as BK) and a cyclic peptide (the decapeptide Gramicidin S = cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$ abbreviated as GS). "Linear" and "cyclic" refer to the primary structure of the peptide and not the secondary structure due to gas phase folding.

2. Experimental

A detailed description of the experimental setup is given in [14]. Briefly, protonated bradykinin and gramicidin S ions, formed by electrospray ionization (ESI), were accelerated to a kinetic energy of $n \times 50$ keV where *n* is the charge state of the ion ($n = 1$ or 2). Spray solutions of 50 μM concentration were used. Precursor ions were mass selected with a magnet and passed though a 3 cm long gas cell containing either helium or dioxygen at a pressure of 2×10^{-3} mbar. The product ions were analyzed with a hemispherical electrostatic analyzer.

In other experiments, we subjected protonated peptides to electron stripping before the magnet by introducing O_2 into this region so that the signal of the protonated peptides was reduced to ~1/3. The acceleration voltage was set to 100 kV for $[GS+H]^+$ such that the formed radical dication has the same kinetic energy as $[GS+2H]^{2+}$ formed in the source and accelerated through 50 kV. An acceleration voltage of 100 kV was also used for the generation of radical trications from diprotonated peptides. The radical cations, formed before the magnet, were then mass selected and collisional activated with helium as described above for the even-electron peptide ions.

ESI also allows the formation of deprotonated bradykinin anions, $[BK–H]^-$. Positive fragment ions formed in collisions between $[BK–H]^-$ and O_2 were monitored; the spectrum so-obtained is denoted a charge-reversal, $^-CR^+$, spectrum.

3. Results and discussion

3.1. Radical polycations of bradykinin and gramicidin

The CID spectra of singly and doubly protonated bradykinin and gramicidin ($[M+nH]^{n+}$, $n = 1, 2$) with O_2 as collision gas are shown in Fig. 1. In all cases electron loss with the concomitant formation of

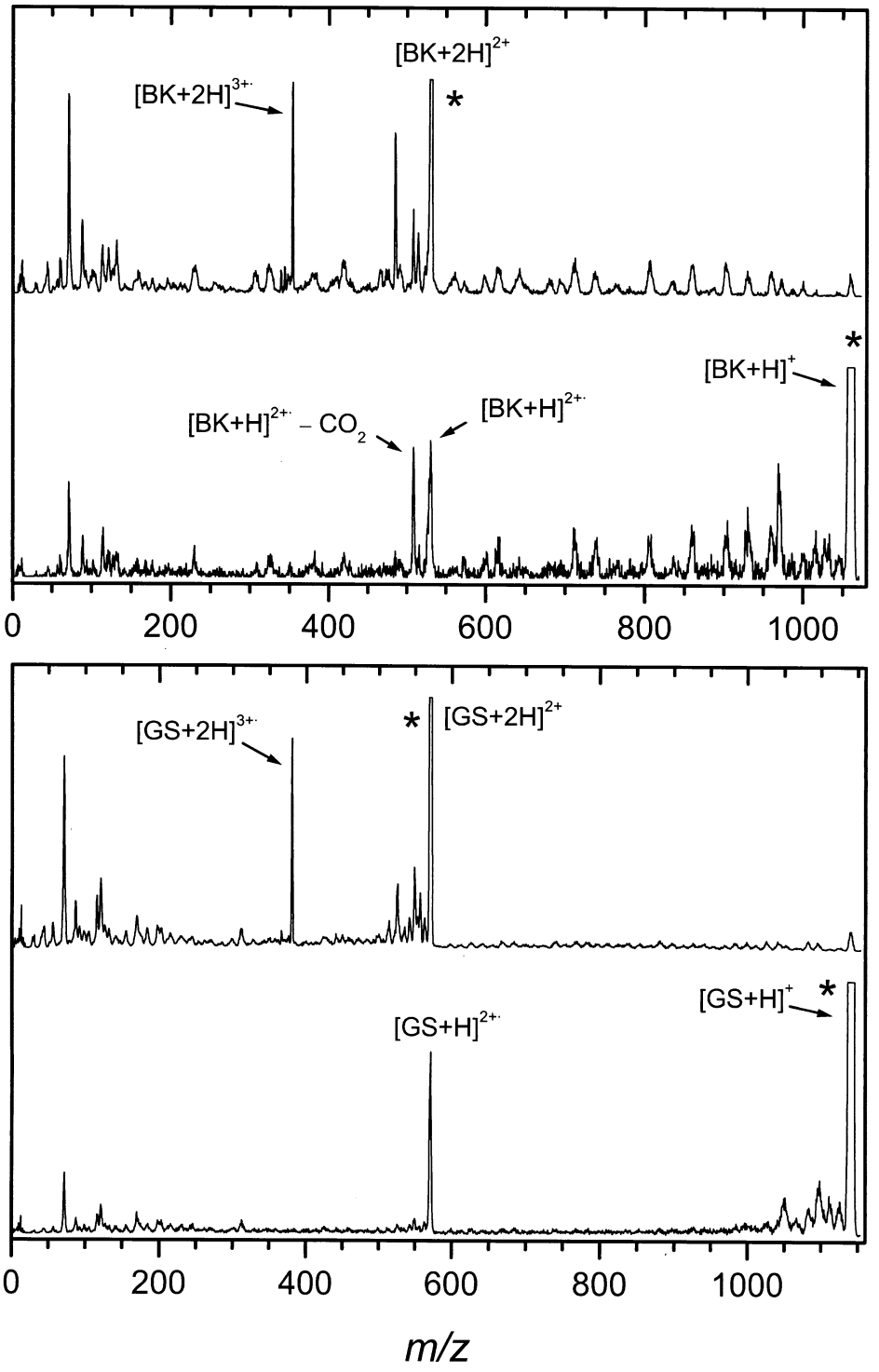


Fig. 1. Spectra obtained from high energy collisions between O_2 and $[BK+nH]^{n+}$ and $[GS+nH]^{n+}$ (O_2 in collision chamber). The parent ions are indicated by an asterisk.

radical cations $[M+nH]^{(n+1)+\cdot}$ is observed. For example, the CID spectrum of singly protonated gramicidin S $[GS+H]^+$ shows a sharp prominent peak at *m/z* 571 which corresponds to the radical dication $[GS+H]^{2+\cdot}$. It is evident from the CID spectra that much less fragmentation occurs for gramicidin S than for bradykinin, and electron loss is actually the most important reaction channel for gramicidin S. We ascribe the lower abundance of fragment ions to the fact that gramicidin S is a cyclic peptide and thus requires cleavage of two bonds to produce a fragment ion.

Electron stripping is an efficient process at very high collision energies (50 and 100 keV). In contrast, electron irradiation of $[BK+H]^+$ in the cell of a Fourier transform ion cyclotron resonance instrument did not yield $[BK+H]^{2+\cdot}$ though it was reported that electron stripping of larger ions is very efficient [15].

Previously, Wu and Fenselau [8] studied collisional ionization of neutral peptides using O_2 as target; however, in contrast to our results, they observed extensive fragmentation of the peptide radical monocations, and in most cases, the intact molecular ion was not obtained. It was found that most fragment ions from a peptide radical monocation and a singly protonated peptide were similar. Using resonance-enhanced multiphoton ionization (REMPI), Grotemeyer and Schlag [10] demonstrated that abundant peptide radical monocations were formed when keeping the photon density low and that increasing the photon density led to fragmentation.

3.2. MS/MS of peptide ions

In order to probe the fragmentation pattern of the radical polycations, we have carried out electron stripping before the magnet, mass-selected the radicals and probed their fragmentation. The $[BK+H]^+$ ion current was too low to form the radical dication for fragmentation experiments. In contrast, $[BK+2H]^{3+\cdot}$, $[GS+H]^{2+\cdot}$, and $[GS+2H]^{3+\cdot}$ were formed in sufficient number (several hundred counts per second) from $[BK+2H]^{2+}$, $[GS+H]^+$, and $[GS+2H]^{2+}$, respectively, for subsequent CID experiments. CID spectra of the precursor even-electron peptides were also acquired for comparisons. In the following, we shall show that increasing the charge state from +2 to +3 induces significant changes in the fragmentation pattern whereas spectra of +1 and +2 peptide cations are similar.

3.3. Bradykinin

The CID spectra of $[BK+H]^+$, $[BK+2H]^{2+}$, and $[BK+2H]^{3+\cdot}$ are shown in Fig. 2. The spectra of $[BK+H]^+$ and $[BK+2H]^{2+}$ are quite similar. For both ions the fragmentation pattern is governed by the presence of Pro residues due to the facile amide bond cleavage on the N-terminal side of a Pro residue (so-called "proline effect"); this leads to the production of y_7-2 and y_8-2 ions in high abundance (Scheme 1).

Provided that protonation occurs at the most basic sites, one or both arginine residues are protonated in $[BK+H]^+$ and $[BK+2H]^{2+}$. However, collisional activation enables the proton to explore less basic sites along the peptide chain ('mobile' proton model) [16]. Protonation of an amide nitrogen weakens the peptide bond thereby promoting cleavage into y- or b-type ions ("charge-directed" cleavage). The proline amide nitrogen is the most basic of the amide nitrogens in a polypeptide and this accounts for the "proline effect".

Abundant a-type ions such as a_7 and a_8 are seen in the CID spectra of $[BK+H]^+$ and $[BK+2H]^{2+}$ (Fig. 2 and Scheme 1). These are believed formed by a charge-remote fragmentation mechanism and typically dominate high-energy CID spectra of protonated peptides [17]. In contrast, most ions observed in low-energy CID spectra are a result of charge-directed fragmentation pathways [16], and a-ions are here formed by loss of CO from b-type ions. The b_7 and b_8 ions are almost absent in our high-energy CID spectra in support of a charge-remote mechanism for the formation of a_7 and a_8.

The CID spectra of $[BK+H]^+$ and $[BK+2H]^{2+}$ obtained at these very high collision energies (50 and 100 keV, respectively) are rather similar to previous reported spectra obtained at 6 and 12 keV collision energies [18,19]. However, the CID spectrum of $[BK+2H]^{3+\cdot}$ is markedly different from those of

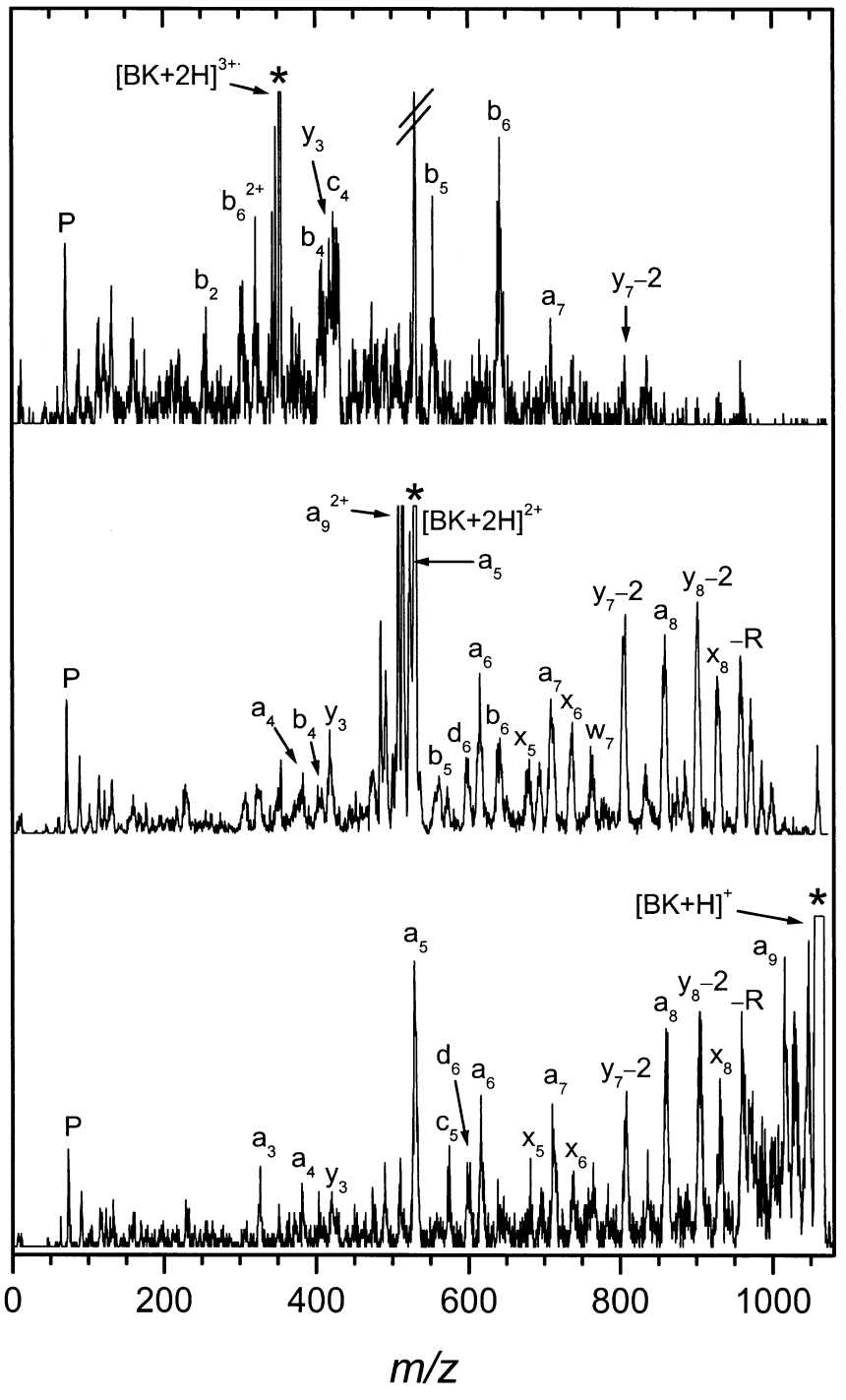
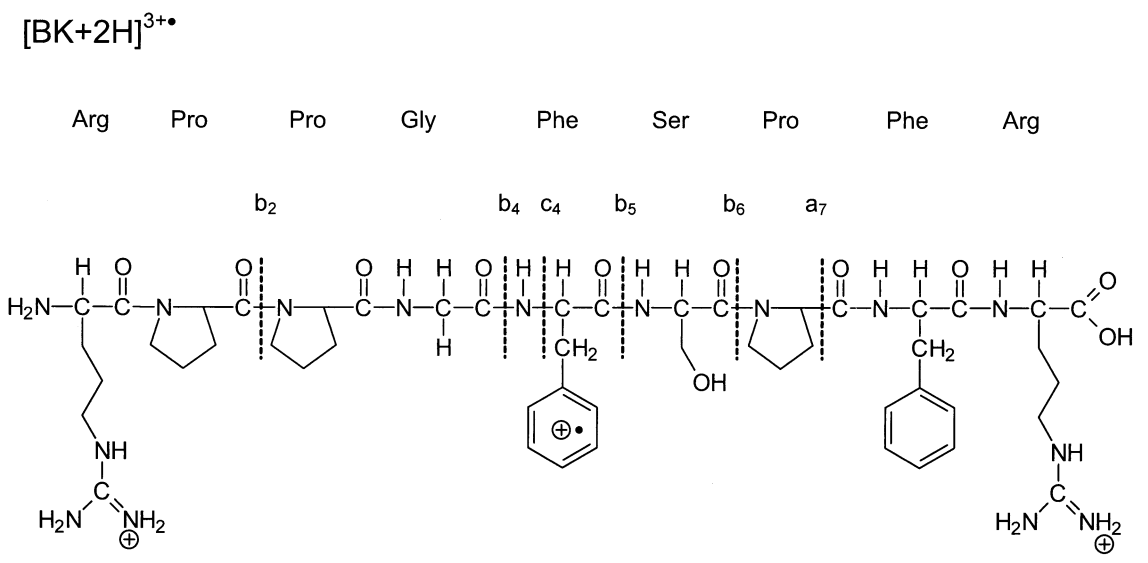


Fig. 2. Spectra obtained from high energy collisions between He and $[BK+H]^+$, $[BK+2H]^{2+}$, and $[BK+2H]^{3+\cdot}$ (formed before the magnet in collisions with O_2). The parent ions are indicated by an asterisk. The // indicates an interfering ion that is also present without letting O_2 in before the magnet; however, a CID spectrum of this ion revealed none of the fragments seen from $[BK+2H]^{3+\cdot}$. Also, the impurity ion is of much smaller abundance than the $[BK+2H]^{3+\cdot}$ ion (less than 5%).



Scheme 1.

$[BK+H]^+$ and $[BK+2H]^{2+}$: the peaks corresponding to y_7-2, y_8-2 are nearly absent in the spectrum of $[BK+2H]^{3+\cdot}$; instead, b-type ions such as b_4, b_5, and b_6 together with c_4 ions are formed in high abundance (Scheme 1). Also, peaks can be assigned to the doubly charged b_6 ion and the complementary y_3 ion.

First, consider the b_6 ion that is formed by cleavage of the amide bond between Ser-6 and Pro-7. Fig. 2 reveals that the b_6 ion abundance increases with increasing charge state; the b_6 ion is nearly absent in the spectrum of $[BK+H]^+$, it appears as a distinct peak in the spectrum of $[BK+2H]^{2+}$, and it is the most abundant fragment ion of $[BK+2H]^{3+\cdot}$. A charge-directed cleavage mechanism was previously used to explain the formation of this ion from $[BK+2H]^{2+}$ [18]. It was argued that when the proton at Arg-9 moves to the Pro-7 amide nitrogen in the activated peptide ion, the b_6 ion is formed after an amide bond cleavage reaction in which the serine side-chain is an important participant. Intuitively, this charge directed cleavage becomes easier when the charge state increases from +1 to +2 because of the higher probability for placing a proton on Pro-7. Our data show that the process is even more favorable for a 3+ ion than for a 2+ ion. Assuming the benzene ring of Phe-5 is the group from which the electron is lost in forming $[BK+2H]^{3+\cdot}$, the benzene radical cation can act as a proton donor to the peptide backbone, thereby facilitating the placement of a proton on the Pro-7 amide nitrogen in favor of the formation of the b_6 ion. Actually, $C_6H_6^{+\cdot}$ is a much better proton donor than $ArgH^+$, cf. PA(Arg) = 1051 kJ mol^{-1} ≫ PA($C_6H_5^{\cdot}$) = 884 kJ mol^{-1}, and the proton affinity of $C_6H_5^{\cdot}$ is almost equal to that of N-methyl-acetamide, PA($CH_3C(O)NHCH_3$) = 888.5 kJ mol^{-1}, which explains in general the high abundance of b-type ions formed after charge-directed cleavages. In order words, the sequestering of protons by the highly basic arginines in $[BK+2H]^{2+}$ is overcome in $[BK+2H]^{3+\cdot}$ in which proton-mobilization is mediated by the less basic phenyl radical.

Since the y_7 and y_8 type ions are also a result of charge-directed cleavage, it is remarkably that they are almost absent in the CID spectrum of $[BK+2H]^{3+\cdot}$. Two plausible explanations for this finding are: 1) since the two proline residues are next to the protonated N-terminal arginine, Coulomb repulsion makes it unfavorable for a proton to bind to the proline amide groups compared to the other amide groups, that is, the PAs of the Pro-2 and Pro-3 amide

groups in $[BK+2H]^{3+\cdot}$ are reduced compared to those in $[BK+H]^{+}$ and $[BK+2H]^{2+}$, and the most basic site is now Pro-7; or 2) the proton movement occurs stepwise from $C_6H_5^{+\cdot}$ to the amide group of Phe-5 to the amide group of Gly-4 to the amide group of Pro-3; however, if amide cleavage is faster than this proton movement the proton will never make it to the amide group of Pro-3. In other words, either the proton affinities of the amide groups in $[BK+2H]^{3+\cdot}$ are different from those of the amide groups in $[BK+2H]^{2+}$ or the kinetics for proton mobilization has changed, resulting in other charge-directed cleavage reactions. Likewise, a low-energy CID spectrum of the triply protonated BK ion, $[BK+3H]^{3+}$, reported by Boyd and co-workers [20] also revealed a high abundance of b and y type ions (b_4, b_5, b_6^{2+}, y_3, y_4) from cleavages in the middle of the peptide and hence a less important proline effect.

Now, we consider in more detail the peak which we tentatively assign to a c_4 ion; this ion is only formed from $[BK+2H]^{3+\cdot}$ and is likely to arise because of the radical cation character of the Phe-5 benzene side-chain. The bond that breaks is the C-N bond of Phe-5, and the presence of c_4 ions and not similar c_7 type ions is indicative of Phe-5 and not Phe-8 being ionized. This finding can be explained on the basis of purely electrostatic arguments: the Phe-5 amino acid residue is just in the middle of the peptide, with maximum distance to the two arginines that carry the charges, whereas Phe-8 is next to Arg-9 and close to a positive charge. Such a picture is however only correct to some extent since bradykinin is not linear but folded in a way which maximizes the distance between the excess charges while maintaining as many hydrogen bonds as possible [21]. Collisional activation causes some unfolding, and the secondary structure of the fragmenting ion is not known.

In electron-capture dissociation of protonated peptides, hydrogen atom transfer accounts for the predominant formation of c-type ions. A similar mechanism may be in play here where a hydrogen atom is donated by the phenyl radical cation [22]. Indeed, Downard and Biemann [23] have shown that the amino acid C-terminal at a cleavage site plays an important role in the formation of a c_n ion, that is, the ability of the amino acid side chain to transfer a hydrogen atom to the N of −C(O)NH–CH– determines the abundance of c_n ions. It requires much more energy to abstract a hydrogen atom from benzene than from a benzene radical cation (4.9 eV vs. 1.3 eV), explaining the lack of c_4 ions from $[BK+2H]^{2+}$ and the facile C-N cleavage in $[BK+2H]^{3+\cdot}$ leading to c_4 ions.

The dissociation in the highly exothermic ECD process occurs through the action of an energetic mobile H atom that induces clevage of the C_α–N bond [5–7]. ECD gives rise to many different c and z ions, only proline C_α–N being immune to cleavage. In contrast, we observe only one specific c-ion as the ionized phenyl ring of Phe presumably acts as a highly local H-atom donor. No b-ions are formed in ECD whereas b-ions are the most abundant fragment ions in our study, which reflects the ability of the ionized phenyl ring to act both as a proton donor and a hydrogen atom donor.

It is worth emphasizing that the fragmentation efficiency is much higher for the radical trication than for $[BK+H]^{+}$ and $[BK+2H]^{2+}$, the abundance of fragment ions relative to the total ion abundance being 0.15, 0.12, and 0.62 for $[BK+H]^{+}$, $[BK+2H]^{2+}$, and $[BK+2H]^{3+\cdot}$, respectively.

In summary, the data clearly show how the fragmentation pathways are changed with the selective enhancement of b and c type ions when the charge state is increased from +2 (even-electron ion) to +3 (uneven electron ion). Hence, this method allows a straightforward assignment of the structurally relevant N-termini b-type ions. The b type ions are a result of proton donation from a phenyl radical cation side-chain whereas the c type ions are formed after hydrogen atom abstraction from the phenyl radical cation. The complementary y type ions are revealed from the lower charge spectra.

3.4. Charge reversal spectra of deprotonated bradykinin

The $^{-}CR^{+}$ spectrum of $[BK–H]^{-}$ is shown in Fig. 3 together with the CID spectrum of $[BH+H]^{+}$ for comparison. The hydrogen-deficient $[BK–H]^{+\cdot}$ cation

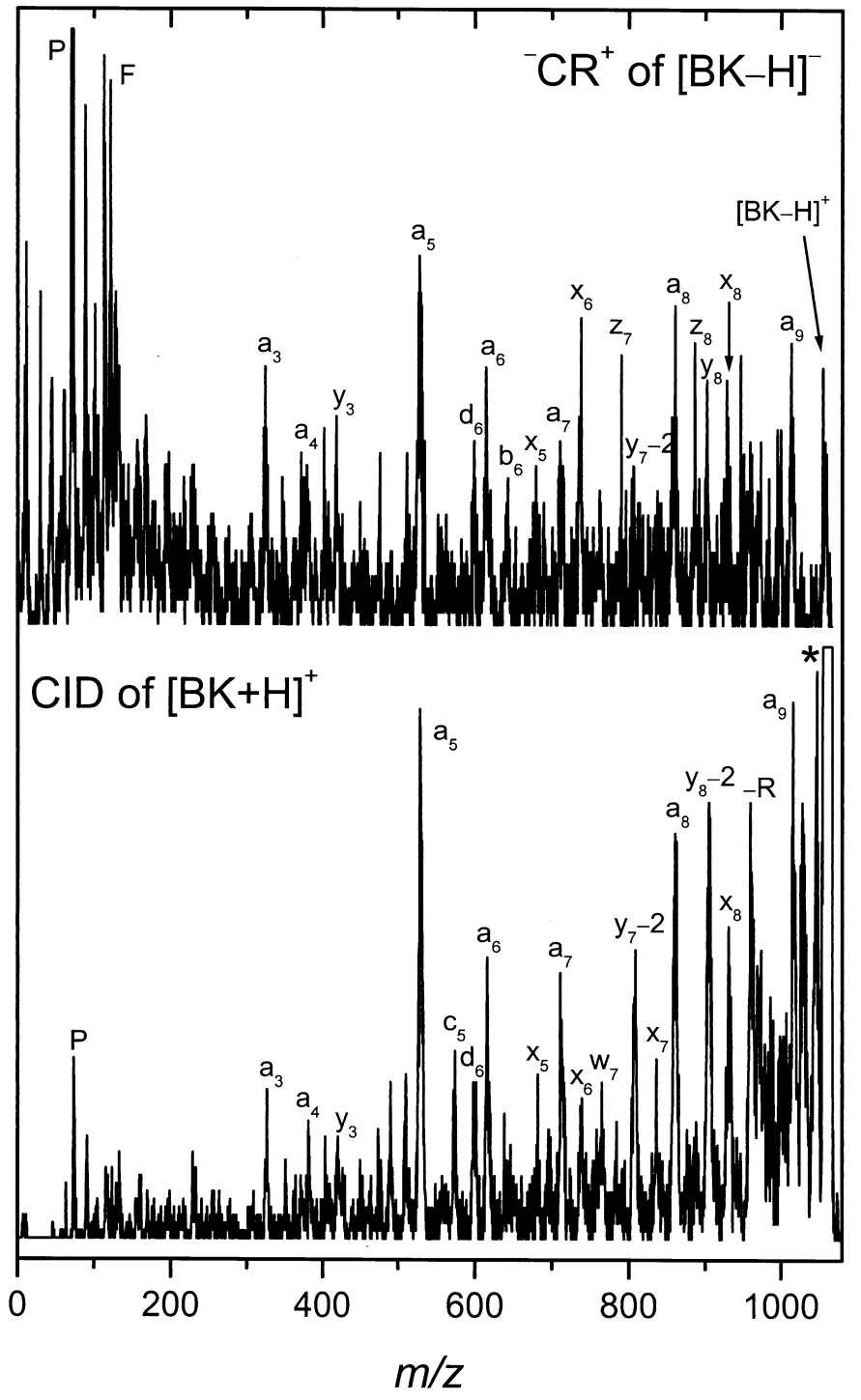


Fig. 3. Fragmentation spectra obtained from high energy collisions between O_2 and $[BK–H]^{-}$ (scanning positive fragments, $^{-}CR^{+}$ spectrum) and He and $[BK+H]^{+}$ (parent ion indicated by an asterisk).

is observed as a clear peak in the $^{-}CR^{+}$ spectrum in contrast to a previous study by Bertrand and Thibault [24] on various peptide anions. In most cases, their spectra did not yield any sequence ions but were completely dominated by immonium ions and fragments hereof. Also, in our study electron stripping of the peptide anion results in a much higher abundance of low mass ions than observed in the CID spectrum of $[BK+H]^{+}$. Cleavage of C–C(O) bonds is a dominant reaction channel, leading to abundant a- and x-type ions. As previously noted, these ions are probably formed by a charge-remote mechanism. If we assume that most of the positive fragments of $[BK–H]^{-}$ are formed from $[BK–H]^{+\cdot}$, charge-remote cleavages are indeed expected to govern the fragmentation pattern due to the deficiency of hydrogen atoms in $[BK–H]^{+\cdot}$ (lacks two hydrogen atoms). The y-type ions are less abundant in the $^{-}CR^{+}$ spectrum of $[BK–H]^{-}$ than in the CID spectrum of $[BK+H]^{+\cdot}$. Finally, abundant z_7 and z_8 ions appear in the $^{-}CR^{+}$ spectrum which we believe is a result of the radical character of $[BK–H]^{+\cdot}$.

3.5. Gramicidin S

The inherent problem of determining the sequence of a cyclic peptide is the lack of free N-termini and C-termini residues. Hence, it is a difficult task to obtain the sequence from a CID spectrum of the parent molecule, since the spectrum is a superposition of spectra from fragmentation of a set of isomeric ring-opened acylium ions of the same mass to charge ratio. Often the peptide is cleaved first by trypsin in solution, followed by mass analysis of the fragments, but it is preferable to avoid enzymatic hydrolysis. Alternatively, CID of selected fragment ions can be used to elucidate the sequence of a cyclic peptide since fragment ions will differ in their composition and sequence if every amino acid residue is different in the cyclic peptide [25]. Nakamura et al. [26] reported in 1986 the structural assignment of the gramicidin sequence using fast atom beam MS/MS (collision energy 5 keV). Above *m/z* 250 their CID spectra are dominated by the following fragment ions *m/z* 311 (PVO), 424 (PVOL), 571 (LFPVO), 684 (LFPVOL) and 831 (LFPVOLF) [Note: four other isobaric linear fragments exist with *m/z* 571].

As anticipated, only little fragmentation occurs for GS compared to BK (Fig. 1 –4). Again, the CID spectra of the singly and doubly protonated even-electron ions, $[GS+H]^{+}$ and $[GS+2H]^{2+}$, are similar, but they differ substantially from the spectrum of $[GS+2H]^{3+\cdot}$ (Fig. 4). For $[GS+H]^{+}$ and $[GS+2H]^{2+}$, the low-mass fragments dominate and the structure-indicative high-mass fragments are unfortunately of low abundance. The most abundant of the sequence ions is PVO (*m/z* 311). Helpful information is gained however from the low-mass immonium ions such as the identification of proline, leucine (or isoleucine if the sequence was not known), and phenylalanine in the peptide based on the characteristic *m/z* 70 (P), 86 (L), and 120 (F) peaks, but to determine the sequence of the peptide, higher-mass fragments are a prerequisite. In this regard, the spectrum of the radical trication is promising because it contains several peaks in the mid-region of the spectrum corresponding to sequence ions. The two excess protons in $[GS+2H]^{2+}$ reside on the ornithine side-chains, and probably one of the two phenyl rings is ionized in collisions with O_2.

The peak at *m/z* 449 in the spectrum of $[GS+2H]^{3+\cdot}$ is especially striking since it is absent in the two other spectra. We assign this peak to the doubly charged VOLFPVOL acylium ion. The second-most intense peak is *m/z* 472 that may correspond to the OLFP acylium ion. The *m/z* 200 peak is assigned to a decarbonylated OL ion. As was the case for the $[GS+H]^{+}$ and $[GS+2H]^{2+}$ parent ions, the *m/z* 311 peak is also evident in the spectrum of $[GS+2H]^{3+\cdot}$ together with the characteristic lower mass fragments. Amide bond cleavages in $[GS+2H]^{3+\cdot}$ account for the two dominant fragment ions, which indicates that the fragmentation mechanism can again be put in the context of the mobile proton model with charge-directed cleavages.

No big difference was observed between the CID spectra of $[GS+2H]^{2+}$ and $[GS+H]^{2+\cdot}$ (spectrum not shown) except for an intense peak at ~*m*/z 1097 in the latter spectrum of $[GS+H]^{2+\cdot}$ corresponding to a 44 ion loss (e.g. $CONH_2^{+}$). This indicates that the differ-

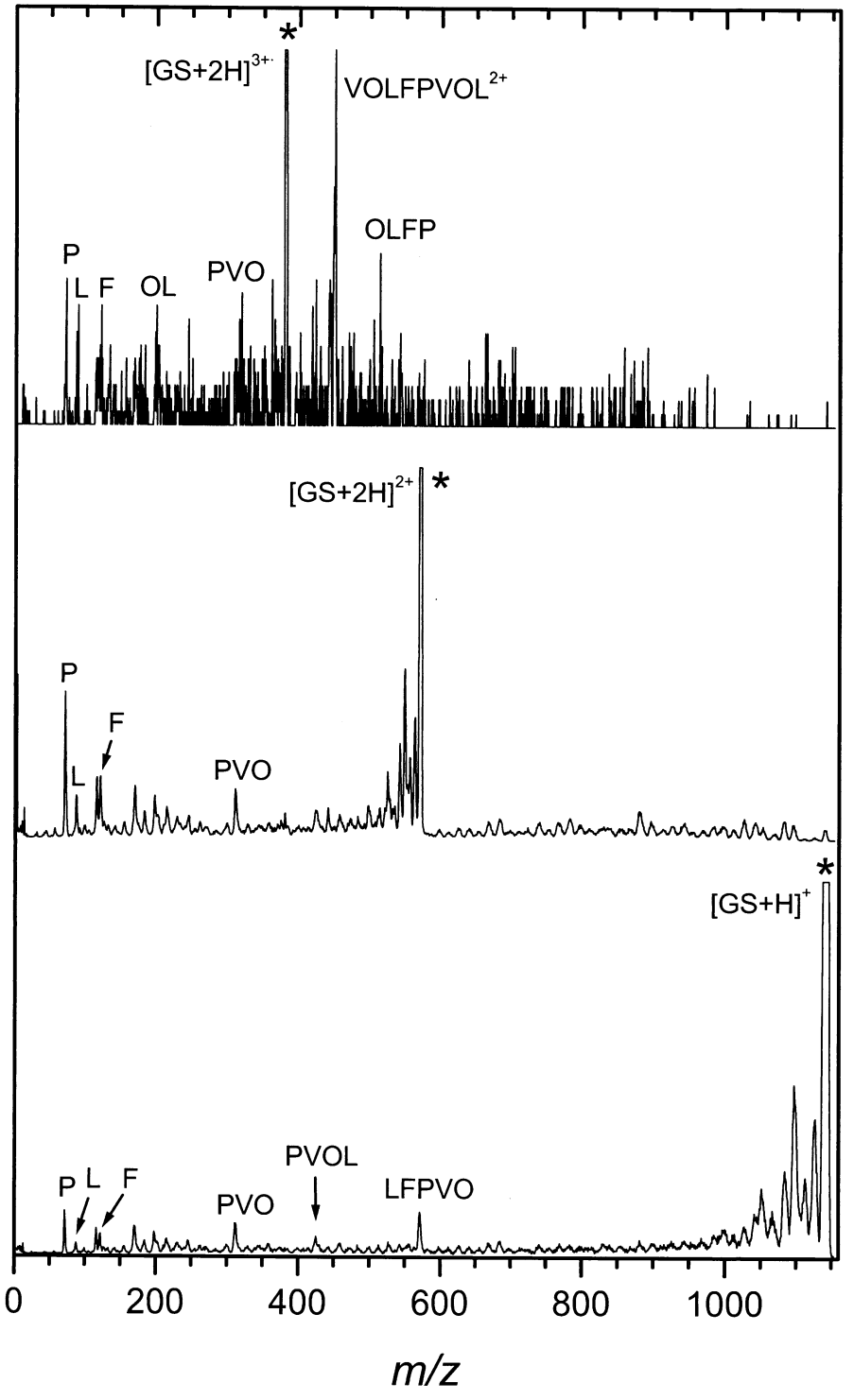


Fig. 4. Spectra obtained from high energy collisions between He and $[GS+H]^+$, $[GS+2H]^{2+}$, and $[GS+2H]^{3+\cdot}$ (formed before the magnet in collisions with O_2). The parent ions are indicated by an asterisk.

ent nature of the cleavage reactions of $[GS+2H]^{3+\cdot}$ is not only due to its radical character but also to its high charge state.

4. Conclusions

We have shown that in high-energy collisions between protonated peptides and molecular oxygen, electron stripping occurs. The method can be used to form a peptide in a charge state of +1 higher than that obtainable from protonation of the available protonation sites. This higher-charge-state ion fragments differently resulting mainly in b and c type ions. Knowledge of the ion types that dominate clearly aids in the spectral assignments. More data are required to consolidate our spectral interpretations, but the important message from this study is that further ionizing of a multiply protonated peptide selectively enhances new fragmentation pathways, thereby providing extra information regarding the sequence. In de-novo sequencing of peptides as much information as possible is desirable to avoid any ambiguity.

The energy required for electron loss is on the order of 10 eV, and multiphoton absorption in the intense field of a femtosecond laser pulse is likely to be a good alternative to collisional electron stripping increasing the applicability of the method.

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